14TH ANNUAL
SYMPOSIUM ON THE CANNABINOIDS

ARISTON HOTEL - PAESTUM, ITALY

JUNE 22 – 27 2004

Program and Abstracts

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International Cannabinoid Research Society

2004 Symposium on the Cannabinoids

June 22-27, 2004

Including a Monothematic One-Day Meeting of the Società Italiana di Farmacologia (SIF), June 26, 2004

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2004 SYMPOSIUM OVERVIEW

Wednesday June 23	Thursday June 24	Friday June 25	Saturday June 26
0700 – Breakfast	**0700 – Breakfast**	**0700 – Breakfast**	**0700 – Breakfast**
0800 – Intro			
0815-1015 Chemical and Structure Activity Relationship Studies on Proteins of the Endocannabinoids System (8 Orals)	**0815-1045** Vanilloid Receptors (3 Orals)	**0815-1045** Reproduction and Brain Development (3 Orals)	**0815-1045** Cannabinoids and Cancer (5 Orals) **SIF Session**
	Evolution of the Endocannabinoid System (3 Orals)	Sensory Nervous System and Pain (7 Orals)	Novel Roles for Cannabinoid CB_2 Receptors **SIF Session** (3 Orals)
	Memory and Cognition (4 Orals)		Cannabinoids in the Eye (2 Orals) **SIF Session**
1015-1045 - Coffee			
1045-1230 Regulation of Endocannabinoid Level: Biosynthesis and Inactivation (7 Orals)	**1045-1100 - Coffee**	**1045-1115 - Coffee**	**1045-1115 – Coffee**
	1100-1415 Visit to Temples Box Lunch at the Ariston Swimming Pool	**1115-1245** Effects on Gastrointestinal, Cardiovascular and Other Peripheral Functions (6 Orals)	**1115-1245** Immune-Modulatory Actions (4 Orals) **SIF Session**
			Reward and Interactions with Other Drugs of Abuse (2 Orals) **SIF Session**

1230-1430 – Lunch			
		1245-1445 - Lunch **NIDA Info Lunch**	**1245-1430 – Lunch**
	1615-1930 Neuromodulatory Role of Endocannabinoids (7 Orals)		
	Neuroprotection and Neurodegenerative Disorders (6 Orals)		
1700-1900 Cannabinoid Receptor Structure, Regulation and Signal Transduction (8 Orals)			
		1715-1845 Stress, Anxiety and Psychiatric Disorders SIF Session (4 Orals)	
		Food-Intake and Energy Control (2 Orals)	**1730-1930** Marijuana Abuse (5 Orals)
			Beneficial Effects of Non-Psychotropic Cannabinoids and Cannabis Extracts (3 Orals)
		1845-1915 Business Meeting	
1930-2030 – Break	**1930-2030 - Break**	**1915 – 2000 - Break**	**1930 – 2030 - Break**

		2000 Dinner	
2030 Dinner	**2030 - Dinner**		**2030 Banquet** **Maximum – 440 Guests**
		2130 Concert	
			2200 Awards

POSTER SESSIONS

WEDNESDAY 1430-1700	THURSDAY 1415-1615	FRIDAY 1445-1715	SATURDAY 1430-1730
Chemical and Structure Activity Relationship Studies on Proteins of the Endocannabinoids System (13 Posters)	Vanilloid Receptors (7 Posters)	Neuroprotection and Neurodegenerative Disorders (15 Posters)	Immune-Modulatory Actions (11 Posters) **SIF Session**
Regulation of Endocannabinoid Level: Biosynthesis and Inactivation (13 Posters) *Includes 1st Controversial Issue Discussion*	Evolution of the Endocannabinoid System (3 Posters)	Reproduction and Brain Development (4 Posters)	Food-Intake and Energy Control (6 Posters)
Cannabinoid Receptor Structure, Regulation and Signal Transduction (14 Posters)	Memory and Cognition (4 Posters)	Sensory Nervous System and Pain (13 Posters)	Cannabinoids and Cancer (3 Posters) **SIF Session** *Includes 2nd Controversial Issue Discussion*
Non-Endocannabinoid Bioactive Fatty Acid Amides	Neuromodulatory Role of Endocannabinoids	Effects on Gastrointestinal, Cardiovascular and Other	Novel Roles for Cannabinoid CB2 Receptors

(4 Posters)	(9 Posters)	Peripheral Functions (8 Posters)	(3 Posters) **SIF Session**
			Cannabinoids in the Eye (3 Posters) **SIF Session**
			Stress, Anxiety and Psychiatric Disorders (2 Posters)
			Reward and Interactions with Other Drugs of Abuse (11 Posters) **SIF Session**
			Marijuana Abuse (1 Poster)
			Beneficial Effects of Non-Psychotropic Cannabinoids and Cannabis Extracts (9 Posters)

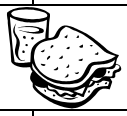
14th Annual Symposium On The Cannabinoids

Including a One-day Monothematic Meeting of the Società Italiana di Farmacologia on "New Advances in Endocannabinoid Research"

2004 Program

Wednesday June 23rd

0700	**Breakfast** 		
0800	***OPENING REMARKS***		
Chemical and Structure Activity Relationship Studies on Proteins of the Endocannabinoid System ➢*Chairs: P. Reggio / M. Mor*			
0815	Thomas, B.F., Fix, S., Gilliam, A.F., Bracken M., Myers, J., Pertwee, R.G., and Seltzman, H.H.	Structure -Activity Relationships for Bivalent Cannabinoid Ligands	1
0830	Reggio, P., Umijiego, U., Hurst, D., Seltzman, H.H., Hyatt, S., Roche, M., McAllister, S., Fleischer, D., Abood, M., Shi, S., Jones, J., and Lewis, D.	Hydrogen Bonding of the SR141716 C-3 Carboxamide Oxygen with K3.28 is Crucial for its Inverse Agonism	2
0845	Huffman, J. W., Joshi, S.N., Bushell, S.M., Wiley, J.L., and Martin, B.R.	2'-Methylalkyl-1-Methoxy-, and 1-Deoxy-Δ^8-Tetrahydrocannabinols: Effect of Sterochemistry and Chain Length Upon CB_1 and CB_2 Receptor Affinity	3
0900	Mahadevan, A., Bourne, C., Roy, S., Wiley, J.L., Martin, B.R., and Razdan, R.K.	Novel, Potent THC / Anandamide (Hybrid) Analogs	4
0915	Lu, D., Fan, P., and Makriyannis, A.	Diterpene Cannabinoids	5
0930	Pertwee, R.G., Thomas, A., Stevenson, L.A., and Mechoulam, R.	(–)-7-Hydroxy-4'-Dimethylheptyl-Cannabidiol Activates a Non-CB_1, Non-CB_2, Non-TRPV1 Target in the Mouse Vas Deferens in a Cannabidiol-Sensitive Manner	6
0945	Mor, M., Rivara, S., Lodola, A., Plazzi, P.V., Tarzia, G., Duranti, A., Tontini, A., Piersanti, G., Kathuria, S., and Piomelli, D.	Structure-Activity Relatioships of Alkylcarbamic Acid Aryl Esters as Fatty Acid Amide Hydrolase Inhibitors	7
1000	Tsuboi, K., Hilligsmann, H., Vandevoorde, S., Lambert, D.M., and Ueda, N.	Characterization of N-Palmitoylethano-lamine-Hydrolyzing Acid Amidase and Development of its Inhibitors	8
1015-1045	**Coffee** 		
Regulation of Endocannabinoid Levels: Biosynthesis and Inactivation ➢*Chairs: B. Cravatt / N. Ueda*			
1045	Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N.	Molecular Cloning and Characterization of N-Acylphosphatidylethanolamine-Hydrolyzing Phospholipase D (NAPE-PLD)	9

1100	Bisogno, T., Howell, F., Williams, G., Minassi, A. Cascio, M.G., Ligresti, A., Williams, E-J., Hobbs, C., Doherty, P., and Di Marzo, V.	Characterization of SN-1-Diacylglycerol Lipases as 2-AG/Endocannabinoid Biosynthesizing Enzymes	10
1115	Hu, S-J., Chen, J., Minassi, A., Roskoski, Jr., R., Di Marzo, V., and Walker, J.M.	Studies of the Biosynthesis of N-Arachidonoyl Dopamine (NADA)	11
1130	Sugiura, T., Oka, S., Gokoh, M., Kishimoto, S., and Waku, K.	2-Arachidonoylglycerol as a Novel Mediator of Inflammation	12
1145	Cravatt, B.F., Saghatelian, A., Hawkins, E.G., Clement, A.B., Bracey, M.H., and Lichtman, A.H.	Functional Disassociation of the Central and Peripheral Fatty Acid Amide Signaling Systems	13
1200	Maccarrone, M., Bari, M., Battista, N., Argirò, G., Finazzi-Agrò, A., Calabresi, P., and Cupini, L.M.	Anandamide Degradation in Headache Patients	14
1215	Vandevoorde, S., Lavand'homme, P., Fowler, C.J., Tsuboi, K., Ueda, N., Rozenberg, R., Habib Jiwan, J-L., and Lambert, D.M.	Oleoylethylamide, an Analgesic FAAH Inhibitor which Modulates Endogenous Anandamide, Oleoylethanolamide and 2-Arachidonoylglycerol Levels in the Brain	15
1230-1430	**Lunch** 		
1430-1700	**Poster Session**		96-138
Cannabinoid Receptor Structure, Regulation and Signal Transduction ➢*Chairs: A. Howlett / T. Rubino*			
1700	Kearn, C.S., Mackie, K., and Glass, M.	Physical Interactions of CB_1 Cannabiniod and D2 Dopamine Receptors	16
1715	McDonald, N., Connolly, C., and Irving, A.	Generation of a N-Terminal CB_1-EGFP Chimera to Study Cannabinoid Receptor Trafficking	17
1730	Niehaus, J.L., Wallis, K.T., Liu, Y., Bhartur, S.G., Elphick, M.R., and Lewis, D.L.	CRIP1a and CRIP1b: Novel CB_1 Cannabinoid Receptor Interacting Proteins	18
1745	Elphick, M.R., Wallis, K.T., Liu, Y., Lewis, D.L., and Egertová, M.	Localization of the CB_1 Cannabinoid Receptor Interacting Protein CRIP1a in the Brain	19
1800	Xie, X.Q., J. Zhao, J., Chen, J.-Z. and Zheng, H.-A.	CB_2 Receptor: Protein NMR from Building Blocks to 3D Structure	20
1815	Breivogel, C.	Beta-Arrestin 2 Affects Cannabinoid Sensitivity to Tetrahydrocannabinol	21
1830	Rubino, T., Viganò, D., and Parolaro, D.	Cannabinoid Receptor Signaling in Ras-GRF1 Knock Out Mice	22
1845	Rao, G.K., and Kaminski, N.E.	Δ^9-Tetrahydrocannabinol (Δ^9-THC) Elicits a Store-Independent Calcium Elevation in T Cells	23
1900-2030	**Break**		
2030	**Dinner** 		

Thursday June 24th

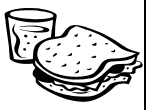
0700	**Breakfast** 		
Vanilloid Receptors ➢*Chairs: G. Appendino / V. Di Marzo*			
0815	Sala, M., Pegorini, S., Guerini-Rocco, C., Verzoni, C., Iosuè, S., and Braida, D.	Capsaicin Exhibits Neuroprotective Effects via Vanilloid Receptor Type 1 (Vr_1) Activation in a Model of Transient Global Cerebral Ischemia in Mongolian Gerbils	24
0830	Costa, B., Giagnoni, G., Trovato, A.E., Franke, C., and Mariapia, C.	Efficacy of Cannabidiol in a Rat Model of Neuropathic Pain: Evidence for VR1-Mediated Anti-Hyperalgesic Effect	25
0845	van der Stelt, Trevisani, M.M., De Petrocellis, L., Moriello, A.S., Campi, B., Geppetti, P., and Di Marzo, V.	Intracellular Anandamide Mediates Store-Operated Calcium Entry by Acting at TRPV1 Channels in Neurons	26
Evolution of the Endocannabinoid System ➢*Chairs: J.M. McPartland / M. Elphick*			
0900	McPartland, J.M., and Guy, G.W.	Numbers with Wings: The Calculus of CB Receptor Evolution	27
0915	Matias, I., Villani, G., and Di Marzo, V.	Occurrence and Biological Role of the Endocannabinoid System in Ciona Intestinalis	28
0930	Soderstrom, K., Tian, Q., Valenti, M., and Di Marzo, V.	Endocannabinoids Link Feeding State and Auditory Perception-Related Gene Expression	29
Memory and Cognition ➢*Chairs: P. Fadda / B. Lutz*			
0945	Varvel, S.A., Stokes, R., Niyuhire, F., and Lichtman, A.H.	Further Evidence for a Role of the Endocannabinoid System in Extinction Processes	30
1000	Cannich, A., Wotjak, C., Lutz, B., and Marsicano, G.	Altered Phosphorylation Levels in the Brain of Cannabinoid Receptor-1 Deficient Mice after Extinction Training	31
1015	Fadda, P., Robinson, L., Fratta, W., Pertwee, R.G., and Riedel, G.	Effects of Δ^9-THC and CBD-Rich Cannabis Extracts on Latent Learning in Rats	32
1030	Allison, C., Brett, R., and Pratt, J.	Deficits in an Attentional Set Shift Task Induced by Repeated Low Dose THC in Rats	33
1045-1100	***Quick* Coffee** 		
1100-1415	**Visit to Temples** ***Box Lunch Provided***		
1415-1615	**Poster Session**		139-161
Neuromodulatory Role of Endocannabinoids ➢*Chairs: B. Szabo / M. Melis*			
1615	Levine, E.S., Trettel, J., and Fortin, D.A.	Physiological Role of Endocannabinoids in the Neocortex	34

1630	Deadwyler, S.A., Zhuang, S.Y., Weiner, J., and Hampson, R.E.	Criteria for Release of Endocannabinoids by Hippocampal Cellular Activity	35
1645	Melis, M., Pistis, M., Perra1,S., Muntoni, A.L., Pillolla, G., Minassi, A., Di Marzo, V., and Gessa, G.L.	Endocannabinoids Mediate Retrograde Signalling at Excitatory and Inhibitory Synapses in the Rat Ventral Tegmental Area	36
1700	Riegel, A.C., and Lupica, C.R.	S_k Ion Channels and Metabotropic Glutamate Autoreceptors Control Endocannabinoid Release from Dopamine Neurons in the Ventral Tegmental Area (VTA)	37
1715	Shivachar, A.	Cannabinoids Inhibit Excitatory Amino Acid Transport in Cultured Rat Cortical Astrocytes	38
1730	Szabo, B., Wallmichrath, I., and Engler, B.	Cannabinoids Depress GABAergic Neurotransmission Between the Caudate-Putamen and Globus Pallidus	39
1745	Tanganelli, S., Ferraro, L., Antonelli, T., Tomasini, M.C., Tattoli, M., and Cuomo, V.	Prenatal Exposure to the CB_1 Receptor Agonist WIN55212-2 Alters Hippocampal and Cortical Glutamatergic Transmission. in Vivo and in Vitro Studies	40
Neuroprotection and Neurodegenerative Disorders ➢*Chairs: D. Baker / M. Glass*			
1800	Pryce, G., and Baker, D.	Inhibition of Experimental Spasticity by CB_1 and FAAH Inhibitors in Cannabinoid Gene Knockout Mice	41
1815	Musty, R.E., Cline, H.M., and Deyo, R.A.	THC Ameliorates Behavioral Deficits in the Spastic Mouse	42
1830	Malfitano, A.M., Matarese, G., Lechler, R.I., Pisanti, S., Laezza, C., Di Marzo, V., and Bifulco, M.	Arvanil Inhibits T-Lymphocyte Activation and Ameliorates the Course and Progression of Experimental Autoimmune Encephalomyelitis	43
1845	Ortega-Gutiérrez,S., Molina-Holgado, E., Arévalo-Martín,A., Viso, A., López-Rodríguez, M.L., and Guaza, C.	Anandamide Uptake Inhibition as Therapeutic Approach in a Murine Model of Multiple Sclerosis	44
1900	Cabranes, A., de Lago, E., Di Marzo, V., Minassi, A., Sánchez, A., García-Merino, A., Ramos, J.A., and Javier Fernández-Ruiz, J.	Beneficial Effects of Inhibitors of the Endocannabinoid Transport in a Rat Model of Multiple Sclerosis: Involvement of Vanilloid TRPV1 Receptors	45
1915	Esposito, G., Iuvone, T., Esposito, R., Santamaria, R., and Izzo, A.A.	Neuroprotective Effect of Cannabidiol on β-Amyloid-Induced Toxicity in PC12 Cells	46
1930	**Break**		
2030	**Dinner** 		

Friday June 25th

0700	**Breakfast** 

Reproduction and Brain Development ➢*Chairs: M. Maccarrone / J.J. Fernandez-Ruiz*			
	Schuel, H., and Burkman, L.J.	**not to be presented**	47
0815	Gómez, M., Hernández, M., de Miguel, R., Ramos, J.A., and Fernández-Ruiz, J.	Relationship of the Endocannabinoid System with Several Key Proteins for Brain Development in Rats	48
0830	Aguado, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., Marsicano, G., Guzmán, M., and Galve-Roperh, I.	Expression and Function of the Endocannabinoid System in Neural Progenitor Cells	49
Sensory Nervous System and Pain ➢*Chairs: S. Maione / M.J. Walker*			
0845	Lichtman,A.H., Shelton, C.C., Jackson, C., Saghatelian, A., and Cravatt, B.F.	Inhibition of FAAH Leads to CB_1-Mediated Analgesia Accompanied by Significant Increases in Endogenous Anandamide in Brain and Spinal Cord	50
0900	Holt, S., Costa, B., and Fowler, C.	FAAH Inhibitors and Indomethacin Reduce Carrageenan Induced Hind Paw Inflammation in the Mouse – Role of Cannabinoid Receptors	51
0915	Malan, Jr., T.P., Ibrahim, M.M., Makriyannis, A., and Porreca, F.	CB_2 Cannabinoid Receptors may Produce Peripheral Anaglesia by Stimulating Local Release of Endogenous Opioids	52
0930	Maione, S., de Novellis, V., Mariani, L., Vita, D., Scafuro, M., and Rossi, F.	Interactive Role of Periaqueductal Gray CB_1 and MGlu5 Receptors in the Formalin-Induced Changes in RVM ON- and OFF-Cells	53
0945	Castañé, A., Celerier, E., Martin, M., Murtra, P., Ledent, C., Parmentier, M., Maldonado, R., and Valverde, O.	Study of CB_1 Cannabinoid Receptor Knockout Mice in a Model of Neuropathic Pain	54
1000	Dogrul, A., Gül, H., Yıldız, O., Bilgin, F., and Güzeldemir, M.E.	Cannabinoids Blocks Tactile Allodynia in Diabetic Mice without Attenuation of its Antinociceptive Effect	55
1015	Duncan, M., Millns, P., Kendall, D., and Ralevic, V.	The Actions of WIN55,212 and THC on the Capsaicin-Evoked Calcium Response in Cultured Rat Dorsal Root Ganglia	56
1030-1045	**Round Table: CB_1 receptor antagonists as new therapeutic drugs** *Coordinators*: Murielle Rinaldi-Carmona, Francis Barth and Gerard Le Fur		
1045-1115	**Coffee** 		
Effects on Gastrointestinal, Cardiovascular and other Peripheral Functions ➢*Chairs: A.A. Izzo / N. Darmani*			
1115	Darmani, N.A., McClanahan, B.A., Trinh, C., Petrosino, S., Valenti, M., and Di Marzo, V.	The Chemotherapeutic Agent Cisplatin Increases Brain 2-Arachidonoyl-Gycerol (2-Ag) Concentrations and Concomitantly Reduces Intestinal 2-AG and Anandamide Levels in a Vomiting Species (The Least Shrew)	57
1130	Izzo, A.A., Capasso, R., Borrelli, F., Mascolo, N., Urbani, P., Di Marzo, V., and Capasso, F.	Inhibitory Effect of N-Arachidonoylserotonin, a FAAH Inhibitor, on Gastric and Intestinal Motility in Mice	58
1145	Demuth, D., Parsons, M., and Molleman, A.	Cannabinoid-Mediated Inhibition of Nicotinic ACH Currents in Myenteric Neurons	59

1200	Grenard, P., Julien, B., Van Nhieu, J.T., Li, L., Ledent, C., Mallat, A., and Lotersztajn, S.	Reduced Liver Fibrosis in Mice Invalidated for CB_1 Receptor	60
1215	Maor, Y., Horowitz, M., and Mechoulam, R.	Atypical Cannabinoids and their Role as Blood Pressure Regulators	61
1230	Rademacher, D.J., Savoie, A.M., Patel, S., Rusch, N.J., and Hillard, C.J.	The Vasoconstrictor U-46619 but not Serotonin Increases Endocannabinoid Content in the Middle Cerebral Artery: Evidence for Functional Relevance	62
1245-1445	**Lunch - NIDA Lunch** "Research Funding Initiatives for Non-US Citizens/Perminant Residents" presented by Dr. Steven Gust, National Institute on Drug Abuse, Extramural Funding, International Funding Initiatives. 		
1445-1715	**Poster Session**		162-201
Stress, Anxiety, and Psychiatric Disorders SIF Session ➢*Chairs: E. Fride / R. Musty*			
1715	Patel, S., Roelke, C., Cravatt, B.F., and Hillard, C.J.	Cannabinoid Modulation of Stress-Induced Amygdala Activation and Neuroendocrine Responses	63
1730	Fride, E., Surise, R. Weidenfeld, J., and Mechoulam, R.	The Endocannabinoid System Lowers the Stress Response	64
1745	Hill, M.N., Karacaeyli, E., and Gorzalka, B.B.	Pharmacological Enhancement of CB_1 Receptor Signaling Elicits an Antidepressant Effect in the Rat Porsolt Forced Swim Test	65
1800	Zavitsanou K., Huang X.-F., and Solowij N.	Significant Correlations Between Cannabinoid and Serotonin/Glutamate Receptor Densities in the Anterior Cingulate Cortex in Schizophrenia: A Site of Functional Interactions?	66
Food-Intake and Energy Control ➢*Chairs: G. Le Fur / G. Kunos*			
1815	Le Fur, G.	Clinical Results with Rimonabant in Obesity	67
1830	McLaughlin, P.J., Swezey, L.A., Winston, K.M., Makriyannis, A., and Salamone, J.D.	Investigation of the Possible Non-Motivational Factors that could Contribute to the Suppression of Feeding Produced by CB_1 Antagonists	68
1845-1915	**Business Meeting**		
1915-2000	**Break**		
2000	**Dinner** 		
2130	**Concert**		

Saturday June 26th

0700	**Breakfast** 		
Cannabinoids and Cancer ***1st Session of SIF Monothematic Meeting*** ➢*Chairs: M. Bifulco / M. Guzman*			
0815	McAllister, S.D., Abood, M.E., Chan, C.L., Luu, T., Taft, R.J., and Yount, G.L.	Δ^9-THC but not WIN55,212-2 Produces Biphasic Effects on the Growth of Multiple Human Glioma Cell Lines	69
0830	Grimaldi, C., Laezza, C., Iacuzzo, I., Eletto, D., Tecce, M.F., Portella, G., Di Marzo, V., and Bifulco, M.	Role of Endocannabinoids in Tumor Progression: Anandamide Inhibits Cell Migration in Human Breast Cancer Cells	70
0845	Velasco, G., Herrera, B., Carracedo, A., Gómez del Pulgar, T., and Guzmán, M.	Mechanism of Cannabinoid-Induced Apoptosis of Leukaemia Cell Lines	71
0900	Ligresti, A., De Petrocellis, L., Moriello, A.S., Portella, G., Bifulco, M., and Di Marzo, V.	Effect of Plant Cannabinoids on Cancer Cell Proliferation	72
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STRUCTURE -ACTIVITY RELATIONSHIPS FOR BIVALENT CANNABINOID LIGANDS

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The existence and pharmacological properties of dimeric G-protein coupled receptors has been the subject of considerable investigation, particularly as regards the structure and functioning of opioid receptors. Evidence suggests that oligomerization of G-protein-coupled receptors may be a relatively common occurrence, serving to modulate their biological activity and control receptor trafficking and internalization processes. In order to investigate the possible existence and functional consequences of cannabinoid receptor dimers, we have begun the synthesis and in vitro testing of bivalent cannabinoid ligands produced by substitution at the 3-position of SR141716, the CB_1 cannabinoid receptor subtype selective antagonist. Our approach allows us to produce a variety of bivalent ligands, ranging from those with virtually no additional carbon spacers between the carbonyls, to those containing spacers extending beyond 12 carbon atoms. By initially varying the spacer length between two SR141716 pharmacophores, we have generated a series of compounds to investigate whether it is possible to produce ligands that can bridge the recognition sites in cannabinoid receptor homodimers. It is plausible that these bivalent ligands, provided they have an appropriate spacer length, can induce or enhance the dimerization of two CB_1 receptors, and by doing so, produce unique pharmacological activity that will be apparent in in vitro assays. It is also our intention to produce bivalent ligands with two distinct receptor recognition pharmacophores, and investigate whether these ligands can induce heterodimers. By altering the level of hetero- or homo-dimerization in cannabinoid receptor signaling, or modifying "normal" receptor processes (e.g. trafficking), it is hoped that these bivalent ligands will possess unique functional properties, such as increased efficacy and potency, and have potential as new drugs with unique clinical value.

Acknowledgements: This work has received support from internal developments funds from RTI International and North Carolina Central University.

HYDROGEN BONDING OF THE SR141716 C-3 CARBOXAMIDE OXYGEN WITH K3.28 IS CRUCIAL FOR ITS INVERSE AGONISM

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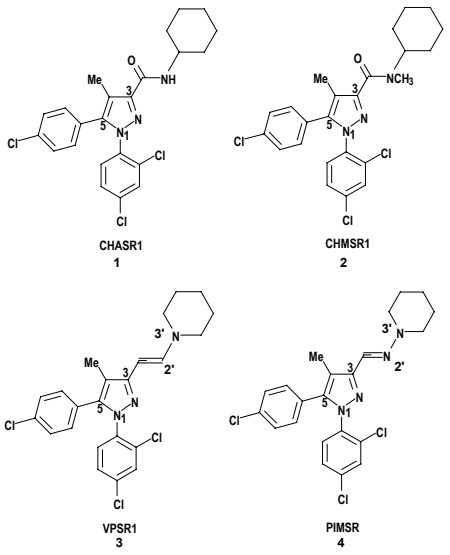

Our recent mutant cycle study of the interaction between K3.28(192) and 141716 (SR) indicated that K3.28 has a direct interaction with the SR C-3 substituent (Hurst et al. Mol. Pharmacol. *62*, 1274, **2002**) and that this interaction is crucial for SRs inverse agonism. The SR C-3 substituent has two moieties that could possibly serve as hydrogen bonding partners with K3.28, the carboxamide oxygen and the piperidine nitrogen. In order to determine which of these may be the interaction site for K3.28, a series of SR analogs were evaluated computationally; then, synthesized and evaluated pharmacologically. Conformational analyses revealed that the global min of **1** is analogous to the second lowest energy min of SR (Delta E= 0.92, HF/631g*). The global min of **2** has its cyclohexyl ring in an analogous conformation to that of **1**, however, the N-methyl group causes the C-3 substituent to rotate 31.5 degrees out of the pyrazole plane. The piperidine ring in the global mins of **3** and **4** has the piperidine ring rotated by 90 degrees about the 2'-3' bond. The energy expense (HF/631g* level) to adopt a piperidine ring conformation that mimicked that of SR was 4.69 kcal/mol for **3** and 0.66 kcal/mol for **4**. Docking studies in a model of the CB_1 inactive state revealed that all ligands could form aromatic stacking interactions with F3.36, Y5.39 and W5.43, residues that we have shown through mutation studies to be part of the SR binding pocket (McAllister et al., J. Med. Chem. *46,* 5139, **2003***).* Only **1** and **2**, however, could form a hydrogen bond between the carboxamide oxygen and K3.28 (with H bond geometry poorer for **2),** while in **4,** the N-2' formed a hydrogen bond with C7.42. CB_1 affinities of **1**-**4** were assessed in membranes from HEK cells expressing human CB_1 vs. [^{3}H]CP or [^{3}H]SR respectively. For **1,** Ki =6.9 nM and 1.7 nM; **2**, 45 nM and 29 nM; **3**, 406 nM and 261 nM; **4**, 57 nM and 6.7 nM. In SCG neurons, expressing the rat CB_1 receptor, **1**-**4** antagonized the effect of WIN55212-2. Compounds **1** (at 1 microM) and **2** (at 10 microM) produced effects when applied alone that were not statistically different from SR, suggesting inverse agonism. In contrast, **3** (at 1 and 10 microM) and **4** (at 1 microM) produced effects on Ca^{2+} current that were statistically different from SR, suggesting neutral antagonism. Taken together, these results support the hypothesis that hydrogen bonding of the SR C-3 carboxamide oxygen with the K3.28 in CB_1 is crucial for its inverse agonist properties.

Acknowledgements: Support: NIDA Grants DA03934 and DA000489 to PR; DA09978 and DA05274 to M.A.; DA10350 to D.L.

2'-METHYLALKYL-1-METHOXY-, AND 1-DEOXY-Δ^8-TETRAHYDROCANNABINOLS: EFFECT OF STEROCHEMISTRY AND CHAIN LENGTH UPON CB_1 AND CB_2 RECEPTOR AFFINITY

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It has been established that a 1',1'- or 1',2'-dimethylheptyl side chain greatly enhances CB_1 receptor affinity and cannabinoid potency. Similarly a 1'- or 2'-methylheptyl group also increases CB_1 receptor affinity and potency (Huffman *et al. Bioorg. Med. Chem.* **1998**, *6*, 2383.). In both the 1',1'-dimethylalkyl-Δ^8-THC series and the homologous series of Δ^8-THC analogs with an unsubstituted side chain those cannabinoids with a side chain of five to eight carbon atoms are most potent (Huffman *et al. Bioorg. Med. Chem.* **2003**, *11*, 1397.). In a series of 1-methoxy- and 1-deoxy-Δ^8-THC analogs, it was found that CB_2 receptor affinity is also enhanced by the presence of a 1',1'-dimethylalkyl side chain, however receptor affinity is much less sensitive to chain length. For instance, the three carbon analog, 3-(1',1'-dimethylpropyl)-1-deoxy-Δ^8-THC has K_i = 14 nM at CB_2 and 1-deoxy-Δ^8-THC-DMH with a seven carbon side chain has only slightly greater affinity with K_i = 3 nM (Huffman *et al. Bioorg. Med. Chem.* **1999**, *7*, 2905.). Nothing is known relative to the effect of side chain substituents other than a 1',1'-dimethyl upon CB_2 receptor affinity. In order to obtain additional data concerning structure-activity relationships at the CB_2 receptor and to investigate the influence of side chain stereochemistry upon receptor affinity we have initiated a program to prepare 1'- and 2'-methylalkyl-1-methoxy- and 1-deoxy-Δ^8-THC analogs.

Highly enantioselective synthetic approaches to both the 1'- and 2'-methyl THC analogs have been developed, and a number of 2'-methylalkyl-1-methoxy- and 2'-methylalkyl-1-deoxy-Δ^8-THCs have been prepared. None of the 1-methoxy compounds synthesized to date have significant affinity for either cannabinoid receptor, but in the deoxy series 1-deoxy-3-(2'*S*-methylpentyl)-Δ^8-THC exhibits 200-fold selectivity for the CB_2 receptor with K_i = 24±9 nM at CB_2 and K_i = 4307±649 nM at CB_1. The 2'*S*-methylhexyl analog has high affinity for both receptors while the 2'-methylpropyl and 2'*S*-methylheptyl compounds have low affinity. The details of the enantioselective synthesis of these compounds, other compounds in these series and their affinities for the CB_1 and CB_2 receptors will be presented.

Acknowledgements: Supported by grants DA03590, DA15340 and DA03672 from the National Institute on Drug Abuse.

NOVEL, POTENT THC / ANANDAMIDE (HYBRID) ANALOGS

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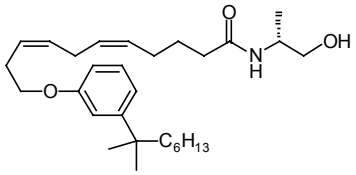
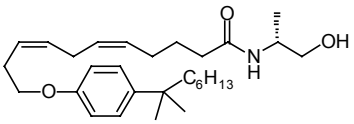
It is well known that the SAR of the end pentyl chain in anandamide (AEA) is very similar to that of THC. Incorporation of a DMH (dimethylheptyl) side chain of THC into a AEA template resulted in AEA analogs which mimic the activity of THC. To further extend this idea we designed hybrid structures **1-3** which incorporated the aromatic ring of THC-DMH and used the oxygen of the phenol of THC to include a part of the AEA moiety as an ether linkage. Hybrid **1 (O-2220)** was found to have very high binding affinity to CB_1 receptors (Ki = 8.5 nM) and it is interesting to note that the orientation of the side chain to the oxygen in the phenol is the same as in THC. Changing the orientation as in hybrids **2-3** was found to decrease the binding affinity.

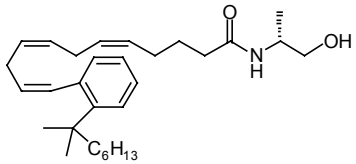
We had previously shown that the binding affinity and potency of THC was enhanced when specific substrates were incorporated at the terminal carbon of the side chain at C-3 in THC (Singer et al., J. Med. Chem. **1998**, *41*, 4400-4407). Hence to further probe the SAR in this series we modified the terminal carbon of the side chain by adding different substituents. Several such analogs were synthesized and tested for their binding affinities and in vivo activity (tetrad tests). The details of the synthesis and the biological activity of these compounds will be presented.



1 (O-2220)



2 (O-2294)



3 (O-2243)

Acknowledgements: Supported by NIDA grants DA-05488, DA-08904, DA-09789 and DA-03672

DITERPENE CANNABINOIDS

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The 3-side chain of classical cannabinoids plays a pivotal role in cannabinergic activity. Modifications of its length and introduction of heteroatoms and different substituents have led to novel ligands possessing enhanced affinity for both CB_1 and CB_2 receptor as well as high potency. However, to date such modifications have led to only modest CB_1/CB_2 selectivity.

To study the effect of side chain conformation on receptor subtype selectivity, we have developed conformationally more defined analogs through the introduction of double and triple bonds as well as carbocyclic rings within the chain. Alternatively, we have shown that cyclic substituents in the 1'-position of the chain can have profound effects on the ligand's properties. To date, our work has shown that both cannabinoid receptors can accommodate ligand conformations in which the side chain is highly folded and result in subtype selective analogs.

Here we report on the design and synthesis of a new class of classical cannabinoids in which the side chain has been substituted with a chiral terpene. Our results indicate that the relative and absolute configuration within the terpene structure can lead to significant receptor subtype selectivity.

Acknowledgments: This work was supported by grant DA3081 and DA7215 from the National Institute on Drug Abuse.

(–)-7-HYDROXY-4'-DIMETHYLHEPTYL-CANNABIDIOL ACTIVATES A NON-CB_1, NON-CB_2, NON-TRPV1 TARGET IN THE MOUSE VAS DEFERENS IN A CANNABIDIOL-SENSITIVE MANNER

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Cannabidiol (CBD) antagonizes the CB_1/CB_2 receptor agonist, *R*-(+)-WIN55212 (WIN), in the mouse isolated vas deferens in a manner that appears to be competitive and yet not to involve direct competition for cannabinoid receptors (Pertwee *et al.,* **2002**). In the the present investigation, we found that a synthetic analogue of CBD, (–)-7-hydroxy-4'-dimethylheptyl-CBD (7-OH-DMH-CBD), shares the ability of WIN to inhibit electrically-evoked contractions of the mouse isolated vas deferens. We then went on to explore the mechanism(s) underlying this inhibitory effect.

In our experiments, vasa deferentia were mounted in organ baths, and CBD, SR141716A, SR144528, capsazepine or DMSO were added to these baths 30 min before 7-OH-DMH-CBD or WIN. Drugs were dissolved in a 50% aqueous solution of DMSO (v/v) (WIN) or in pure DMSO. Other experimental details are given elsewhere (Pertwee *et al.,* **2002**). Values have been expressed as means with 95% confidence limits.

7-OH-DMH-CBD produced a concentration-related inhibition of electrically-evoked contractions of the mouse isolated vas deferens with an EC_{50} of 13.3 nM (4.6 and 37.9 nM; n=5 to 11), which is below its reported K_i values for CB_1 (4.4 μM) or CB_2 receptors (0.67 μM) (Bisogno et al., **2001**). At 1 μM, CBD produced a dextral shift of 5.5 (2.8 and 11.7; pA_2 = 6.65; n=7) in the log concentration response curve of 7-OH-DMH-CBD that did not deviate significantly from parallelism (P>0.20). In contrast, 7-OH-DMH-CBD was not antagonized by 32 nM SR141716A (unlike WIN), or by 32 nM SR144528 or 10 μM capsazepine, suggesting that 7-OH-DMH-CBD was not acting through cannabinoid CB_1 or CB_2 receptors or through vanilloid TRPV1 receptors. At 100 nM, 7-OH-DMH-CBD did not attenuate contractile responses of the vas deferens to phenylephrine hydrochloride or α,β-methylene-ATP, supporting the hypothesis that it inhibits electrically-evoked contractions of this tissue by acting on prejunctional neurones.

In conclusion, 7-OH-DMH-CBD appears to act on a prejunctional non-CB_1, non-CB_2, non-TRPVR1 target in the vas deferens in a manner that is sensitive to antagonism by CBD. This antagonism is produced at 1 μM, a concentration below any at which CBD binds to CB_1 or CB_2 receptors (Bisogno et al., 2001). Further experiments are required to identify the pharmacological target(s) at which 7-OH-DMH-CBD and CBD interact.

Acknowledgements: Supported by NIDA and GW Pharmaceuticals.

Bisogno, T. *et al.* (**2001**) *Br. J. Pharmacol.* 134**,** 845-852.
Pertwee, R.G. *et al.* (**2002**) *Eur. J. Pharmacol.* 456**,** 99-106.

STRUCTURE-ACTIVITY RELATIOSHIPS OF ALKYLCARBAMIC ACID ARYL ESTERS AS FATTY ACID AMIDE HYDROLASE INHIBITORS

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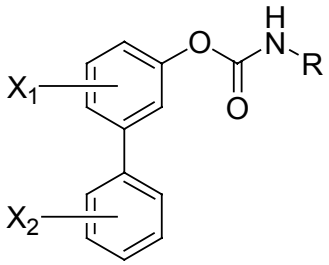
Inhibition of fatty acid amide hydrolase (FAAH), a membrane-associated enzyme responsible of inactivation of fatty acid amides (FAAs) including anandamide, oleoylethanolamide and palmitoylethanolamide, can be achieved by compounds belonging to traditional classes of serine hydrolase inhibitors, such as sulfonyl or phosphonyl fluorides and activated carbonyl derivatives. Recently, a new class of *N*-alkylcarbamic acid aryl esters able to irreversibly inhibit FAAH activity with good in vitro and in vivo potency has been described by our groups (Kathuria et al. Nat. Med. **2003**, 9, 76). These compounds, whose most potent terms are characterized by a biphenyl moiety, probably mimicking the bound conformation of the FAA fatty acid chain (Tarzia et al., J. Med. Chem. **2003**, 46, 2352), were submitted to QSAR analysis and structural optimization, leading to the 3'-carbamoyl derivative URB597 (X_1=H, X_2=$CONH_2$, IC_{50}=4.6±1.6 nM) as the most potent one in the in vitro tests. The publication of FAAH crystal coordinates by Bracey et al. (Science **2002**, 298, 1793) allowed molecular modelling studies, aimed at reproducing the recognition step between these inhibitors and their putative binding site by docking and molecular dynamics calculations. Inhibitor potency data on more than 70 derivatives, in combination with theoretical investigation, allowed a detailed description and interpretation of structure-activity relationships for this class of compounds. In particular, substitution at the distal ring of the biphenyl moiety gave the most interesting results at the meta position, where a polar interaction with the enzyme cavity was postulated by QSAR analysis, indicating an inverse correlation between potency and lipophilicity, and further supported by modelling investigation, which pointed out possible hydrogen bonds of polar substituents with backbone and side chain polar groups of the enzyme. Interestingly, a 3D-QSAR model, built from IC50 data of inhibitors, showed high similarity with the shape and polarity of an enzyme cavity where the biphenyl moiety may be accommodated by docking. The relation between carbamate group reactivity and potency was also explored by a series of compounds substituted at the ortho and para position of the proximal phenyl ring. While a steric restraint was observed to limit the potency of para-substituted derivatives, electron withdrawing substituents led to less active compounds.



CHARACTERIZATION OF *N*-PALMITOYLETHANOLAMINE-HYDROLYZING ACID AMIDASE AND DEVELOPMENT OF ITS INHIBITORS

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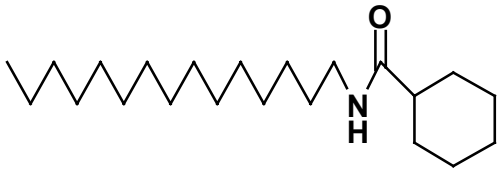
Bioactive *N*-acylethanolamines, including anandamide and *N*-palmitoylethanolamine, are degraded to their corresponding fatty acids and ethanolamine by the catalysis by fatty acid amide hydrolase (FAAH), which exhibits an alkaline pH optimum. In addition, we have found another amidohydrolase catalyzing the same reaction (Ueda *et al.*, J. Biol. Chem. **2001**, 276, 35552). This enzyme, termed "*N*-palmitoylethanolamine-hydrolyzing acid amidase (NPAA)", was characterized by the highest activity with *N*-palmitoylethanolamine among various *N*-acylethanolamines, the pH optimum around 5, and the stimulation by dithiothreitol and Triton X-100. In attempts to find selective inhibitors of NPAA, we synthesized a large number of derivatives of palmitic acid, 1-hexadecanol, and 1-pentadecylamine, and examined their inhibitory effect on rat lung NPAA with *N*-palmitoylethanolamine as substrate. Among the tested compounds, *N*-cyclohexanecarbonylpentadecylamine (1) was the most potent with an IC_{50} of 4.5 μM. In contrast, compound 1 did not inhibit FAAH at concentrations up to 100 μM. The inhibition was reversible and non-competitive. Compound 1 also functioned as an NPAA inhibitor in intact rat alveolar macrophages. Moreover, by the use of the combination of compound 1 and methyl arachidonyl fluorophosphonate (a potent FAAH inhibitor), we could show that rat basophilic leukemia (RBL-1) cells possess not only FAAH but also NPAA. Thus compound 1 may be a useful tool to distinguish NPAA from FAAH in various tissues and cells and to clarify the physiological role of NPAA.



N-Cyclohexanecarbonylpentadecylamine (1)

MOLECULAR CLONING AND CHARACTERIZATION OF *N*-ACYLPHOSPHATIDYLETHANOLAMINE-HYDROLYZING PHOSPHOLIPASE D (NAPE-PLD)

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Previously we reported the partial purification and characterization of a membrane-bound *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) from rat heart (Ueda *et al*. Biochim. Biophys. Acta **2001**, *1532*, 122; Liu *et al*. Chem. Phys. Lipids **2002**, *115*, 77). We further purified the enzyme from rat heart, and using peptide sequences obtained from the purified enzyme, cloned cDNAs of NAPE-PLD from mouse, rat and human. The deduced amino acid sequences from cDNAs of three animal species were composed of 393 or 396 residues. The sequence of NAPE-PLD showed no homology with those of the reported PLD, but was suggested to belong to the zinc metallo-hydrolase family of the ß-lactamase fold. As was overexpressed in COS-7 cells, the recombinant enzyme generated anandamide, *N*-palmitoylethanolamine, *N*-oleoylethanolamine, and *N*-stearoylethanolamine from their corresponding NAPEs with similar Km (2-4 μM) and Vmax (73-101 nmol/min/mg protein), confirming that the enzyme does not exhibit selectivity for *N*-acyl groups of NAPEs. In contrast to typical PLD, the enzyme did not react with phosphatidylcholine or phosphatidylethanolamine, and did not catalyze transphosphatidylation in the presence of ethanol. The enzyme assay, RT-PCR analysis, and Western blotting of various mouse tissues indicated that the enzyme is ubiquitously expressed with higher levels in brain, testis and kidney. These results demonstrate that NAPE-PLD is a novel enzyme, structurally and functionally distinguishable from the known PLD, and emphasize the physiological importance of *N*-acylethanolamines including anandamide.

CHARACTERIZATION OF SN-1-DIACYLGLYCEROL LIPASES AS 2-AG/ENDOCANNABINOID BIOSYNTHESIZING ENZYMES

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A diacylglycerol (DAG) lipase selective for the sn-1 position of diacylglycerol substrates was proposed to synthesize the most abundant endogenous cannabinoid receptor ligand, 2-arachidonoyl-glycerol (2-AG). We have recently reported the cloning and preliminary enzymatic characterization of two specific Ca^{2+}-sensitive sn-1 DAG lipases, DAGLαand β (Bisogno et al., J. Cell. Biol., **2003**). We now present further data on the properties of these enzymes, their regulation and pharmacological inhibition and, most importantly, their crucial role in the biosynthesis of that part of 2-AG that acts as an endocannabinoid. Full length human DAGLα and mouse DAGLβ constructs were expressed in COS-7 cells and the clones with the highest levels of the gene products (clones α12 and β15) were selected for enzymatic characterization. Using sn-1-stearoyl-2-[^{14}C]-arachidonoyl-glycerol as substrate we found that both enzymes are mostly expressed in the 10,000 x g membrane fraction, and exhibit optimal activity at pH=7, with K_m of 154.7±19.1 and 74.1±4.9 μM for the α and β isoform, respectively. Using four different series of radiolabelled diacylglycerols we established enzyme preference for the sn-1 position of DAGs. Substrate specificity was further investigated by using 1-oleoyl-2-acyl-glycerols, which showed that the β isoform prefers linoleic=oleic> arachidonic>stearic acid on the 2 position, whereas the α form worked equally well with all fatty acids. Ser443 and Asp495 on DAGLβ were found to be necessary to enzymatic activity by site-directed mutagenesis. Quaternary structure and regulation by protein kinases were also investigated. Finally, both enzymes were inhibited by RHC80267 and, more importantly, by tetrahydrolipstatin (THL) (IC_{50}=60 and 100nM for the α and β isoform, respectively), a more specific DAG lipase inhibitor that also inhibited Ca^{2+}-dependent, but not basal, 2-AG levels in intact cells.

In agreement with the proposed role of 2-AG and CB_1 receptors in retrograde signalling in depolarisation-induced suppression of excitatory (DSE) or inhibitory (DSI) neurotransmission, THL (500 nM, either in the assay medium or injected in the post-synaptic neuron) was found to inhibit DSI in rat Purkinje's cerebellar neurons, and both DSI and DSE in rat anterior VTA dopaminergic neurons.

In conclusion, we have reported the further characterization of the two cloned 2-AG-biosynthesizing enzymes. Our findings open the way to the development of new therapeutic opportunities for a wide range of disorders, and contribute to clarifying the mechanisms for the regulation of synaptic transmission in the central nervous system.

Acknowledgements: The authors are very grateful to Dr. Bela Szabo, Dr. M. Melis and Prof. G.L. Gessa for the experiments with THL in DSE and DSI.

STUDIES OF THE BIOSYNTHESIS OF *N*-ARACHIDONOYL DOPAMINE (NADA)

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N-Arachidonoyl dopamine (NADA) acts as an endogenous ligand for the cannabinoid CB_1 receptor and capsaicin (TRPV1) receptor (Bisogno et al., **2000**; Huang et al., **2002**) and is concentrated in the striatum, hippocampus and cerebellum of the rat brain (Huang et al., **2002**). Two alternative pathways have been proposed to explain the biosynthesis of NADA: one through the direct condensation of dopamine with arachidonic acid, and the other indirectly first via a putative *N*-arachidonoyltyrosine, which then could be converted to *N*-arachidonoyl-L-DOPA, and finally to NADA (Huang et al., **2001**; **2002**). We addressed these two hypotheses in the following experiments.

By using LC/MS/MS, we first found evidence for the existence in brain of *N*-arachidonoyltyrosine (NATyr). Multiple reaction monitoring of 3 prominent fragments of synthetic NATyr with the same precursor mass and column retention time were found in a partially purified rat brain extract, suggesting that the metabolism of this compound could lead to the formation of NADA. We further observed that lesions of midbrain dopamine cells with 6-hydroxydopamine (6-OHDA) produced an 85% depletion of dopamine along with a drop below our detection limit (<1 fmol) of NADA in the ipsilateral striatum suggesting that NADA biosynthesis in striatum requires dopamine-producing cells. In the second experiment, rats injected (i.p.) with the tyrosine hydroxylase (TH) inhibitor DL-α-methyltyrosine methyl ester HCl (AMPT) showed a time-dependent decrease (>270 min after injection) in both striatal dopamine and NADA. However, the amount of *N*-arachidonoyltyrosine in the striatum also decreased at 270 mins after the AMPT injection. When incubated with TH (16.5 μg) separately in cell-free assays, 40% of 100 μM L-tyrosine was converted to L-DOPA, whereas only 0.04% of 100 μM [$^2H_8$]*N*-arachidonoyltyrosine was converted to [$^2H_8$] *N*-arachidonoyl- L-DOPA. By contrast, incubating brain membranes with arachidonic acid and dopamine yielded NADA. These results suggest that NADA is formed by the condensation of dopamine and arachidonic acid rather by metabolism of NATyr. +

2-ARACHIDONOYLGLYCEROL AS A NOVEL MEDIATOR OF INFLAMMATION

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2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid receptor ligand (Sugiura *et al*., (**1995**) *Biochem. Biophys. Res. Commun*. 215, 89; Mechoulam *et al*., (**1995**) *Biochem. Pharmacol*. 50, 83). Apparently, 2-AG is a noticeable molecule from a variety of viewpoints, yet not much attention has been directed toward 2-AG until recently. We have focused on 2-AG and studied intensively its physiological significance. We obtained evidence that 2-AG is the true natural ligand for the cannabinoid receptors (CB_1 and CB_2) (Sugiura *et al*., (**1999**) *J. Biol. Chem*. 274, 2794; Sugiura *et al*., (**2000**) *J. Biol. Chem*. 275, 605). We have proposed that both CB_1 and CB_2 receptors are primarily 2-AG receptors. As for the physiological significance of these cannabinoid receptors, evidence is gradually accumulating which shows that the CB_1 receptor is involved in the attenuation of synaptic transmission. On the other hand, the physiological roles of the CB_2 receptor still remain rather obscure. Previously, we found that 2-AG induces the activation of p42/44 and p38 MAP kinases and c-Jun N-terminal kinase, enhanced formation of chemokines such as IL-8 and MCP-1 in HL-60 cells and the migration of HL-60 cells differentiated into macrophage-like cells and human monocytes. In the present study, we first examined the effects of 2-AG on human eosinophils. We found that the CB_2 receptor is abundantly expressed in both EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. We also found that 2-AG induces the migration of EoL-1 cells and human peripheral blood eosinophils. The migration induced by 2-AG was abolished by treatment of the cells with either SR144528, a CB_2 receptor antagonist, or pertussis toxin, suggesting that the CB_2 receptor and Gi/o are involved in the 2-AG-induced migration. In contrast to 2-AG, anandamide did not induce the migration. We then examined whether the level of 2-AG is augmented in inflamed tissues. We found that the level of 2-AG in mouse ear was dramatically elevated following treatment with TPA which induces acute inflammation. The level of 2-AG in the ear was also markedly augmented following the challenge with oxazolone in sensitized mice. Noticeably, inflammation induced by TPA or oxazolone was blocked by treatment with SR144528 or Δ^9-THC. These results, together with the results of previous studies, strongly suggest that 2-AG plays essential roles in both acute and allergic inflammation.

Category key words: Endogenous cannabinoids (anandamide or 2-arachidonoylglycerol(2-AG), Endocannabinoids

FUNCTIONAL DISASSOCIATION OF THE CENTRAL AND PERIPHERAL FATTY ACID AMIDE SIGNALING SYSTEMS

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Fatty acid amides (FAAs) constitute a large class of endogenous signaling lipids that modulate several physiological processes, including pain, feeding, blood pressure, sleep, and inflammation. FAAs have been proposed to evoke their behavioral effects through both central and peripheral mechanisms; however, these distinct signaling pathways have remained experimentally challenging to uncouple. By crossing mice that lack FAA hydrolase ($FAAH^{-/-}$ mice) with transgenic mice that express FAAH under the neural specific enolase promoter, we report here a mouse model (FAAH-NS) that possesses wild type levels of FAAs in the nervous system, but significantly elevated concentrations of these lipid transmitters in peripheral tissues. This anatomically restricted biochemical phenotype correlates with a reversion of the reduced pain sensitivity of $FAAH^{-/-}$ mice, consistent with the FAA anandamide producing this effect by acting on cannabinoid receptors in the nervous system. Interestingly, however, FAAH-NS mice still exhibit reduced inflammation similar in magnitude to $FAAH^{-/-}$ mice, indicating that this activity is mediated by peripheral FAAs. These data suggest that the central and peripheral FAA systems regulate discrete behavioral processes and may be targeted for distinct therapeutic gain.

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ANANDAMIDE DEGRADATION IN HEADACHE PATIENTS

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Endocannabinoids are lipid mediators that modulate pain. Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol are the most biologically active endocannabinoids, which bind to both central and peripheral cannabinoid (CB) receptors. The level of AEA in the extracellular space is controlled by cellular uptake via a specific AEA membrane transporter (AMT), followed by intracellular degradation by the enzyme AEA hydrolase (fatty acid amide hydrolase, FAAH). Recently, functional CB receptors, AMT and FAAH have been shown in human platelets (Maccarrone M., Thromb. Haemost, **2003**, 89, 771), which are involved in the control of pain sensation and headaches. Head pain is an almost universal human experience, yet primary headache disorders, such as migraine without aura (MWoA) or episodic tension-type headache (ETTH), can determine a serious life impact when frequent and disabling. We have analysed possible anomalies of the endocannabinoid system in MWoA or ETTH patients, and compared them with healthy controls. In particular, we measured the activity of FAAH, as well as that of AMT and the level of CB receptors, in platelets isolated from our subjects. Sixty-nine headache patients and thirty-six controls were selected.

We show that the endocannabinoid system in human platelets is altered in female but not male migraneurs. Our results suggest that in MWoA women AMT activity and FAAH activity in platelets increase ~2-fold over the healthy controls, whereas CB receptors are not affected. No changes in AMT, FAAH or CB receptors were observed in ETTH women, nor were they observed in MWoA or ETTH male patients. These data are suggestive of a reduced concentration of AEA in the blood of MWoA subjects, which might reduce the pain threshold and possibly explain the prevalence of migraine in women compared to men.

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OLEOYLETHYLAMIDE, AN ANALGESIC FAAH INHIBITOR WHICH MODULATES ENDOGENOUS ANANDAMIDE, OLEOYLETHANOLAMIDE AND 2-ARACHIDONOYLGLYCEROL LEVELS IN THE BRAIN

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Oleoylethylamide (OEt) was synthesized in our laboratory and investigated in *in vitro* and *in vivo* assays. The affinity of OEt for cannabinoid receptors was determined in radioligand binding assays using homogenates of Chinese hamster ovary (CHO) cell membranes selectively expressing either the human CB_1 or the human CB_2 receptor. OEt did not inhibit the specific binding of [^{3}H]-CP55940 (for CHO-hCB_1) or [^{3}H]-WIN55,212-2 (for CHO-hCB_2) and it was characterized as a compound devoid of CB_1 or CB_2 affinity. The inhibitory potency of OEt against fatty acid amide hydrolase (FAAH) was evaluated using rat brain homogenates as source of FAAH and [^{3}H]-anandamide as substrate. In this assay, OEt potently inhibited FAAH with a pI_{50} value of 5.25. The inhibitory potency of OEt was also investigated against *N*-palmitoylethanolamide-hydrolyzing acid amidase (NPAA), another endocannabinoid hydrolyzing enzyme for which palmitoylethanolamide (PEA) is the most active substrate. Inhibition of NPAA was evaluated using solubilized proteins from rat lung homogenates and [^{14}C]-PEA as substrate. In this assay, OEt (at 100 µM) only weakly inhibited NPAA (20 % inhibition). These *in vitro* assays allowed the characterization of OEt as a selective FAAH inhibitor devoid of CB_1 and CB_2 affinity.

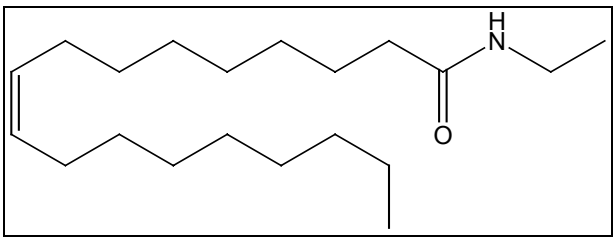

Fig. 1 : Structure of oleoylethylamide

Consistently with the data on the analgesic properties of FAAH inhibitors[a], the antinociceptive effect of OEt was investigated in an acute pain model of noxious heat stimuli applied to the rodent paw. In this model, OEt induced an antinociceptive effect which is reversed by the CB_1 antagonist SR141716A but not by the CB_2 antagonist SR144528. The measurement of the endocannabinoid levels after injection of OEt indicated that OEt elevated the endogenous levels of anandamide (from 4.4 ± 0.8 ng/g wet tissue to 7.4 ± 0.4 ng/g wet tissue), oleoylethanolamide (from 153.6 ± 9.1 ng/g wet tissue to 215.4 ± 12.4 ng/g wet tissue), and 2-arachidonoylglycerol (from 3707.8 ± 84.5 ng/g wet tissue to 4471.6 ± 198.9 ng/g wet tissue) , but did not influence PEA levels (163.1 ± 18.4 ng/g wet tissue vs 162.5 ± 22.6 ng/g wet tissue).

[a] Cravatt B.F. and Lichtman A.H., Fatty acid amide hydrolase : an emerging therapeutic target in the endocannabinoid system. *Curr Opin Chem Biol.*, **7**, 469-475 (**2003**)

PHYSICAL INTERACTIONS OF CB_1 CANNABINIOD AND D2 DOPAMINE RECEPTORS

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The focus of these studies is to develop an understanding of the interrelationship between cannabinergic and dopaminergic signaling in neurons. We have identified a molecular mechanism that may underlie the novel interplay between CB_1 and D2 receptors. Our data suggest that signaling by each receptor is profoundly altered by a regulated interaction between the two receptors.

Evolving evidence strongly supports the concept of G-protein coupled receptor (GPCR) complexes as unique signaling entities. Indeed, homo-oligomeric and hetero-oligomeric GPCR complexes can alter receptor trafficking and may possess unique binding and signaling properties distinct from their constituent components. At this time, the physiological importance of many of these signaling complexes remains vague.

When studied in isolation, the CB_1 and the D2 receptor each couple to pertussis toxin sensitive G-proteins of the $G\alpha_i/G\alpha_o$ family to inhibit adenylyl cyclase. Both receptor types are co-expressed on medium spiny neurons of the striatum and nucleus accumbens. Data obtained from primary cultures of striatal neurons strongly suggest that co-stimulation of CB_1 and D2 receptors activates a signaling cascade quite distinct from that activated by CB_1 or D2 receptors in isolation. This results in a distinct signal cascade which alters the apparent net output of the system (Glass and Felder, **1997**).

To investigate the molecular mechanisms of the receptor interactions we have established a model heterologous expression system in HEK293 cells stably expressing epitope tagged CB_1 and D2 receptors. Utilizing this system, we have determined that the receptors form a physical association consistent with an oligomeric complex under basal conditions. Additionally, we have found that the receptor complex is dynamic and agonist regulated with highest complex levels detected when both receptors are stimulated with sub-saturating concentrations of agonist. Following a similar concentration response, concurrent receptor activation results in an increase in the second messenger cAMP, reversing an initial synergistic inhibition of adenylyl cyclase activity seen at sub-threshold concentrations of cannabinoid agonist. Thus, concurrent receptor stimulation promotes a hetero-oligomeric receptor complex and an apparent shift of CB_1 signaling from inhibition to stimulation of adenylyl cyclase.

Reference: M. Glass and C. C. Felder. J. Neurosci. 17(14):5327-33. **1997**.

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GENERATION OF A N-TERMINAL CB_1-EGFP CHIMERA TO STUDY CANNABINOID RECEPTOR TRAFFICKING

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The majority of the CNS actions of cannabinoids are thought to be mediated by the CB_1 subtype of cannabinoid receptor. However, little is know about the trafficking and regulation of CB_1 receptor expression. The generation of fusion proteins incorporating a fluorescent moiety (eGFP) are powerful tools for studying such issues. Not only does this allow for the direct measurement of receptor translocation in living cells, but it can also be used to study receptor insertion and turnover within the plasma membrane. We have used, therefore, molecular biological approaches to generate a CB_1-eGFP chimera, with eGFP fused to the N-terminal (extracellular) region of the CB_1 receptor. We have expressed this construct in HEK293 and COS7 cells and show that it is functional and undergoes agonist-induced internalization.

The CB_1 receptor cDNA was subcloned into pCDNA-ss-eGFP using PCR. An N-terminal signal sequence (ss) was introduced upstream of the eGFP to facilitate the correct (and efficient) insertion of CB_1-eGFP into the plasma membrane. The cDNA was transfected (by electroporation) into HEK293 and COS7 cells. The CB_1-eGFP is expressed at high levels at the plasma membrane with some intracellular labeling evident. Surface localization of CB_1-eGFP was verified by incubating live cells with an anti-GFP antibody. Cells were then fixed in 4% paraformaldehyde and immunolabelling detected using a Cy3-coupled secondary antibody. Strong surface immunolabelling was associated with the plasma membrane, including filopodial projections.

Treatment of HEK293 cells with WIN55212-2 (1 μM) or HU210 (10-100 nM) for 1-12 hours (37^oC) resulted in a reduction in cell surface CB_1-eGFP receptor expression (~60%; measured using the anti-GFP antibody). In live imaging experiments carried out on a Zeiss confocal microscope (LSM510), treatment with cannabinoids (30-60 min; room temp) resulted in a striking loss of membrane/filopodial fluorescence and the accumulation of CB_1-eGFP within intracellular vesicles. The effects of WIN55212-2 and HU210 on agonist-induced CB_1 trafficking were prevented in the presence of the cannabinoid receptor antagonists AM251 (1 μM) or SR141716A (1 μM).

In conclusion, this CB_1-eGFP construct may provide a useful tool for the study of CB_1 receptor trafficking in living cells. It appears to function as the wild type receptor with respect to agonist-induced internalization, as reported previously (Hsieh *et al*., **1999** J. Neurochem 73, 493-501; Keren & Sarne, **2003** Brain research 980, 197-205).

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CRIP1a AND CRIP1b: NOVEL CB_1 CANNABINOID RECEPTOR INTERACTING PROTEINS

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The C-terminal tail of G protein-coupled receptors (GPCRs) has been shown to be an important region for interaction with other intracellular components and may explain the functional specificity of GPCRs. The C-terminal tail of the CB_1 cannabinoid receptor is known to be involved in selectivity of G-protein binding, tonic activity, desensitization and internalization; however, proteins interacting with CB_1 have not been investigated in detail.

Using a yeast two-hybrid assay, we have discovered a human brain cDNA encoding a 128aa Cannabinoid Receptor Interacting Protein (CRIP) that binds with the C-terminal tail of CB_1 (aa 418-472). Subsequently, we cloned a rat brain cDNA encoding a related 164aa protein, which is also found in humans. Analysis of human genome sequence data indicates that the 128aa and 164aa proteins are encoded by a gene comprising four exons (1, 2, 3a, 3b) that is alternatively spliced to generate cDNAs that encode the 164aa (1-2-3a) or the 128aa (1-2-3b) proteins. Moreover, comparative analysis of the chimpanzee, mouse and rat genomes reveals that while exons 1, 2 and 3a occur in all three species, exon 3b is only found in chimpanzee and humans. Based on these findings, we now refer to the 164aa protein as CRIP1a and the 128aa protein as CRIP1b (formerly known as CB_1-IP1). Orthologues of CRIP1a are also present in chick and puffer fish. Therefore, CRIP1a probably occurs throughout the vertebrates; whereas, CRIP1b is unique to a subset of mammals including humans and chimpanzees.

To confirm that CRIP1a and CRIP1b interact with CB_1, bacterially expressed CRIPs fused to an S-tag were pulled-down by the C-terminal tail of CB_1 fused to GST, as detected by an S-tag probe. Co-immunoprecipitation experiments utilizing epitope tags are in progress to determine whether CRIP1a and CRIP1b interact with CB_1 receptors in cultured mammalian cells. RT-PCR was used to identify CRIP1a and CRIP1b in various cell lines and primary neuronal cultures. CRIP1a, but not CRIP1b, has been detected in HEK 293, AtT20, N18TG2, rat superior cervical ganglion neurons and rat cerebellar granule neurons. Western blot analysis will be used to verify the presence or absence of CRIP1a and CRIP1b proteins in these cells. Based on the known functions of the CB_1 C-terminal tail, we hypothesize that CRIP1a and CRIP1b may play a role in G-protein binding, CB_1 tonic activity, ion channel modulation or receptor trafficking to presynaptic sites.

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LOCALIZATION OF THE CB_1 CANNABINOID RECEPTOR INTERACTING PROTEIN CRIP1a IN THE BRAIN

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CRIP1a is a novel 164 amino-acid protein that interacts with the CB_1 cannabinoid receptor in vitro by binding to its C-terminal tail, as reported by Niehaus et al. (ICRS, **2004**). To investigate interaction of CRIP1a with CB_1 in vivo, we have produced rabbit antisera to a peptide comprising the C-terminal 17 amino-acid sequence of mouse/rat CRIP1a. Dot-blot analysis of sera revealed the presence of antibodies to the CRIP1a antigen peptide. Moreover, Western blot analysis of a bacterial lysate containing recombinant CRIP1a revealed the presence of a single ~ 18 kDa immunoreactive band, consistent with the expected molecular mass for CRIP1a. Western blot analysis of mouse brain homogenates with CRIP1a antisera also revealed the presence of a single ~ 18 kDa immunoreactive band, indicating that we have succeeded in producing antibodies that specifically recognise CRIP1a.

Immunocytochemical analysis revealed CRIP1a-immunoreactivity in many regions of the mouse and rat brain, including the olfactory bulbs, cortex, hippocampus, striatum, thalamus, cerebellum and brain stem. In some brain regions, CRIP1a was clearly co-localized with CB_1 (e.g. in parallel fibres of cerebellar granule cells), demonstrating that interaction between CRIP1a and CB_1 could occur in vivo. Intense CRIP1a-immunoreactivity was evident in the somata of cortical neurons in layers 5 and 6. Pyramidal cells in these layers project to subcortical regions, including the striatum, thalamus and brain stem and therefore CRIP1a-immunoreactivity in these regions may be associated with the axons of layer 5/6 cortical pyramidal cells. Intense CRIP1a-immunoreactivity was also present in the cell bodies of hippocampal CA3 pyramidal cells and in the stratum radiatum of the CA1 region where the axons of CA3 neurons terminate. Interestingly, low level CB_1 mRNA expression also occurs in hippocampal CA3 pyramidal cells (Matsuda et al., **1993**; J. Comp. Neurol. 327, 535-550) but little or no CB_1 protein is detected in the axons of these neurons. Conversely, CRIP1a-immunoreactivity was not evident in neurons in which the highest levels of CB_1 expression have been reported, which include striatal GABAergic projection neurons and local GABAergic interneurons in the hippocampus, cerebellar cortex and neocortex (Egertová & Elphick, **2000**, J. Comp. Neurol. 422, 159-171).

Collectively, our data indicate that CRIP1a may interact with CB_1 in some, but not all, CB_1-expressing neurons in the brain. Furthermore, our data lead us to speculate that the relative abundance of CRIP1a and CB_1 in neurons may influence the number of CB_1 receptors targeted to axons and CRIP1a may regulate axonal targeting of CB_1 receptors.

CB_2 RECEPTOR: PROTEIN NMR FROM BUILDING BLOCKS TO 3D STRUCTURE

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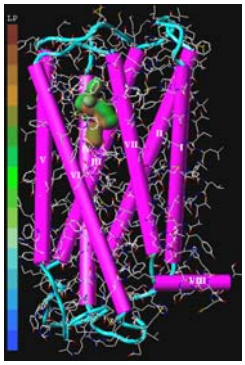
Lack of 3D GPCR structures and drug binding information is still one of the key roadblocks in novel GPCRs drug research today. Due to their intrinsic transmembrane property, GPCR proteins are difficult to be crystallized, or to be functionally expressed in other media for structural studies. We are developing a recombinant GPCR protein-engineering/isotope-editing NMR structural proteomics approach, i.e., "from peptide building blocks to a 3D structure" by targeting a cannabinoid receptor (CB_2) receptor. CB_2 receptor was selected because it is expressed in high quantities in human spleens and tonsils, is involved in the signal transduction processes in immune systems, and can potentially be a drug target for immuno-treatments. CB_2 receptor, a 39.7 kDa of large membrane protein, was engineered into transmembrane peptide fragments or helix bundles in $^{15}N/^{13}C$ isotope-enriched M9 media. The target protein fragments (e.g, $CB_{2180\text{-}233}$) were cloned and over-expressed in a preparative scale as a fusion protein with a modified TrpΔLE leading sequence and a nine-histidine tag at the N-terminal. A purification protocol was also developed using affinity column, Factor Xa or CNBr cleavages and reverse phase HPLC. The process of cleavage and purification was monitored by SDS-PAGE, HPLC, and MS. The prepared proteins were reconstituted into helical structures in solution and confirmed by CD spectrometer. The developed and optimized preparation scheme provided a sufficient amount of protein samples for 2D/3D NMR structural analysis and other biophysical studies. The NMR-determined helix structures were then incorporated into the homology-constructed CB_2 model to refine the computational 3D CB_2 structure in aide of receptor-based drug design. The present study will contribute towards cannabinoid drug research and NMR structural biology, as well as GPCRs in general



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BETA-ARRESTIN 2 AFFECTS CANNABINOID SENSITIVITY TO TETRAHYDROCANNABINOL

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Cannabinoid drugs exert their effects on the central nervous system through activation of G-protein coupled receptors (i.e. CB_1). The regulation of G-protein coupled receptors is thought to be due, at least in part, to proteins known as beta-arrestin 1 and/or 2. Beta-arrestin 2, in particular, has been implicated in desensitization of CB_1 receptors in cell culture models (W Jin, S Brown, JP Roche, C Hsieh, JP Celver, A Kovoor, C Chavkin and K Mackie, **1999**; J Neurosci 19: 3773-80). Mouse brain naturally expresses both CB_1 receptors and beta-arrestin 2, making it a suitable tissue to study these interactions. Previous studies have shown that transgenic mice lacking beta-arrestin 2 are more sensitive to morphine (LM Bohn, RR Gainetdinov, FT Lin, RJ Lefkowitz and MG Caron, **2000**, Nature 408, 720). The current study examines the effects of beta-arrestin 2 on cannabinoid receptor sensitivity by comparing the behavioral and biochemical response of transgenic mice lacking beta-arrestin 2 (Barr2-/-) and wild type (w.t.) mice to various cannabinoid compounds. For the behavioral assay, warm water tail immersion analgesia tests and change in rectal temperature measurements were performed. The two groups showed no difference in response to CP55940. In contrast, the Barr2-/- mice showed greater and prolonged responses to Δ^9-tetrahydrocannabinol (THC). For THC, statistically significant differences in percent maximum possible effect (%MPE) for the tail flick assay ($p = 0.0140$) and in rectal temperature change ($p = 0.0030$) were observed between groups. Since CP55940 has been shown to be a full agonist and THC a weak partial agonist, two other partial agonists, JWH-073 and O-1812, and one full agonist, methanandamide, were assayed. The results showed no significant difference between Barr2-/- and w.t. mice in either %MPE or change in rectal temperature for any of these ligands. At the biochemical level, receptor binding and G-protein activation were analyzed in cerebellar and hippocampal membranes from each group. [^{3}H]SR141716A binding showed a B_{max} of 1.6 pmol/mg in Barr2-/- mice, and 1.5 pmol/mg in w.t. mice, indicating no significant difference in receptor levels. Also, no difference was observed between the groups in [^{35}S]GTPγS binding for any ligand including THC. It was noteworthy that though JWH-073 and O-1812 exhibited very similar potency and efficacy to THC for G-protein activation, the two genotypes of mice showed a difference in responses only to THC and only in the behavioral assays. Ongoing studies will examine the potential effects of deleting beta-arrestin 2 on CB_1 localization and MAPK activation.

CANNABINOID RECEPTOR SIGNALING IN RAS-GRF1 KNOCK OUT MICE

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One of the major challenges in drug addiction research is to understand the cellular and molecular mechanisms associated with the behavioural manifestation of drug addiction. Surprisingly, many of the mechanisms implicated in such adaptations have been found similar to those involved in learning and long-term memory formation. Ras/ERK signaling seems to play a crucial role in these neuroadaptive processes and in long-term neuronal plasticity, thus the ability of cannabinoids to stimulate Ras/ERK cascade could play a pivotal role in triggering long-lasting neuronal adaptations underlying the compulsive drug use and its remarkable persistence.

The aim of the present work is to study the role of Ras/ERK pathway in cannabinoid dependence. We will take advantage of Ras-GRF1 mutant mice that are lacking of the GRF1 exchange factor that represents the molecular switch through which the G protein-coupled receptors (such as the cannabinoid receptor) can activate the Ras signaling pathway ending in ERK activation.

Since we have recently shown that *in vivo* acute injection of THC in rats increased ERK activation in the striatum and cerebellum, we first checked the ability of in vivo THC to induce higher pERK levels in these areas of wild type and knockout mice. As expected, ERK activation was present in wild type mice but it lacked in Ras/GRF1 deficient mice. When THC (10 mg/kg sc) was acutely injected, both wild type and knockout animals presented a significant reduction in spontaneous locomotor activity and showed a significant analgesia, suggesting that Ras signalling pathway is not necessary for triggering the acute effect of THC. However, after chronic THC administration (10 mg/kg, twice a day, sc for five days), wild type mice developed tolerance to these effects, wheras in KO mice a significant hypomotility and analgesia were still present. Moreover cannabinoid abstinence syndrome produced by injecting the cannabinoid antagonist SR141716 (5 mg/kg ip) in THC chronically treated mice was reduced in Ras/GRF1 deficient animals vs wild type ones. In order to elucidate the molecular underpinnings of the behavioural diversity between KO and wild type mice, we performed autoradiographic binding studies to check the level of CB_1 receptors in THC chronically treated mutant mice and littermates. As expected, wild type brains exhibited significant CB_1 receptor downregulation, whereas KO mice did not. Moreover, GTPγS binding assays revealed a different picture between the two groups not only after the stimulation with the cannabinoid agonist CP-55,940 but also in basal conditions. These results seem to indicate that phosphorylation events mediated by ERK could play a role in modulating CB_1 receptor functionality. To further address this item we investigated on the possible cytoplasmatic and nuclear targets of ERK phosphorylation and the presence of alterations in GRK and β-arrestin for cytoplasmatic events and transcription factors (pCREB, c-Fos and Fos B) for the nuclear ones was evaluated.

Concluding our results indicate that the phosphorylation events triggered by ERK could play a pivotal role in the induction of synaptic plasticity due to cannabinoid chronic exposure.

Δ^9-TETRAHYDROCANNABINOL (Δ^9-THC) ELICITS A STORE-INDEPENDENT CALCIUM ELEVATION IN T CELLS

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Cannabinoid compounds are widely known to alter leukocyte function. Previous studies from this laboratory have shown T cells to be a sensitive target to cannabinoid treatment. The objective of the present studies was to examine the effect of Δ^9-THC, the primary psychoactive constituent in *Cannabis sativa*, on the regulation of intracellular calcium ($[Ca^{2+}]_i$) in HPB-ALL cells, a human CB_2 expressing T cell line. Δ^9-THC (10-12.5 μM) induced a robust rise in $[Ca^{2+}]_i$ in resting HPB-ALL cells (600-800 nM), which was antagonized by the pretreatment with the CB_2 receptor antagonist, SR144528. Studies performed with the intracellular calcium chelator, BAPTA, or in the absence of extracellular calcium revealed that the Δ^9-THC-mediated elevation in $[Ca^{2+}]_i$ was due to influx of extracellular calcium. Furthermore, pretreatment with thapsigargin to deplete intracellular calcium pools did not abrogate the Δ^9-THC-mediated elevation in $[Ca^{2+}]_i$, indicating that Δ^9-THC-mediated rise in $[Ca^{2+}]_i$ was independent of intracellular calcium store-depletion. However, SKF96365 and 2-aminoethoxydiphenyl borate but not $LaCl_3$, compounds previously described as calcium release activated calcium (CRAC) channel blockers, attenuated the Δ^9-THC-mediated elevation in $[Ca^{2+}]_i$. Together these data suggest that Δ^9-THC elicits an elevation in $[Ca^{2+}]_i$ in resting T cells in a CB_2 receptor-dependent manner, which is independent of store-depletion and involves an influx of extracellular calcium putatively through receptor-operated calcium (ROC) channels.

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CAPSAICIN EXHIBITS NEUROPROTECTIVE EFFECTS VIA VANILLOID RECEPTOR TYPE 1 (VR_1) ACTIVATION IN A MODEL OF TRANSIENT GLOBAL CEREBRAL ISCHEMIA IN MONGOLIAN GERBILS

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VR_1 receptor might have a role as a modulator of nerve terminal excitability in the CNS. Pathological changes in brain temperature or pH, for example after a severe stroke, may influence VR_1 activity (Marinelli et al., J. Physiol. **2002**, 543, 531), suggesting an important role in events occurring after ischemia such as modulation of glutamate release and consequent activation of excitatory aminoacids receptors. Recently, either capsaicin or capsazepine have been shown to lead to neuroprotective effects against ouabain-induced *in vivo* excitotoxicity (Veldhuis et al., J. Neurosci., **2003**, 23, 4127).

The aim of the present work was to investigate the effect of post-ischemic treatment with capsaicin on transient global cerebral ischemia in gerbils using a range of doses (0.01 - 0.6 mg/kg) reported to be devoid of toxicity *in vivo* (Di Marzo et al., FEBS Lett. **1998**, 436,449).

Starting from 30 min to 7 days after recirculation, gerbils, previously submitted to bilateral carotid occlusion, were monitored for different parameters, known to be hardly influenced by cerebral ischemia: electroencephalographic (EEG) mean total spectral power, spontaneous motor activity, cognitive function, rectal temperature and hippocampal neuronal count. Capsaicin was given 5 min after recirculation. VR_1 activation was investigated by means of s.c. pre-treatment with the selective antagonist capsazepine (0.01 mg/kg), given 15 min before ischemia.

Starting from 0.05 to 0.6 mg/kg of capsaicin, a dose-dependent recovery of mean total spectral power against ischemia-induced EEG flattening, was obtained. Within the same range of doses, capsaicin antagonized ischemia-induced hyperlocomotion. A recovery of ischemia-induced memory impairment with 0.05 - 0.2 mg/kg, was shown. Capsaicin (0.01-0.2 mg/kg) produced a dose-dependent decrease of body temperature A complete recovery from neuronal death in the CA1 hippocampal field, after treatment with the maximal neuroprotective dose of capsaicin (0.2 mg/kg), was observed. Pre-treatment with capsazepine blocked all capsaicin-induced antischemic effects.

In summary, the present findings demonstrate that capsaicin exhibits antischemic properties via VR_1 receptor interaction.

EFFICACY OF CANNABIDIOL IN A RAT MODEL OF NEUROPATHIC PAIN: EVIDENCE FOR VR1-MEDIATED ANTI-HYPERALGESIC EFFECT

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The anti-hyperalgesic and anti-allodynic properties of THC and synthetic cannabinoids in animal models of neuropathic pain are already established. Despite their efficacy, the clinical employment of these cannabinoids is limited by the difficulties in separating the psychotropic effects from therapeutic ones. On the contrary, cannabidiol (CBD) is devoid of psychoactive effects, because it has very low affinity for either cannabinoid receptor subtypes CB_1 and CB_2. CBD displays a wide range of pharmacological activities, but little is known about its molecular mechanism(s). In this research we report that the repeated oral administration of CBD in rats with chronic constriction injury of the sciatic nerve (CCI model of neuropathic pain), starting from day 7 after the injury, evoked a dose-related relief of both thermal hyperalgesia (assessed by plantar test) and mechanical allodynia (assessed by Randall-Selitto test). We also evaluated whether this CBD-induced effect was accompanied with alteration in the production of some mediators known to be increased and involved in the development and maintenance of neuropathic pain. CBD did not affect the enhanced plasma concentration of prostaglandinE2 (PGE2), while it was able to inhibit both the overproduction of nitric oxide (NO) and the lipoperoxidation, that can occur as the result of interaction between NO and other reactive oxygen species. Furthermore, the enhanced production of NO found in CCI animals was due to an increased expression of neuronal isoform of nitric oxide synthase (nNOS) in sciatic nerve which was reversed after CBD repeated treatment. Like PGE2 and NO, proinflammatory cytokines are involved in the generation of pain; among these, tumor necrosis factor alpha (TNFα) plays a pivotal role, not only in the development of neurogenic inflammation, but also in the demyelination of the sciatic nerve. As expected enhanced level of TNFα was found in the spinal cord of CCI animals; repeated treatment with CBD did not affect this increase. All these findings seem indicate CBD as an anti-hyperalgesic compound unable to affect some of the mediators sustaining the pathology. The main objective was then to study a possible mechanism of action of CBD. Considering that it was a very weak ligand for both cannabinoid receptor subtypes, our attention focused on vanilloid receptor type 1 (VR1), for which CBD is a full agonist. VR1 receptor is a non-selective cation channel mostly expressed in sensory neurons and acts as ligand-, proton- and heat- activated molecular integrator of nociceptive stimuli. It has been reported that VR1 receptor sensitivity and expression are increased during neuropathic pain. However, activation of VR1 by agonists, such as capsaicin, is almost immediately followed by desensitisation, so that VR1 agonists have been proposed as analgesic compounds. The injection of the selective VR1 receptor antagonist, capsazepine (10 mg/kg i.p.) simultaneously to the last CBD administration to CCI rats completely reversed the anti-hyperalgesic and anti-allodynic efficacy of CBD. Our findings for the first time highlight the vanilloid system as the molecular target for CBD action and provide evidence that vanilloid receptor contributes to analgesic properties of CBD.

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INTRACELLULAR ANANDAMIDE MEDIATES STORE-OPERATED CALCIUM ENTRY BY ACTING AT TRPV1 CHANNELS IN NEURONS

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Calcium signalling is fundamental to life and involved in the control of a vast array of cellular functions. Intracellular Ca^{2+} levels are kept at very low concentrations (~100 nM), and Ca^{2+} required for cellular processes is stored in specialized compartments of the ER. To maintain cell viability and function, depleted Ca^{2+}-stores signal to store-operated channels in the plasma membrane to gate extracellular Ca^{2+}, however, the molecular mechanism of this process is unresolved. Cation channels of the TRP family, including TRPV1, are candidates to be store-operated channels. Anandamide activates TRPV1 at an intracellular site. However, the physiological relevance of this interaction is unclear, because no cellular processes are known in which endogenous anandamide activates TRPV1. Therefore, we hypothesized that anandamide may function as store-operated intracellular messenger signalling to TRPV1 to gate extracellular Ca^{2+}.

Here, we show that mobilisation of intracellular Ca^{2+} by thapsigargin, muscarinic and P_2Y purinergic receptor stimulation leads to formation of intracellular anandamide and subsequent TRPV1 activation in HEK293 cells and sensory neurons of rat dorsal root ganglion. Anandamide metabolism and efflux from the cell tonically limit TRPV1 activation. We suggest that TRPV1 acts as a molecular sensor for the intracellular milieu that detects depletion of Ca^{2+}-stores via intracellular anandamide, and responds by gating extracellular Ca^{2+}. Thus, endogenous anandamide produced via the PLC/IP_3-pathway acts as a diffusible intracellular messenger, which mediates store-operated Ca^{2+} entry in cells expressing TRPV1 receptors. Our data represent the first example of how endogenous anandamide can act at TRPV1 channels also under physiological conditions to control a basic cellular function. Furthermore, our results set the example to search for other endogenous ligands for other TRP channels, which may contribute to store-operated Ca^{2+}-entry.

NUMBERS WITH WINGS: THE CALCULUS OF CB RECEPTOR EVOLUTION

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The endocannabinoid system is a genetic module consisting of receptors, ligands, and ligand-metabolizing enzymes. Genes have been cloned and sequenced for the receptors (CB_1, CB_2, VR1), ligand-catabolizing enzymes (FAAH, MGL, COX2), and ligand-synthesizing enzymes (NAPE-PLD which cleaves anandamide from NAPE, and DAGLα and DAGLβ which cleave 2-AG from diacylglycerol).

We searched for homologous genes (orthologs) of these nine sequences in eight organisms whose entire genomes have been sequenced. Orthologs make it possible to reconstruct evolutionary history; combining genomic and evolutionary studies has created "phylogenomics". Genome sequences were obtained from GenBank (www.ncbi.nlm.nih.gov); BLAST 2.0 served as a sequence alignment search engine. Phylogenomic gene trees were constructed using two algorithms, sequence agreement (percentage identity) or sequence divergence (neighbor-joining method, ClustalX); the gene trees provided "molecular clocks" for the timing of evolutionary events. We validated our molecular methods with corroborative pharmacological methods (functional mapping of sequences, using point mutation studies that identified amino acid motifs that impart enzyme or receptor specificity) and chemotaxonomic methods (meta-analysis of ligand extraction studies).

Our results re CB_1-CB_2 divergence are currently uncertain because of conflicting molecular evidence – either prior to 590 million years ago (MYA) based on the leech CB_1 sequence (Stefano et al., **1997**) or prior to 400 MYA based on fugu fish sequences (Yamaguchi et al., **1996**; Elphick, **2003**). We propose that VR1 may predate CB receptors but its affinity for endocannabinoids is a recent acquisition, appearing after vertebrate divergence. Among the catabolic enzymes, MGL appears to be ancient (with orthologs in bacteria), COX2 evolved with the deuterostomes, and FAAH appeared with the vertebrates. The ligand-synthesizing enzymes also evolved separately, NAPE-PLD may be as old as MGL, DAGLα appeared during animal evolution, and DAGLβ diverged recently, with vertebrates.

Ligands and receptors often coevolve together, in order to maintain their structural and energetic interactions (Park et al., **2002**). We aligned gene trees to search for phylogenomic evidence of coevolution between CB receptors and ligand-metabolizing enzymes. Gene trees can also be used to measure the rate of gene evolution. Our caculations indicate the human CB_1 gene is undergoing a rapid rate of evolution, an indicator of positive selection.

OCCURRENCE AND BIOLOGICAL ROLE OF THE ENDOCANNABINOID SYSTEM IN *CIONA INTESTINALIS*

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Recently an orthologue of the cannabinoid receptors (CiCBR) has been identified in the genome of *Ciona intestinalis* (Elphick et al., Gene, **2003**, 302, 95). We have been interested in establishing the presence of a complete endocannabinoid system (receptors, endogenous ligands and enzymes for their biosynthesis and degradation) in this organism in order to gain further information on the evolution of the endocannabinoid system in chordates.

First, we have investigated CiCBR structure and tissue distribution by using RT-PCR and sequence analysis. After densitometric analysis of the CiCBR transcript bands, normalized to the respective bands of the housekeeping calmodulin transcript, CiCBR was found to be mostly expressed in the branchial pharynx, heart and testis. Since CiCBR contains the amino acids necessary for its activation by the cannabinoid receptor agonist HU-210, we investigated the effect of HU-210 on a typical behavioural response in *Ciona*, i.e. mouth reopening after physical stimulation-induced mouth closure. HU-210 time-dependently and dose-dependently mouth re-opening by an unknown mechanism that was maximal with a bolus of 300 nmol and after 2h30 from administration, and was significantly attenuated by antagonists of CB_1 and CB_2 receptors.

We looked at the presence and tissue distribution of endocannabinoids in *C. intestinalis* by isotope-dilution liquid-chromatography mass spectrometry analyses. Anandamide was found with the highest concentrations in the brain and in the heart, at intermediate concentrations in the branchial pharynx, testis, and intestine, and in lowest levels in the stomach and ovaries. 2-AG was found with the highest levels in stomach and intestine, in intermediate levels in the brain and in the branchial pharynx and to a lower extent in the heart and ovaries. However 2-AG was 5-fold more abundant than anandamide in the brain and in all the others tissues investigated. The endocannabinoid related fatty acid-derived endogenous compound, *N*-palmitoyl-ethanolamine, was also detected in all *Ciona* tissues analysed.

Finally, we realized a phylogenomic analysis by blasting the genome of *Ciona* and have identified a FAAH orthologue gene (CiFAAH). The alignment of the rat FAAH protein with this orthologue FAAH shows a high degree of homology (40%) using the BLAST or LALIGN programs. We investigated CiFAAH structure and tissue distribution by using RT-PCR and sequence analysis as well as enzymatic activity assays. We found that CiFAAH contains all the amino acids necessary to functional FAAH enzyme as well as the amidase sequence signature, and exhibits similar pH-dependency, cellular and tissue distribution as mammalian FAAH's.

We are now investigating the presence of other endocannabinoid anabolic and catabolic enzymes cloned so far in mammals, in order to assess if a complete endocannabinoid system is present in *Ciona intestinalis.*

ENDOCANNABINOIDS LINK FEEDING STATE AND AUDITORY PERCEPTION-RELATED GENE EXPRESSION

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Singing by adult male zebra finches is a learned behavior important for courtship and territory maintenance and is inhibited by both brief periods of limited food availability and systemic injection of cannabinoids. These similar effects on singing, combined with increasing evidence for endocannabinoid involvement in feeding behavior, led us to evaluate a possible shared mechanism. We have found that limited food availability increases levels of the endocannabinoid 2-arachidonoylglycerol within zebra finch caudal telencephalon, a region that contains auditory telencephalon including L2 subfield of L (L2) and caudal medial nidopalluim (NCM). Development and use of an anti-zebra finch CB_1 antibody demonstrates dense cannabinoid receptor expression within L2. NCM receives L2 projections and is implicated in integration of auditory information. Activity in this area, determined through expression of the transcription factor ZENK, is increased upon exposure to unfamiliar song. Because these novel song-stimulated increases in NCM activity are mitigated by cannabinoid exposure, we tested and found that similar effects on ZENK expression are produced by limiting food. Limited food-related reductions in activity of NCM neurons were reversed by the cannabinoid antagonist SR141716A, implicating CB_1 cannabinoid receptor involvement. Taken together these experiments indicate a link between feeding state and gene expression related to auditory perception that is mediated by endocannabinoid signaling

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FURTHER EVIDENCE FOR A ROLE OF THE ENDOCANNABINOID SYSTEM IN EXTINCTION PROCESSES

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Recent reports from our lab and others suggest that the endocannabinoid system may be tonically active in extinction processes. Specifically, mice treated repeatedly with the CB_1 antagonist SR 141716 or CB_1 knockout mice display deficits in extinguishing certain learned behaviors, such as the spatial bias in the Morris water maze (Varvel et al. **in press**) and a conditioned freezing response (Marsicano et al. **2002**). We have recently replicated this effect of SR in a passive-avoidance model. Using standard procedures, C57bl/6 mice were trained to not enter a dark chamber which had been associated with three successive .3 mAMP shocks delivered through the floor grid the first time they entered the chamber. On subsequent days, mice were pretreated with veh or one of several doses of SR and placed in the start box and the latency to enter the dark chamber was recorded. Once each mouse entered the dark chamber (if it failed to enter within 240 seconds it was manually placed there) it was allowed to remain for two minutes with no shock administered. Over successive sessions, the conditioned aversion extinguished and mice again freely entered the dark chamber. A significant statistical interaction demonstrated that mice treated with SR extinguished the avoidance response more slowly than did vehicle mice, $F(27, 239) = 2.1$, $p < 0.01$, and latencies were higher in the 3 mg/kg SR when compared to vehicle on extinction days 8, 9, and 10. The corollary of the hypothesis that blocking the endocannabinoid system should attenuate extinction is that enhancing this endogenous system should actually facilitate extinction. We can now positively report this phenomenon in FAAH knockout mice and OL-135, a reversible FAAH inhibitor. Standard procedures were used to train mice to locate a hidden platform in a Morris water maze (four daily 120 second trials, platform remained in a constant position). No significant differences were found between FAAH knockouts and wildtypes, $F(2,271) = 0.26$, $p = 0.77$, or between vehicle or 30 mg/kg OL-135 treated mice, $F(1,127) = 0.63$, $p = 0.44$, on the acquisition of this task. However, when the platform was removed and mice were given weekly extinction trials, both FAAH knockouts, and OL-135 treated mice, extinguished more quickly than their respective controls. These results support the hypothesis that the endocannabinoid system may be tonically involved in extinction processes, and raise the possibility that enhancement of the endocannabinoid system via FAAH inhibition may represent a novel approach to the treatment of a wide range of behavioral disorders in which facilitation of extinction would be beneficial.

ALTERED PHOSPHORYLATION LEVELS IN THE BRAIN OF CANNABINOID RECEPTOR-1 DEFICIENT MICE AFTER EXTINCTION TRAINING

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Cannabinoid receptors type 1 (CB_1) and the endogenous cannabinoid system play a central role in the extinction of fear behaviour as assessed in fear conditioning experiments, using freezing as indicator of fear (Marsicano et al., Nature 418:530, **2002**). However, the molecular mechanisms underlying this function are still unknown. Contrary to wild-type littermates (WT), CB_1-KO mice do not decrease freezing during acute presentation of a tone that was previously paired with a footshock (short-term extinction). This acute impairment is reflected also in following exposures to the tone (long-term extinction). Therefore, it is conceivable to hypothesise that the acute activation of the endogenous cannabinoid system during tone presentation triggers intracellular cascades, which eventually lead to the long-term changes necessary for the maintenance of extinction behaviour in following extinction trials (extinction memory).

Whereas a lot is known concerning the molecular pathways involved in acquisition and consolidation of fear memory, only limited information is available about intracellular mechanisms mediating the long-term maintenance of extinction behaviour. Pharmacological and biochemical studies indicated extracelluar regulated kinases (ERKs), PI-3 kinase pathway and the phosphatase calcineurin as potential candidates for this role. Considering that CB_1 is able to regulate some of these intracellular pathways, we asked whether these mechanisms are altered in CB_1-KO mice after an acute extinction trial.

WT and CB_1-KO mice were conditioned (a 20-sec tone co-terminating with a 2-sec footshock at 0.7 mA) and the day after they were subjected to an extinction trial consisting in a 3-min tone presentation (extinction group). Control animals were treated in the same way, except that they did not receive any tone during the trial (no extinction group). 30 minutes after the trial, the animals were sacrificed and different brain regions (known to be involved in extinction of fear behaviour) were dissected and processed for immunoblotting. Phosphorylation levels of ERKs and AKT, and protein levels of calcineurin were measured by densitometric analysis and the effect of the tone presentation on these levels were evaluated in WT and CB_1-KO mice.

The results show that tone presentation induce significant differences in the activation of intracellular pathways in WT as compared to CB_1-KO mice in different brain regions, including basolateral amygdala, central amygdaloid nucleus, medial prefrontal cortex and hippocampus.

Therefore, the tight regulation of intracellular signalling pathways might represent one of the mechanisms through which the endogenous cannabinoid system controls extinction of fear behaviour.

EFFECTS OF Δ^9-THC AND CBD-RICH CANNABIS EXTRACTS ON LATENT LEARNING IN RATS

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Cannabinoid CB_1 receptors are present in memory-mediating brain areas and the endogenous cannabinoid system plays a modulatory role in learning and memory formation, especially working and short-term memory. Among others, administration of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main chemical constituent of cannabis extract, leads to delay-dependent performance deficits in working memory tasks in rats and mice. Unexplored, to date, is whether plant extracts rich in Δ^9-THC or other cannabinoids, such as cannabidiol (CBD), have similar consequences on memory formation. In using a delayed-matching-to-position (DMTP) procedure, we assessed effects of both compounds on spatial working/short-term formation in rats and furthermore established dose-response relationships.

Ten young adult hooded Lister rats (300g) were trained in a DMTP paradigm in the open-field water maze (1.5m in diameter). The platform was submerged and remained at the same place within each daily session, but was moved to a different place between sessions. In the first trial, rats were placed onto the platform for 120s and a memory trial (trial 2) was given after a delay of either 30s or 4 h followed by a further two trials in quick succession. After initial training, animals were repeatedly tested in this latent learning protocol and exposed to the different extracts and vehicle.

Δ^9-THC-rich extracts impaired performance in the memory trial (trial 2) of the DMTP task in a dose-dependent but delay-independent manner. Deficits appeared at doses of 2 or 5mg/kg (i.p.) at both 30s and 4h delays and were similar in severity relative to pure Δ^9-THC. By contrast, CBD-rich extracts had no effect on working/short-term memory, even at doses of up to 50mg/kg. When given concomitantly, CBD-rich extracts had no effect or enhanced the Δ^9-THC-rich extract-induced deficit (2mg/kg Δ^9-THC + 10mg/kg CBD), but showed no signs of reversal. Effects were not due to non-specific sensorimotor side effects of the drugs and catalepsy was mild and of no consequence on the ability to swim.

Our data confirm previous suggestions that Δ^9-THC, either pure or extracted from cannabis plants, compromises working/short-term memory formation in rats. Similarly, CBD was ineffective in numerous behavioural tasks, as is the CBD-rich extract. A complication arises from the amount of Δ^9-THC present as a contaminant in the CBD extract. In 50mg/kg CBD, this would amount to 4mg/kg Δ^9-THC, yet there was no obvious effect on latent learning suggesting reversal of Δ^9-THC-effects by CBD, at least for some CBD/Δ^9-THC dose ratios. Therefore, CBD-rich extracts may hold promise in some treatments (for example schizophrenia), in which Δ^9-THC exaggerates the negative symptoms of the disease.

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DEFICITS IN AN ATTENTIONAL SET SHIFT TASK INDUCED BY REPEATED LOW DOSE THC IN RATS

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Cannabis use in humans has been associated with impairments in cognitive function, including deficits in the ability to focus, sustain and also shift attention. Birrell and Brown (J. Neurosci., **2000**, 20, 4320) have developed an attentional set shifting task (ASST) in rodents, analogous to the human Wisconsin Card Sort Test, and have demonstrated the dependency of task performance on prefrontal cortex integrity. Previous results from our laboratory using this task (Brett R, Egerton A, and Pratt J (**2003**) **2003** Symposium on the Cannabinoids, Burlington Vermont, International Cannabinoid Research Society, 53) have demonstrated impairments in particular aspects of cognitive flexibility following acute administration of Δ^9-tetrahydrocannabinol (THC). However the human literature suggests that cannabis-induced cognitive deficits are more apparent following chronic use. We therefore employed the ASST to test the hypothesis that a chronic intermittent THC treatment regime would lead to deficits in executive function which are different from those induced by acute treatment.

Rats were administered THC (1mg/kg i.p.) or vehicle once per week for 4 weeks and tested in the ASST 30 minutes after the last dose of THC or vehicle. The test itself involved the rats discriminating between two different stimuli (digging medium or odour) to obtain a food reward. The ability to shift attention either within (intradimensional shift (IDS)) or between (extradimensional shift (EDS)) perceptual dimensions and also the ability to reverse exemplar-reward associations (reversal learning) were measured by scoring the number of trials to reach criterion in each of the discriminations. Data was analysed using three-way GLM mixed factor ANOVA with repeated measures, followed by planned comparisons to test the source of significant interactions.

Results revealed that chronic intermittent THC produced significant deficits in the ability to shift attention both within [(IDS), trials to criterion: VEH: 7.9 ± 0.38, THC: 10.4 ± 1.02; p=0.03] and between [(EDS), trials to criterion : VEH: 8.7 ± 0.76, THC: 11.2 ± 0.84; p=0.04] perceptual dimensions in the ASST. THC treatment did not significantly affect performance in any of the other discriminations comprising the task, including the reversal discriminations.

This is in contrast to our previous work with acute THC in the ASST which demonstrated deficits in IDS and reversal learning, but no impairment of EDS. The present results indicate that with chronic intermittent THC treatment there is apparent tolerance to the acute deficit in reversal learning, no tolerance to the acute deficit in IDS and that an EDS deficit emerges in the chronically treated rats. This indicates that after chronic intermittent treatment with THC, there is an emerging deficiency in the ability to effectively switch attention between perceptual dimensions in the task. THC-induced alterations in prefrontal circuitry may underlie these effects of chronic intermittent treatment on behavioural flexibility.

PHYSIOLOGICAL ROLE OF ENDOCANNABINOIDS IN THE NEOCORTEX

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The cerebral cortex mediates a diverse range of sensory, motor, and cognitive processes. These functions rely on the activity of pyramidal neurons (PNs) - the sole output cells of the cortex. Pyramidal neuron activity, in turn, is tightly controlled by distinct classes of GABAergic inhibitory interneurons, which innervate functionally segregated domains on PNs to regulate action potential timing, the efficacy of synaptic inputs, and synchronous activity. These interneurons maintain high firing rates *in vivo* and provide potent inhibition to PNs, thus modulation of this inhibitory tone plays a critical role in shaping the responsiveness and firing patterns of these cells. Little is known, however, about the impact of different classes of interneurons on distinct aspects of PN activity. Whole-cell recordings from layer 2/3 PNs indicate that endocannabinoids act retrogradely to mediate depolarization-induced suppression of inhibition (DSI) in sensory neocortex, similar to findings in the hippocampus and cerebellum. Using focal drug application, we found that endocannabinoids selectively suppressed perisomatic afferents that gave rise to synaptic currents with fast rise times and large amplitudes. Moreover, cortical DSI was reliably induced by a brief train of action potentials (APs), and the magnitude and time course of DSI was directly related to the frequency and number of postsynaptic APs. Reuptake of endocannabinoids also appeared to play a role in regulating the kinetics of DSI. To explore the functional consequences of suppressing perisomatic inhibition, we examined the responses of PNs to synaptic input following a brief AP train. The expression of AP-induced DSI coincided with an increase in the effectiveness of excitatory inputs and the probability of PN firing. We are also investigating the possibility that endocannabinoid-mediated disinhibition regulates back-propagating APs in PNs, which could facilitate synaptic plasticity at excitatory synapses and modulate the integrative properties of these neurons. Thus, the endocannabinoid system may participate in information processing by PNs in the neocortex.

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CRITERIA FOR RELEASE OF ENDOCANNABINOIDS BY HIPPOCAMPAL CELLULAR ACTIVITY

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Endocannabinoids have been shown to be released from hippocampal neurons when they are sufficiently depolarized for a brief period of time. This is evidenced by the reduction in release of GABA from presynaptic terminals of interneruons. The linkage of this depolarization induced suppression of inhibition (DSI) with release of endocannabinoids provides the importance evidence that endocannabinoids may have a functional role in altering hippocampal activity during behavior.. We tested this hypothesis in a direct manner by first characterizing the exact conditions whereby DSI (and endocannabinoids release) was produced by depolarizing pulses, and then assessing whether trains of depolarizing pulses or trains of action potentials elicited by constant depolarizing currents, similar to those recorded under behavioral conditions, could produce DSI. Pyramidal cells were recorded in slices of hippocampus under both voltage and current clamp conditions while evoked and spontaneous gabaergic inhibitory potentials (IPSCs ,IPSPs) were assessed. In addition, sodium dependent actions potentials were blocked by TTX and underlying calcium spikes evaluated. Results in current clamp showed in current clamp showed a definitive relationship between frequency of pulses to elicit DSI and amplitude of summed calcium mediated depolarizations. This assessment indicated that pulse or action potential frequencies that exceeded 30 Hz could produce significant DSI, however, pulse trains below this frequency were ineffective indicating that release of endogenous cannabinoids by hippocampal neurons was dependent on a pulse or action potential separation < 20 ms. Given this dependence, spike trains of single neurons recorded during hippocampal-dependent tasks (such as place fields and delayed nonmatch to sample paradigms) achieved this frequency of spike occurrence less than 10% of the time in which increased activity was recorded. These findings suggest that DSI mediated by endocannabinoid release occurs only under conditions in which underlying calcium spikes are of sufficient amplitude suggests that facilitation of synaptic and cellular activity in hippocampus by endocannabiniod release is dependent upon neurons being either 1) significantly depolarized for a long time period or 2) receive a high frequency train of depolarizing events. The significance of DSI for hippocampal function may relate to the increase in the probability to release endocannabinoids under these specific conditions.

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ENDOCANNABINOIDS MEDIATE RETROGRADE SIGNALLING AT EXCITATORY AND INHIBITORY SYNAPSES IN THE RAT VENTRAL TEGMENTAL AREA

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Accumulated evidence suggests that endocannabinoids act as diffusible and short-lived intercellular messengers that modulate synaptic transmission. We recently found that depolarization of postsynaptic ventral tegmental area (VTA) dopamine (DA) neurons induces a Ca^{2+}-dependent transient release of endocannabinoids that, by acting retrogradely, can activate CB_1 receptors located onto presynaptic terminals, and suppress glutamate release (depolarization-induced suppression of excitation, DSE). Importantly, DSE was partially blocked by the D_2DA antagonist eticlopride, and enhanced by the D_2DA agonist quinpirole without changing the presynaptic cannabinoid sensitivity. In the present study we performed whole cell patch-clamp recordings from VTA DA neurons in an in vitro slice preparation to examine the effect of postsynaptic depolarization on both excitatory and inhibitory synapses. We found that DSI can also be induced in the VTA, and is abolished by pre-treatment with the CB_1 receptor antagonist AM281. Interestingly, DSI is more prominent when D_1-like DA receptors are blocked, thus suggesting that somatodendritic released DA counteracts DSI by facilitating presynaptic GABA release, which in turn might activate presynaptic $GABA_B$ receptors located on glutamatergic terminals. Further, application of the DA reuptake inhibitor, GBR12909, enhanced DSE, and this effect was partially blocked by eticlopride. Additionally, DSE was partially blocked following selective inhibition of the 2-AG biosynthesis with tetrahydrolipstatin (THL), a blocker of the *sn-1* diacylglycerol lipase (DAGL). This effect was not enhanced in the presence of eticlopride. On the other hand, inhibition of intracellular DAGL with THL completely abolished DSI. The present results suggest that: i) 2-AG fully mediates DSI, while it only contributes to DSE together with other endocannabinoids; ii) somatodendritic released DA counteracts DSI via D_1-activated receptors, while it promotes DSE through both presynaptic $GABA_B$ and postsynaptic D_2 receptors; iii) activation of postsynaptic D_2 receptors might trigger an intracellular cascade accounting for 2-AG production. As a result, prolonged depolarization of DA neurons leads to the release of both endocannabinoids and DA which, either directly or indirectly, account for a decreased drive onto VTA DA cells to prevent pathological overexcitation.

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S_K ION CHANNELS AND METABOTROPIC GLUTAMATE AUTORECEPTORS CONTROL ENDOCANNABINOID RELEASE FROM DOPAMINE NEURONS IN THE VENTRAL TEGMENTAL AREA (VTA)

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Dopamine (DA) neurons in the ventral midbrain are implicated in various neurological diseases as well as in drug abuse. Consistent with this, the active ingredients in marijuana (cannabinoids, CBs) can inhibit motor activity and reward operant behavior via actions on this DAergic circuitry. A mechanism that may underlie the effects of the CBs is increased DA release from nerve terminals, resulting from increased firing of midbrain DA neurons. Since electrophysiological findings consistently demonstrate the presynaptic inhibition of GABA release by CB_1 receptors throughout the brain, the excitation of DA neurons in the ventral midbrain may reflect this disinhibition. In the VTA, DA neuron activity is regulated by $GABA_B$ receptors that receive input from an anatomically distinct pathway, arising from the GABAergic medium spiny output neurons of the nucleus accumbens. Here we test the hypothesis that this GABAergic pathway to VTA DA neurons can be regulated by CB receptors and endocannabinoids. Whole-cell voltage clamp recordings from physiologically identified DA neurons in the VTA were performed using video-microscopy in rat brain slices. $GABA_B$-mediated inhibitory postsynaptic currents (IPSCs) were evoked using trains of electrical stimuli, and were pharmacologically isolated from other synaptic currents using ion channel blockers and receptor antagonists. The remaining outward currents were blocked by the $GABA_B$ antagonist CGP35348 (100 μM). The $GABA_B$ IPSC was concentration-dependently inhibited (EC_{50} 365 nM) by the CB agonist WIN55,212-2 (maximum = 44 ± 2.7%, n = 10), and this was completely reversed by the CB_1 receptor antagonist AM251 (2 μM). In contrast, outward $GABA_B$ currents produced in response to direct application of GABA (20 mM) to the DA neurons were not inhibited by WIN (3 ± 11.7 %, n = 9), indicating that WIN activated presynaptic CB_1 receptors to inhibit evoked $GABA_B$ IPSCs. Because the WIN-related inhibition of evoked IPSCs could also be prevented by blockade of small conductance Ca^{2+}-activated K^+ channels (S_K; 2 ± 6.6%, n = 10) with apamin (100 nM), and metabotropic glutamate receptor (mGluR) antagonism (-2 ± 6.9%, n = 10), we determined whether these targets regulated endocannabinoid release. Consistent with this idea, AM251 significantly increased $GABA_B$ IPSC amplitudes when S_K channels were blocked by apamin (36 ± 8.3%, n = 10) or when mGluRs were blocked by LY341495 (200 μM; 63 ± 8.2%, n = 4), a nonselective antagonist. Furthermore, AM251 applied during treatment with both apamin and LY341495 produced an additive increase in the IPSCs (113 ± 19.1%, n = 4), that was blocked by chelation of intracellular Ca^{2+} with BAPTA in the patch pipette (3 ± 6.4%, n = 13). These data suggest that endocannabinoid release from DA neurons is regulated by S_K channels and mGluRs, and that endocannabinoid activation of CB_1 receptors inhibits GABA release from afferents targeting $GABA_B$ receptors on DA neurons in the VTA. This likely disinhibits DA neurons, and contributes to an increase in DA release in the nucleus accumbens.

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CANNABINOIDS INHIBIT EXCITATORY AMINO ACID TRANSPORT IN CULTURED RAT CORTICAL ASTROCYTES

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Activation of cannabinoid CB_1 receptors induces a variety of behavioral effects in animals, including locomotion. However, a biphasic excitatory/inhibitory effect has been reported: an increase in motor activity associated with relatively low doses or immediately after administration of higher doses of cannabinoids. In the brain, enhanced motor activity occurs as a consequence of excitatory neurotransmitter glutamate accumulation and subsequent activation of presynaptic metabotropic glutamate receptors. The persistent activation of which will inhibit the presynaptic release of glutamate. Impairment of glutamate uptake is known to inhibit synaptic transmission via facilitation of metabotropic glutamate receptor-mediated signaling pathway. In agreement with this concept, a recent study demonstrates that cannabinoid receptor CB_1 activation in the striatum decreases glutamate transporter activity and that the resulting increase in synaptic cleft glutamate concentration causes the activation of presynaptic metabotropic glutamate receptors, which then inhibit glutamate release.

In the brain, extra cellular glutamate is normally removed by a Na^+-dependent high-affinity glutamate transport system in neurons and neighboring astrocytes. The latter cell types, by possessing two high affinity glutamate transporters, excitatory amino acid transporter-1 (EAAT-1) and EAAT-2, are responsible for clearing most of the extra cellular glutamate from the synaptic cleft.

Here we investigated whether cannabinoid agonists affect these high affinity sodium-dependent astroglial excitatory amino acid transporters. Primary cultures of neonatal rat cortical astrocytes were incubated for 20 min at 37C in oxygenated HEPES-PBS, pH 7.4 with or without the cannabinoids under investigation. The incubation was continued for 10 min after adding D- [3H] aspartate (10 micro M), a stable analog of glutamate and the uptake was terminated by rapid aspiration of the medium and subsequent washing of the monolayer with ice-cold, sodium-free HEPES-PBS buffer. D- [3H] Aspartate uptake kinetics in cortical astrocytes were measured in HEPES-PBS containing various concentrations of non-radioactive D-aspartate and D- [3H] aspartate. Our results show that cortical astrocytes possess a sodium-dependent high-affinity excitatory amino acid transport system. A 20 min exposure of cultured rat cortical astrocytes to various cannabinoids resulted in the inhibition of D-aspartate uptake with HU210 > CP55, 940 = delta^9-tetrahydrocannabinol (delta^9-THC) = anandamide >> HU211, the less active isomer of HU210. Among the cannabinoid analogs tested, HU210 showed a biphasic effect: stimulation of D-aspartate uptake at very low concentration and inhibition at higher concentration. The cannabinoid CB_1 receptor antagonist SR141716A partially reversed HU210 inhibited D-aspartate uptake. The molecular mechanisms of cannabinoid interaction with astroglial excitatory amino acid transporters will be discussed. Thus cannabinoid inhibition of astroglial excitatory amino acid transport may contribute to both behavioral and cellular effects of cannabinoids on locomotion.

CANNABINOIDS DEPRESS GABAERGIC NEUROTRANSMISSION BETWEEN THE CAUDATE-PUTAMEN AND GLOBUS PALLIDUS

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The density of CB_1 receptors in the globus pallidus (GP) is high. GP neurons receive a dense GABAergic input from the caudate-putamen, and anatomical studies indicate that the majority of CB_1 receptors in the GP is localized on axon terminals of this input. Accordingly, the hypothesis of the present work was that activation of CB_1 receptors depresses neurotransmission between the caudate-putamen and the GP with a presynaptic mechanism.

Sagittal slices were prepared from brains of young mice. Electrical stimulation in the caudate-putamen elicited GABAergic inhibitory postsynaptic currents (IPSCs) in the GP. The synthetic CB_1/CB_2 cannabinoid receptor agonist WIN55212-2 ((R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl] pyrrolo [1,2,3-de] - 1,4-benzoxazin-yl] - (1-naphtalenyl) - methanone mesylate; 0.3 and 10 μM) concentration-dependently decreased the amplitude of IPSCs. The selective CB_1 receptor antagonist SR141716A(N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4methyl-3-pyrazole-carboxamide; 1 μM) prevented this inhibition. In a series of experiments, GP neurons were depolarised for 5 s via the patch pipette for inducing the synthesis of endocannabinoids. The depolarisation slightly suppressed IPSCs elicited by stimulation in the caudate-putamen; this suppression was not influenced by SR141716A (1 μM).

Several kinds of experiments were carried out to determine the mechanism by which WIN55212-2 inhibits neurotransmission. 1) WIN55212-2 (10 μM) had no effect on currents elicited by pressure ejection of muscimol in the vicinity of recorded GP neurons. 2) GABA was superfused in a chemically bound ("caged") form and was abruptly released by an UV flash pulse. The released GABA elicited a $GABA_A$ receptor-mediated current in GP neurons. The amplitude and duration of this current was not modified by WIN55212-2 (10 μM). 3) The influence of cannabinoids on action potential-evoked increases in calcium concentration in axon terminals was also studied. At first, terminals of axons projecting from the caudate-putamen to the GP were labelled: Oregon green 488 BAPTA dextran, a calcium sensitive fluorescent dye, was injected into the caudate-putamen of anaesthetized mice. During survival for 2-5 days, the dye was transported into axon terminals in the GP. Mice were then killed, and brain slices prepared. Axons were stimulated in the caudate-putamen and the evoked increases in calcium concentration in the GP were determined fluorometrically. Tetrodotoxin (1 μM) and Cd^{2+} (100 μM) eliminated the electrical stimulation-evoked increase in calcium concentration in axon terminals. WIN55212-2 (10 μM), however, did not change the calcium response.

These findings suggest that activation of CB_1 receptors by exogenous cannabinoid agonists inhibits GABAergic neurotransmission between the caudate-putamen and the GP. No inhibition mediated by endocannabinoids released from depolarised GP neurons occurs at this synapse. Lack of effect of cannabinoids on currents evoked by direct stimulation of postsynaptic $GABA_A$ receptors by muscimol or by GABA released by flash photolysis shows that GABAergic neurotransmission was depressed by presynaptic inhibition of GABA release from axon terminals. Lack of effect of cannabinoids on the action potential-evoked increase in axon terminal calcium concentration suggests that the transmitter release process is inhibited at a site after calcium entry into the terminal, probably at the level of the vesicular release machinery.

PRENATAL EXPOSURE TO THE CB_1 RECEPTOR AGONIST WIN55212-2 ALTERS HIPPOCAMPAL AND CORTICAL GLUTAMATERGIC TRANSMISSION. IN VIVO AND IN VITRO STUDIES

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In spite of marijuana is widely used during pregnancy and negative effects exerted by cannabinoids on learning and memory is widely recognized, scientific data concerning the possible long term cognitive outcomes on offspring remain scarce. To investigate on the possible long-term consequences of gestational exposure to cannabinoids on cognitive functions, pregnant rats were administered with the CB_1 receptor agonist WIN55,212-2 (WIN), at a dose (0.5 mg/kg) that causes neither malformations nor overt signs of toxicity. Prenatal WIN exposure induced a disruption of memory retention in 40- and 80-day old offspring subjected to a passive avoidance task. A hyperactive behaviour at the age of 12 and 40 days was also found. The memory impairment caused by the gestational exposure to WIN was correlated with alterations of hippocampal long-term potentiation (LTP) and glutamate release. *In vivo* microdialysis showed a significant decrease in basal, and K^+-evoked, extracellular glutamate levels in the hippocampus of juvenile and adult rats born from WIN-treated dams. A similar reduction in glutamate outflow was also observed in primary cell cultures of hippocampus obtained from pups born from mothers exposed to WIN. The decrease in hippocampal glutamate outflow appears to be the cause of LTP disruption which, in turn, might underlie, at least in part, the long-lasting impairment of cognitive functions caused by the gestational exposure to this cannabinoid agonist.

These findings obtained in hippocampus, together with recent data reporting that CB_1 receptors regulate glutamatergic transmission in rat prefrontal cortex, lead to hypothesise that, amongst other mechanisms, permanent changes in cortical glutamate signals could contribute to the impairments of cognitive performances induced by perinatal marijuana exposure. In cortical cell cultures obtained from 1 day-old rats born from mothers exposed during pregnancy to WIN55,212-2 a significant reduction in basal and K^+-evoked glutamate extracellular levels was found in respect to cell cultures obtained from pups born from vehicle-treated dams. In both groups of cultures, WIN55,212-2 (1 nM) significantly increased glutamate levels. In cortical cell cultures of animals born from mothers exposed to WIN55,212-2 during pregnancy, the selective CB_1 receptor antagonist SR141716A (100 nM) did not affect the WIN55,212-2-induced increase of glutamate levels. On the contrary, in cortical cell cultures of animals born from mothers exposed to the vehicle during pregnancy, the CB_1 antagonist prevented the enhancement induced by WIN55,212-2. In the cerebral cortex of adult (90 days old) rats born from WIN55,212-2-treated dams a significant reduction in basal and K^+-evoked glutamate extracellular levels was found in respect to the offspring born from vehicle-treated mothers. In both groups of animals the injection of WIN55,212-2 (0.1 mg/kg) increased dialysate glutamate levels. Interestingly, SR141716A (0.1 mg/kg) counteracted the WIN55212-2-induced increase of glutamate levels in the offspring exposed during gestation to the vehicle but not in the animals born from WIN55,212-2 treated dams.

Taken together, these *in vivo* and *in vitro* findings demonstrate an impairment of glutamatergic transmission in hippocampus and in cerebral cortex after prenatal WIN55,212-2 exposure and could provide an explanation of cognitive alterations observed in children born from women who use marihuana during pregnancy.

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INHIBITION OF EXPERIMENTAL SPASTICITY BY CB_1 and FAAH INHIBITORS IN CANNABINOID GENE KNOCKOUT MICE

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Multiple sclerosis (MS) is increasingly is a major demyelinating disease of the central nervous system and causes the accumulation of disability and the development of troublesome symptoms such as spasticity, that greatly diminish "quality of life". In response to anecdotal claims that cannabis can alleviate spasticity, we investigate the action of cannabinoids in the control of experimental spasticity in a chronic relapsing experimental allergic encephalomyelitis model of MS. This indicated the tonic cannabinoid control of experimental spasticity and efficacy was found using cannabinoid receptor agonists (Methanandamide, JWH133, WIN-55-212-2 and endocannabinoid degradation inhibitors (AM404, AM374 and VDM11) (Baker et al. Nature **2000**, 404: 84; Baker et al. FASEB J **2001**; 15:300). Efficacy was found with CB_1 agonists and surprising some CB_2 agonists (Baker *et al* **2000**). However all cannabinoids are not specific for their targets and the number of potential cannabinoid receptors and endocannabinoid degradation pathways are not yet completely known. Likewise CB receptor antagonists have additional specificities, which make interpretation of the pharmacology difficult. Therefore we investigated the action of anti-spastic agents using cannabinoid system gene knockout mice.

JWH 133 is one of the most selective CB_2 agonists, but it has some affinity for CB_1, therefore we examined JWH-056, which has very low affinity for CB_1. This failed to inhibit spasticity in wildtype mice. Likewise the rapid inhibition of spasticity induced by CP55, 940 and WIN-55-212-2 in wildtype mice was not present in CB_1 receptor deficient mice, indicating that CB_1 was the major target for anti-spastic activity. This also indicates that CB_2 and probably the "putative CB_3" (G-protein WIN-55,212-2 binding receptor) were not targets for treatment of spasticity and suggests that anti-spastic activity of CB_2 agonists may be due to cross-reactivity with the CB_1 system. However, there are clearly non CB_1-dependent anti-spastic mechanisms as arvanil (CB_1, VR-1, endocannabinoid degradation inhibitor) can inhibit spasticity and induce "tetrad effects" in CB_1 deficient mice (Brooks et al. Eur J Pharmacol. **2002**; 439:83). Likewise there have been many questions concerning the specificity of endocannabinoid inhibitors, including concern over the existence of specific anandamide re-uptake transporters, which may also bind to some extent with the anandamide hydrolytic fatty acid amide hydrolase (FAAH. Hillard et J Neurochem. **2000**; 74:2597). Recently potent FAAH inhibitors have been generated including compound 29 and 53 (Boger et al. Proc Natl Acad Sci U S A. **2000**; 97:5044) and URB524. (Tarzia et al. J Med Chem. **2003**; 46:2352). These were found to inhibit spasticity during EAE. However whilst the anti-spastic effect of compound 53 was lost in FAAH-deficient mice, URB524 exhibited anti-spastic activity in FAAH-deficient mice, suggesting additional activities of this compound. Although cannabinoid system inhibitors appear to be useful in the control of signs associated with MS, due to the lack of truly specific agonists/antagonists compounds and knowledge of the extent of the cannabinoid system, gene knockout animals are important in definitively identifying the target for therapy.

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THC AMELIORATES BEHAVIORAL DEFICITS IN THE SPASTIC MOUSE

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A number of relatively rare motor disorders affecting both humans and animals has been associated with a defect in the function or number of glycine receptors in the brain stem and spinal cord. These include genetic abnormalities such as stiff baby syndrome (Lingam, Wilson & Hart, **1981**), and the startle disorders such as hyperekplexia. In addition, similar motorr disorders are caused by as tetanus toxin, DDT poisoning and brainstem encephalophathy (which result in in similar reductions in glycine receptor function). Since THC extracts have been reported to reduce muscle spasms and spasticity associated with Multiple Sclerosis (Wade DT, Robson P. et al. Clin Rehabil. **2003**;17:21-9) we tested the therapeutic effects of THC in the mutant spastic mouse (B6C3Fe a/a-Glrbspa/J),which show behavioral and anatomical deficits that match many human disorders, Animals and Drugs. Heterozygotes of the B6C3Fe a/a-Glrbspa/J spastic mouse were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Homozygotes were obtained by mating heterozygotes. Three hundred and twenty-nine offspring were screened for inclusion in the experiment on day 42 postpartum, using an inclusion criteria based on previous reports of an impaired righting reflex in the spastic mouse (Chai, C. K, J. Heredity, **1961**;52, 241-243) to assign subjects to groups. Mice were placed on their backs and the amount of time required to return to a standing position was recorded. Mice with a righting reflex of > 3 seconds were retained and randomly assigned to one of five drug conditions (0, 0.5, 2 or 4 mg/0.01cc/g body weight THC (GW Pharmaceuticals, Porton Down, UK) or 2 mg/0.01cc/g body weight R-(+)-baclofen (Sigma Chemical Co., St. Louis, MO, USA).

Behavioral Method. The following behaviors were recorded: righting reflex hindlimb clenching, startle responses, spontaneous activity and rearing.

Results. A significant drug effect was found on the amount of time required for the animals to right themselves $F(4,25)= 3.99$, $p < .05$. Post-hoc analyses were completed with Dunnett's test for contrasts involving a control mean. Analyses indicated that the 4 mg/kg/0.01cc/g body weight dose of THC extract significantly reduced the amount of time required to return to all four paws. Baclofen, however, significantly lengthened the righting reflex. Clenching ($F(4,25) = 1.581$, $p > .05$) and startle responses ($F(4,25) = 2.525$, $p > .05$) were not effected significantly by either baclofen or THC. Spontaneous activity ($F(4,25) = 0.218$, $p > .05$) and rearing ($F(4,25) = 0.123$, $p > .05$) in the open-field were also unaffected by the type of drug administered.

Discussion. These data suggest that THC extracts may be useful for the treatment of some of the behavioral symptoms associated with glycine receptor defects, but it is not likely to treat all of the symptoms. THC based extracts may, however, prove beneficial if combined with current treatments thereby allowing the use of lower dosages and a reduction in the likelihood of side effects.

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ARVANIL INHIBITS T-LYMPHOCYTE ACTIVATION AND AMELIORATES THE COURSE AND PROGRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Arvanil (*N*-arachidonoyl-vanillyl-amine) is a synthetic capsaicin-anandamide hybrid molecule, that exerts biological activities through VR1/CB_1 receptors (Melck et al., *Biochem. Biophys. Res. Commun.*, **1999**). Arvanil is a potent analgesic and inhibitor of spasticity in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (Brooks et al., *Eur. J. Pharmacol.*, **2002**). Anandamide exerts anti-proliferative effects on lymphocytes and inhibits mammal and prostate tumour cell proliferation trough the CB_1 receptor. We analysed *in vitro* and *in vivo* the immunomodulatory effect of arvanil. We showed that arvanil inhibited mitogen-induced human lymphocyte proliferation in a dose dependent manner. In order to understand the mechanism of this inhibitory effect, we analysed by flow cytometry the phenotype of anti-CD3 (OKT3) activated T cells treated with arvanil. We observed a down regulation of T cells activation marker expression on CD4+Tcells expressing CD134/OX40, CD25 and HLA-DR. In murine cells treated with arvanil we observed inhibition of proliferation and decreased levels of interferon (IFN)- γ. In EAE, arvanil (0.5mg/kg/dose i.p. administration) ameliorated the clinical course of the disease. Administration of the CB_1 receptor antagonist SR141716A (0.7mg/kg/dose i.p. administration) to EAE mice treated with arvanil attenuated the immunomodulatory effects of arvanil as well as the first phase of its beneficial effects on EAE clinical score. These data suggest a possible involvement of the CB_1 receptor in the mechanism of action of arvanil *in vivo*. These results suggest that the antiproliferative effect of arvanil is associated to a decrease of lymphocyte activation; the decreased level of IFN- γ suggests an effect on T helper 1 (Th1) type 1 cells. From our findings, arvanil exerts immunosuppressive properties that can be relevant to the use of this molecule for new immunotherapeutic approaches for the treatment of autoimmune pathologies of the central nervous system.

ANANDAMIDE UPTAKE INHIBITION AS THERAPEUTIC APPROACH IN A MURINE MODEL OF MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is a human chronic inflammatory disease of the central nervous system characterized by autoimmune responses against myelin proteins that eventually impair the normal neurotransmission leading to sensory deficits and deteriorated motor coordination. Theiler´s murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) is a well-characterized murine model of human MS which closely resembles the chronic and progressive clinical form of the disease.

Recent studies have described the potential of the endogenous cannabinoid system (ECS) as a promising therapeutic target for the treatment of MS and the benefits associated to the administration of cannabinoid agonists have been reported (*J. Neurosci.* **2003**, *23*, 2511). However, the treatment with direct agonists, specially those acting at CB_1 receptor, could be related with the appearance of psychotrophic symptoms, so a therapeutic alternative to overcome these undesirable side effects could be the reinforcement of the physiological endocannabinoid tone by administration of indirect agonists.

With the objective to study whether such indirect agonists can mimic the effects induced by direct agonists, we administered the potent and selective anandamide uptake inhibitor UCM707 (*J. Med. Chem.* **2003**, *46*, 1512) to TMEV infected mice (5 mg/kg/day, 12 days, i. p.). Our results indicate that treatment during established disease significantly improves the motor functionality of the diseased mice measured in the rotarod test. Besides, at a histological level, UCM707 is able to reduce microglial activation, diminish the major histocompatibility complex class II antigen expression and decrease the infiltrating leukocytes in the spinal cord. These results, therefore, suggest that agents able to enhance the levels of endocannabinoids could constitute a new series of drugs for the treatment of MS.

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BENEFICIAL EFFECTS OF INHIBITORS OF THE ENDOCANNABINOID TRANSPORT IN A RAT MODEL OF MULTIPLE SCLEROSIS: INVOLVEMENT OF VANILLOID TRPV1 RECEPTORS

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Recent studies have addressed the changes in endocannabinoid ligands and receptors that occur in the brain of animal models of multiple sclerosis (Baker et al., FASEB J. 15 : 300-302, **2001**; Berrendero et al., Synapse 41 : 195-202, **2001**), as a way to explain the efficacy of compounds acting at the endocannabinoid system to alleviate spasticity, pain, tremor and other signs of this autoimmune disease (Baker and Price, Expert Opin. Investig. Drugs 12 : 561-567, **2003**). Using Lewis rats with experimental autoimmune encephalomielitis (EAE), an animal model of multiple sclerosis generated by inoculation of guinea-pig myelin basic protein, we recently found a decrease in cannabinoid CB_1 receptors in several brain regions (Berrendero et al. Synapse 41 : 195-202, **2001**). More recently, we have observed that the changes in receptors are accompanied by a reduction of brain endocannabinoid levels (Cabranes et al., ICRS **2003**). Based on these observations, we hypothesize that the elevation of the endocannabinoid activity, for instance after the inhibition of the endocannabinoid transport, might be beneficial in EAE rats. In these rats, the appearance of neurological signs takes place from days 9-10 post-inoculation reaching a maximal value on day 13. Based on this pattern, EAE rats and their controls were daily treated with different endocannabinoid uptake inhibitors or vehicle from day 9 up to day 13 post-inoculation, being the animals daily subjected to neurological analysis during that period. We found that the administration of the endocannabinoid transport inhibitor and vanilloid TRPV1 receptor agonist AM404 (5 mg/kg) was effective to reduce the magnitude of the neurological decline in EAE rats, as also observed with other compounds used in this disease such as rolipram. Another transport inhibitor that also shows TRPV1 receptor activity, arvanil (2 mg/kg), was also effective to reduce neurological decline in EAE rats, while VDM11 (5 mg/kg) and UCM707 (5 mg/kg; see abstract by de Lago et al., ICRS **2004**), which block the transporter with no direct effects on TRPV1 receptor, were not effective. By contrast, OMDM2 (5 mg/kg), another endocannabinoid transport inhibitor which is also devoid of activity at TRPV1 receptors, did reduce neurological decline in EAE rats, possibly because this compound is more metabolically stable than VDM11 in vitro (Ortar et al., Biochem. Pharmacol. 65 : 1473-1481, **2003**), and its effects are more long-lasting in vivo (de Lago et al., Eur. J. Pharmacol. 484 : 249-257, **2004**). In summary, our data support the notion that vanilloid TRPV1 receptors, in addition to cannabinoid CB_1 receptors, may represent a novel pharmacological target to reduce the neurological decline experienced by EAE rats.

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NEUROPROTECTIVE EFFECT OF CANNABIDIOL ON β-AMYLOID-INDUCED TOXICITY IN PC12 CELLS

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Alzheimer's disease is widely held to be associated with oxidative stress due, in part, to the membrane action of β-amyloid peptide aggregates. Here, we studied the effect of cannabidiol, a major non-psychoactive component of the marijuana plant (Cannabis sativa) on β-amyloid peptide-induced toxicity in cultured rat pheocromocytoma PC12 cells. Following exposure of cells to β-amyloid peptide (1 μg/ml), a marked reduction in cell survival was observed. This effect was associated with increased reactive oxygen species (ROS) production and lipid peroxidation as well as caspase-3 (a key enzyme in the apoptosis cell-signalling cascade) appearance, DNA fragmentation and increased intracellular calcium. Treatment of the cells with cannabidiol (10^{-7}-10^{-4} M) prior to β-amyloid peptide exposure significantly elevated the cell survival while it decreased reactive oxygen species production, lipid peroxidation, caspase-3 levels, DNA fragmentation and intracellular calcium. Our results indicate that cannabidiol exerts a combination of neuroprotective, antioxidative, and anti-apoptotic effects against β-amyloid peptide toxicity and that inhibition of caspase-3 appearance from its inactive precursor, pro-caspase-3 by cannabidiol is involved in the signalling pathway for this neuroprotection.

THE NEURON IS A SPERM CELL WITHOUT A TAIL: A PERSPECTIVE ON ENDOCANNABINOID SIGNALING

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Sea urchin and human sperm contain receptors for numerous neurotransmitters and psychoactive drugs, including cannabinoid receptors [CBR]. Anandamide, arachidonoylethanolamide [AEA], is an endogenous agonist for CBR in neurons and non-neural cells in the brain and peripheral organs, including the reproductive system. AEA is rapidly released from membrane phospholipids when neurons are stimulated. Sperm and neurons are highly differentiated excitable cells which contain voltage- and ligand-gated ion channels for rapid electrical signaling.

Retrograde AEA signals from depolarized postsynaptic neurons inhibit neurotransmitter release at excitatory synapses in mammalian brain. Analogous processes regulate sperm functions during fertilization in sea urchins. Both AEA and (-) Δ^9tetrahydrocannabinol [THC], the major psychoactive constituent of *Cannabis*, inhibit fertilization in sea urchins. They reversibly inhibit fertilization by blocking the egg-jelly-stimulated acrosome reaction [AR] which enables sperm to penetrate and activate the egg. The acrosome is a Golgi-derived secretory granule in sperm that is analogous to synaptic vesicles and granules in neurons. Consistent with observations that AEA and THC inhibit release of neurotransmitters at synaptic endings, ultrastructural studies on sea urchin sperm show that THC prevents the membrane fusion step in acrosomal exocytosis. AEA and THC do not block ionophore-induced-AR. These data suggest that cannabinoids inhibit the AR by modulating signal transduction event(s) prior to the opening of ion channels, findings consistent with results obtained with neurons. Unfertilized sea urchin eggs have enzymes required to release AEA from membrane phospholipids. These results indicate that sea urchin eggs may release AEA after activation by the fertilizing sperm. Released AEA may then activate sperm CBR to prevent other sperm in the vicinity from undergoing AR, thereby helping to prevent polyspermic fertilization. With respect to sea urchin gametes, the sperm is functionally equivalent to a presynaptic neuron in the brain while the egg is equivalent to a postsynaptic neuron. AEA is present in human seminal plasma, mid-cycle oviductal fluid, and follicular fluid. Sperm are sequentially exposed to these fluids as they move from the vagina to the site of fertilization in the oviduct. R-methanandamide [AM-356], a potent and metabolically stable AEA analog, and THC modulate capacitation and fertilizing potential of human sperm *in vitro*.

These findings suggest that AEA-signaling: directly affects sperm functions required for fertilization in sea urchins and humans; (2) has an ancient origin in evolutionary history; (3) may modulate sperm capacitation and fertilizing potential in human reproductive tracts; (4) and provide additional evidence for common signaling processes in neurons and sperm.

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RELATIONSHIP OF THE ENDOCANNABINOID SYSTEM WITH SEVERAL KEY PROTEINS FOR BRAIN DEVELOPMENT IN RATS

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Cannabinoid CB_1 receptors emerge early in brain development and are abundantly expressed in certain brain regions that play key roles in neural development. This observation implies that the endocannabinoid signaling system, in addition to well-described adult functions in the brain, might also play a modulatory role in the regulation of specific processes during brain development. Three processes have been proposed so far: (i) to regulate the gene expression of key proteins for specific neurotransmitters, such as the enzyme tyrosine hydroxylase and the opioid precursor proenkephalin, (ii) to participate in the apoptotic death of specific groups of neurons with a neurotrophic role, and (iii) to modulate the gene expression and/or function of neural adhesion molecules. Moreover, this system, if it is active during development, provides several targets for the pharmacological action of the psychoactive compounds present in cannabis derivatives when these are consumed during the perinatal period, so that this might be the way for plant-derived cannabinoids to affect basic developmental processes leading to the disturbances in the development of specific neurotransmitters and their related functions. In the present study, we wanted to explore the influence of prenatal cannabinoid exposure on a key protein for neural development, the neural adhesion molecule L1, which plays an important role in processes of cell proliferation and migration, neuritic elongation and guidance, and synaptogenesis. To this end, pregnant rats were daily treated with Δ^9-tetrahydrocannabinol (Δ^9-THC) since the 5th day of gestation up to the day before birth (GD21), day at which pups were removed and sacrificed for analysis of mRNA levels for L1 in different brain structures. Our results confirmed that the levels of L1 transcripts were increased after prenatal Δ^9-THC exposure in several regions such as the fimbria, stria terminalis, stria medullaris and corpus callosum, which share the properties of being white matter regions and containing, exclusively during development, an abundant population of cannabinoid CB_1 receptors, the major targets for the action of plant-derived cannabinoids. L1-mRNA levels were also increased in grey matter structures such as the septum nuclei and the habenula, but remained unchanged in most of grey matter structures analyzed (cerebral cortex, basolateral amygdaloid nucleus, hippocampus, thalamic and hypothalamic nuclei, basal ganglia and subventricular zones) and also in a few white matter structures (fornix and fasciculum retroflexum). An important aspect of these observations is that the increases in L1-mRNA levels reached statistical significance only in the case of Δ^9-THC-exposed males but not in the case of Δ^9-THC-exposed females where only trends or no effects were detected, thus supporting previous evidence on a sexual dimorphism, with greater effects in male fetuses, for the action of cannabinoids in the developing brain. We are presently exploring the influence of perinatal cannabinoid exposure on another two key proteins for neural development, bcl-2 and bax, which play regulatory roles in the physiological apoptosis occurring during brain development, in particular at early postnatal ages. In summary, cannabinoids seem to influence the expression of L1 in specific brain structures during the perinatal period, which, considering the role played by this protein in different events related to neural development, might represent a physiological mechanism for the endocannabinoid system to regulate processes such as cell proliferation and migration, neuritic elongation and guidance, and synaptogenesis.

EXPRESION AND FUNCTION OF THE ENDOCANNABINOID SYSTEM IN NEURAL PROGENITOR CELLS

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The discovery of multipotent neural progenitor (NP) cells has provided strong support to the existence of neurogenesis in restricted adult brain areas. However, the signals controlling their proliferation and differentiation remain elusive. Endocannabinoids, in addition to their neuromodulatory role, play an important role in the control of neural cell death and survival. Here we show that NPs express a functional endocannabinoid system that actively regulates cell proliferation and differentiation. Specifically, NPs produce endocannabinoids and express the CB_1 receptor and the major endocannabinoid-inactivating enzyme - fatty acid amide hydrolase (FAAH). CB_1 receptor activation promotes NP proliferation and similarly adult FAAH knock-out mice show increased proliferation of hippocampal neural progenitors when compared to wild-type animals. Moreover, endocannabinoid signaling modulates NP differentiation leading to increased astrogliogenesis and reduced neurogenesis, both in vitro and in adult FAAH knock-out mice. Our results demonstrate that endocannabinoids constitute a new group of lipid signaling cues involved in the control of NP proliferation and differentiation.

INHIBITION OF FAAH LEADS TO A CB_1-MEDIATED ANALGESIA ACCOMAPANIED BY SIGNIFICANT INCREASES IN ENDOGENOUS ANANDAMIDE IN BRAIN AND SPINAL CORD

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The goal of these experiments was to investigate whether highly selective and potent inhibitors of fatty acid amide hydrolase (FAAH), the enzyme predominantly responsible for the biodegradation of the endogenous cannabinoid anandamide and other fatty acid amides (FAAs), could serve as potential analgesics. In preliminary studies, FAAH inhibitors were screened for their propensity to augment anandamide-induced hypothermia and analgesia in mice. Whereas anandamide (50 mg/kg, i.p.) given alone failed to elicit significant pharmacological effects, pretreatment with an unfused pyridyl oxazole FAAH inhibitor OL-135 elicited CB_1-mediated analgesic and hypothermic effects in anandamide-treated mice with ED_{50} (95% confidence intervals) values of 1.9 (1.0-3.7) mg/kg and 1.7 (1.0-2.6) mg/kg, respectively. We then evaluated whether OL-135 administered alone would produce analgesic effects in the tail withdrawal, hot plate, and formalin nociceptive tests. In both the tail withdrawal and hot plate tests, OL-135 produced modest but significant analgesic effects within 15 min, with effects peaking between 1 and 2 h, and the analgesia completely dissipated by 4 h. These results indicate that the analgesic effects of OL-135 are reversible. This FAAH inhibitor also produced a dose-related analgesia in both phase 1 and phase 2 of the formalin test with ED_{50} (95% confidence intervals) values of 7.9 (6.6-9.4) and 7.5 (6.2-9.0) mg/kg, respectively. In each assay, SR 141716 (3 mg/kg, i.p.) pretreatment completely blocked OL-135-induced analgesia, indicating a CB_1 receptor mechanism of action. On the other hand, OL-135 appeared to be devoid of overt motor effects, as it failed to affect locomotor behavior and coordination as assessed in the inverted screen test. Finally, OL-135 caused significant increases in endogenous anandamide levels in both brain and spinal cord 1 h after treatment. Taken together, these findings indicate that OL-135 elicits CB_1 mediated analgesia in at least three pain assays, accompanied by elevated levels of endogenous anandamide in the CNS. These results are remarkably reminiscent to those found in FAAH (-/-) mice and suggest that selective and reversible FAAH inhibitors, such as OL-135, may offer a unique strategy for the treatment of pain disorders.

FAAH INHIBITORS AND INDOMETHACIN REDUCE CARRAGEENAN INDUCED HIND PAW INFLAMMATION IN THE MOUSE – ROLE OF CANNABINOID RECEPTORS

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Recent data have indicated that inhibition of fatty acid amide hydrolase (FAAH) by acidic non-steroidal anti-inflammatory agents such as indomethacin (Holt *et al., Br J Pharmacol* 133 [**2001**] 513-20; Fowler *et al., J Enz Inhib Med Chem* 18 [**2003**] 55-8) might contribute to their effects upon inflammatory pain (Gühring *et al. Eur J Pharm-acol* 454 [**2002**] 153–63, Ates *et al., Eur J Neurosci* 17, [**2003**] 597–604). Here, we have determined whether the anti-oedema effect of indomethacin in the carrageenan model inflammation is prevented by cannabinoid receptor antagonists, and whether FAAH inhibitors can mimic the effect of indomethacin. Male C57BL/6J pentobarbital-anaesthetised mice were given an intraperitoneal injection of test compounds or vehicle (1:20 ethanol:carboxymethylcellulose, 1.5 % w/v in saline) 30 min before intraplantar injection of λ-carrageenan (1 % w/v in saline) in the hind paw. The CB_1-antagonist AM251 (10 mg/kg), or the CB_2-antagonist SR144528 (3 mg/kg) were given 15 min prior to the test compounds. The resulting oedema was estimated four hours later in the awake animals by measuring the volume of the hind paw in a plethysmometer (Ugo Basile, Varese, Italy) and subtracting the volume of the contralateral paw. A robust oedema response to carrageenan was seen. Oedema formation was prevented completely by indomethacin (5 mg/kg) and partially by the non selective and selective FAAH inhibitors PMSF (30 mg/kg) and URB597 (0.3 mg/kg), respectively. The COX-2 inhibitor nimesulide, which does not affect FAAH activity *in vitro*, produced a partial inhibition of the oedema at a dose of 10 mg/kg. No significant potentiation of the response to nimesulide was produced by concomitant administration of URB597. SR144528 treatment *per se* was without effect on the oedema produced by carrageenan, but significantly antagonised the effect of indomethacin and URB597, so that the oedema observed was not significantly different from that seen for animals treated with carrageenan alone. AM251 had a significant effect *per se*, producing a 31% ($P<0.01$) reduction in the response to carrageenan, thereby complicating interpretation of these data. However, the oedema response to indomethacin was also significantly reduced by AM251 treatment. These data are consistent with the suggestion that cannabinoid receptors may be involved in the anti-oedema actions of indomethacin and URB597. Whilst URB597 is selective for FAAH (Kathuria *et al., Nat Med* 9 [**2003**] 76–81), it is in theory possible that inhibition of both FAAH and monoacylglycerol lipase (the enzyme responsible for 2-AG metabolism) may contribute to the actions of indomethacin. However, at pH 7.2, indomethacin was a weak inhibitor of rat brain monoacylglycerol lipase, producing 30% inhibition at the highest concentration tested (500 μM). In contrast, indomethacin inhibited FAAH with an IC_{50} value of 33 μM. In conclusion, FAAH inhibition is sufficient to produce an anti-inflammatory effect in the carrageenan model of inflammation, and may contribute to the actions of indomethacin in this model.

CB_2 CANNABINOID RECEPTORS MAY PRODUCE PERIPHERAL ANAGLESIA BY STIMULATING LOCAL RELEASE OF ENDOGENOUS OPIOIDS

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Introduction: CB_2 cannabinoid receptor-selective agonists inhibit pain responses in models of acute, inflammatory, and neuropathic pain by acting in peripheral tissues. The mechanisms underlying the antinociceptive effects of CB_2 receptor activation are unclear, since CB_2 receptors are not normally found on neurons of the central or peripheral nervous systems. Therefore, we postulated that CB_2 receptor agonists may exert antinociceptive actions indirectly, by affecting the release of substances that modulate the responsiveness of primary afferent neurons to noxious stimuli. This study tests the hypothesis that CB_2 cannabinoid receptor agonists produce antinociception by stimulating the release of the endogenous opioid β-endorphin. *Methods:* Approval for these experiments was obtained from the University of Arizona Institutional Animal Care and Use Committee. Except where noted, male Sprague Dawley Rats (250-350 g) were used. Nociceptive thresholds were assessed by measuring withdrawal latency to application of radiant heat to the hindpaw. The CB_2 receptor-selective agonist AM1241 was administered intraperitoneally (i.p.) in a volume of 500 µl 15 min before nociceptive testing. Other drugs were injected locally in the dorsal surface of the hindpaw (i.paw) in a volume of 50 µl 20 min before nociceptive testing. Beta-endorphin release from skin tissue was measured by incubating 8 mm punch biopsy samples of skin taken from the dorsal hindpaw in buffer (with gentle agitation) and measuring the β-endorphin content in buffer after 30 min. Beta-endorphin release from cultured keratinocytes (HaCat cells) was measured by incubating cells in culture medium and measuring β-endorphin content in the medium after 30 min. Beta-endorphin was measured by enzyme immunoassay. Statistical analysis consisted of ANOVA followed, where indicated, by Student's t-test with Bonferroni's correction. *Results:* AM1241 produced the maximum possible antinociception. The effect of AM1241 was completely blocked by naloxone or by sequestration of β-endorphin using an antiserum directed against the peptide. AM1241 produced antinociception in wild-type, but not in µ-opioid receptor knockout mice. Beta-endorphin produced equivalent antinociception to that produced by AM1241 and the effects of β-endorphin were also blocked by naloxone and by β-endorphin antiserum. AM1241 stimulated β-endorphin release from skin tissue and from cultured keratinocytes *in vitro*. AM1241-stimulated β-endorphin release was completely inhibited by the CB_2 cannabinoid receptor-selective antagonist AM630 and was not observed in CB_2 receptor knockout mice. *Conclusions:* These results are consistent with the hypothesis that CB_2 receptor activation produces antinociception by stimulating release of endogenous opioids, which then inhibit the responsiveness of primary afferent neurons.

INTERACTIVE ROLE OF PERIAQUEDUCTAL GRAY CB_1 AND MGLU5 RECEPTORS IN THE FORMALIN-INDUCED CHANGES IN RVM ON- AND OFF-CELLS

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Introduction. This study investigated in the rat whether formalin (50 μl, 5%) injected into the hind paw was associated with: i) changes in the firing rate of both ON- and OFF-cells in the rostral ventral medulla (RVM) and ii) whether or not these changes were modified by either CB_1 or mGlu1/5 receptors stimulation or inhibition into the periaqueductal grey (PAG).

Methods. The formalin test, a model of persistent pain was combined with *in vivo* extracellular recording in urethane anaesthetised rats.

Results. Fifty microliters of a 5% formalin solution (or vehicle) was injected into the subcutaneous space at a dorsal side of the right hind paw of rats while, at the same time, basal spontaneous activities of either ON- or OFF-cells RVM were recorded. Formalin, but not vehicle, induced few seconds after injection a persistent activation of RVM ON-cells (42±7%) and inhibition of OFF-cells (35±4%). Both ON- or OFF-cells recovered basal firing rates (pre-formalin injection) 30-45 min after formalin in the order of 80% and 45%**,** respectively. Intra-PAG microinjection of WIN 55,212-2 (2-10 nmol/rat), a cannabinoid receptors agonist, induced (2-3 min after injection) a dose-dependent and reversible inhibition of RVM ON-cells (55±7%) and stimulation of OFF-cells (60±4%). The WIN-induced effect was prevented by SR141716A (0.5 mg/kg i.p.), a selective antagonist of CB_1 receptors, by MPEP (10 nmol/rat), a selective antagonist of mGlu5 glutamate receptors, but not by CPCCOEt (20 nmol/rat), a selective mGlu1 glutamate receptors antagonist. Intra-PAG microinjection of WIN55,121-2 (1 nmol/rat **),** a dosage which was unable per se to modify both ON- or OFF-cells activities, prevented the formalin-induced effects in both ON- or OFF-cells.

Conclusions. These data show that PAG CB_1 receptors modulate ON- and OFF-cells RVM activities with the involvement of mGlu5 glutamate receptors. Moreover, subcutaneous formalin modified spontaneous activities in ON- and OFF-cells RVM and these effects were prevented by selective stimulation of CB_1 receptors into the PAG matter.

STUDY OF CB_1 CANNABINOID RECEPTOR KNOCKOUT MICE IN A MODEL OF NEUROPATHIC PAIN

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Neuropathic pain is a clinical manifestation characterized by the presence of spontaneous pain, as well as, allodynia and hyperalgesia. Here we studied the involvement of CB_1 cannabinoid receptors in the development and expression of neuropathic pain. For this purpose, partial ligation of the sciatic nerve was performed in CB_1 knockout mice as well as in wild-type littermates and the development of allodynia and hyperalgesia was evaluated using Von Frey, cold-plate, and plantar tests. In the absence of nerve injury, paw withdrawal responses to the different tests were similar in both genotypes. Partial ligation of the sciatic nerve led to a neuropathic pain syndrome characterized by a marked reduction in the paw withdrawal thresholds in Von Frey (mechanical allodynia) and plantar (thermal hyperalgesia) tests, and an increase of the paw withdrawal responses in cold-plate test (thermal allodynia). These manifestations were developed in a similar way in both genotypes. We have also investigated the antinociceptive properties of gabapentine in this model of neuropathic pain. The administration of gabapentine (50 mg/kg, i.p.) induced a similar suppression of mechanical and thermal allodynia in wild-type and CB_1 knockout mice. Mild differences were observed between genotypes when the gabapentine was tested in plantar test. Finally, the neurochemical consequences of sciatic nerve injury were evaluated in CB_1 knockout and wild-type mice by using c-fos expression in the spinal cord. Preliminary results showed no differences between genotypes. Taken together our results indicate that CB_1 cannabinoid receptors do not seem to be implicated in the development and expression of neuropathic pain nor in the antinociceptive effects of gabapentine in this neuropathic model of pain.

CANNABINOIDS BLOCKS TACTILE ALLODYNIA IN DIABETIC MICE WITHOUT ATTENUATION OF ITS ANTINOCICEPTIVE EFFECT

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Many studies showed that diabetes alter pain sensitivity and analgesic response to several drugs and diabetic neuropathic pain considered as one of the difficult types of pain to treat. Although hyperglisemia, neuronal loss, or alteration of receptors or neurotransmitters have been reported to be responsible for change in pain perception in diabetes, exact mechanism involved are not clear. Cannabinoids and opiods are different psychoactive drug classes and both of them induce antinociception. It is well known that diabetic animals are less sensitive to the analgesic effect of morphine and opioids are found to be ineffective in the treatment of painful diabetic neuropathic pain. However, it has been reported that cannabinoid analgesia remained intact and cannabinoids found to be effective in some models of nerve injury. So, in this study, we aimed to investigate antinociceptive efficacy of cannabinoids by using WIN 55, 212-2, a cannabinoid agonist, in streptozotocin (STZ)-induced diabetic mice and the effects of cannabinoid system on behavioral sign of diabetic neuropathic pain. Diabetes was induced by i.p. STZ (200 mg/kg) and antinociception were assested by radiant tail-flick test. Mechanical and thermal sensitivites was measured by Von Frey filaments and hot plate test, respectively, following 60 days after STZ administration.Tactile allodynia, but not thermal hyperalgesia was developed in diabetic mice. Systemic WIN 55, 212-2 (1, 5 and 10 mg/kg, i.p.) dose dependently produce antinociceptive effects in both diabetic and control mice. The antinociceptive effects of WIN 55, 212-2 in tail-flick test was similar in diabetic mice when compared to control mice suggesting efficacy of cannabinoid antinociception was not diminished in diabetic mice. WIN 55, 212-2 (1, 5 and 10 mg/kg, i.p.) also exerted dose dependently antiallodynic effects in diabetic mice. In conclusion, cannabinoids have clearly important roles in the antinociceptive mechanism both in control and diabetic stiuation. The antinociceptive potency of cannabinoid is preserved in diabetes. WIN 55, 212-2 has also antiallodynic effect in diabetic neuropathic pain. Given that painful neuropathy is an important diabetic complication, which severely affects quality of life, our study support a role of cannabinoid system in diabetic neuropathic pain and suggest further investigations cannabinoids as potential therapeutic targets in diabetic neuropathic pain.

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THE ACTIONS OF WIN55,212 AND THC ON THE CAPSAICIN-EVOKED CALCIUM RESPONSE IN CULTURED RAT DORSAL ROOT GANGLIA

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Rat dorsal root ganglion (DRG) neurones express both inhibitory CB_1 receptors and excitatory TRPV1 receptors (Ralevic *et al*., *Life Sciences,* **2002**, 418, 117-25). HU210 has been reported to inhibit capsaicin-evoked calcium responses, which is reversed by SR141716A indicating CB_1 involvement (Millns *et al*., *Br. J. Pharmacol.,* **2001**, 132, 969-971). However, anandamide alone produces transient increases in $[Ca^{2+}]_i$ which is reversed by SR141716A but not the TRPV1 antagonist capsazepine (Millns *et al*., **2001,** *Br. J. Pharmacol.,* 135, 256P). In the present study we investigated if the cannabinoid agonists WIN55,212 and THC modulate the capsaicin-evoked calcium response.

DRG were isolated from adult Wistar rats and neurones cultured as described by Lindsay (*J.Neurosci*., **1988**, 8, 2394-2405). Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in individual neurones in fields of up to 80 cells were calculated as the ratios of peak fluorescence intensities (measured at 500 nm) at excitation wavelengths of 340 and 380 nm respectively. DRG neurones were then superfused (2 ml min^{-1}) with capsaicin (100nM, 60 s) followed by a 45 min washout. THC/WIN55,212 (1μM) was then added for 4 min, capsaicin in combination with THC/WIN55,212 was then added for 60 s. Responses were calculated as percentage of initial capsaicin response.

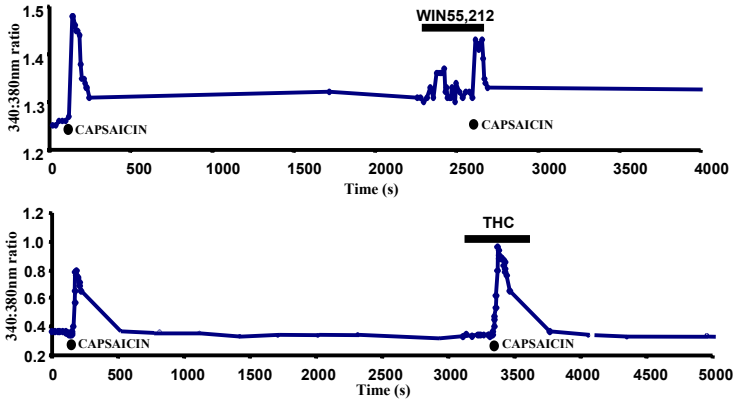

Figure 1 Representative trace of the effect of 1μM A) WIN55,212 and B) THC on the capsaicin-induced calcium response (100nm) in cultured adult dorsal root ganglion cells.

In the presence of 1μM WIN55,212 the capsaicin-evoked calcium response was significantly inhibited (65.6 ± 2.4%, n=80) compared with control experiments (79.1 ± 3.8%; n=35). However, 1μM THC augmented to capsaicin response (115.0 ± 8.3%, n=78) compared to control experiments (79.1 ± 3.8%, n=35). WIN55,212 alone produced a transient increase in $[Ca^{2+}]_i$, 47.7 ± 3.3% of the initial capsaicin response; THC alone produced no response.

These preliminary data indicate that WIN55,212 attenuates the capsaicin-evoked calcium response in cultured DRG neurones. WIN55,212 also evokes a calcium response when applied to DRG cells which may indicate a mechanism different to that of HU210 and THC. The ability of THC to enhance the capsaicin-evoked calcium response demonstrates opposing actions of cannabinoids in this system.

THE CHEMOTHERAPEUTIC AGENT CISPLATIN INCREASES BRAIN 2-ARACHIDONOYL-GYCEROL (2-AG) CONCENTRATIONS AND CONCOMITANTLY REDUCES INTESTINAL 2-AG AND ANANDAMIDE LEVELS IN A VOMITING SPECIES (THE LEAST SHREW)

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Cisplatin produces vomiting via release of a number of emetic neurotransmitters including dopamine and serotonin. Antagonists of dopamine D_2- and serotonin 5-HT_3-receptors are useful antiemetics against cisplatin-induced emesis. Previously, we have shown that cannabinoid CB_1/CB_2 agonists (Δ^9-THC, WIN 55, 212-2 and CP 55, 940) prevent cisplatin-induced vomiting via cannabinoid CB_1 receptors (1, 2, 3). In addition, exogenously administered 2-AG and its major metabolite arachidonic acid (AA) appear to be potent emetic agents, while anandamide is known to possess mild antiemetic activity (4) but may induce non-dose-dependent emesis (4). Furthermore, both the cyclooxygenase inhibitor, indomethacin, and Δ^9-THC prevent vomiting produced by exogenous administration of 2-AG or AA (4). The purpose of this study was to investigate whether: i) intraperitoneal administration of cisplatin can alter brain and intestinal levels of endogenous 2-AG and anandamide, and ii) selective inhibitors of endocannabinoid catabolic enzyme (fatty acid amide hydrolase = FAAH) (i.e. AA-5-HT and URB-597) and uptake inhibitors (i.e. OMDM1) would prevent emesis produced by either cisplatin (20 mg/kg, i.p.) or 2-AG (10 mg/kg, i.p.) or apomorphine (2 mg/kg, i.p.). Thus, different groups of shrews were treated with varying doses of cisplatin (0, 5, 10 and 20 mg/kg, i.p., n = 4-6 per group) for either 30 or 60 minutes. Following cisplatin exposure, shrew whole brain and intestine were isolated and frozen. Endocannabinoid concentrations were determined by the use of an isotope-dilution LC/MS technique following tissue extraction. Relative to vehicle-treated control groups, cisplatin caused dose- and time-dependent increases in brain 2-AG levels while the concentration of intestinal 2-AG was reduced correspondingly. On the other hand, while anandamide brain levels exhibited some enhancements in a non-dose-dependent manner following cisplatin administration, the chemotherapeutic agent dose-dependently reduced intestinal anandamide levels. However, a 10 min pretreatment with the cited inhibitors of FAAH and endocannabinoid uptake (2.5 - 10 mg/kg, i.p.), failed to modify emesis produced by either cisplatin (20 mg), 2-AG, or apomorphine. The cannabimimetic agents induced emesis by themselves at doses greater than 10 mg/kg. The results suggest that cisplatin increases central endocannabinoid levels, in particular brain 2-AG concentration, but simultaneously decreases their intestinal concentrations. The exact role(s) of endogenous 2-AG and anandamide in cisplatin-induced emesis in the least shrew remains to be elucidated.

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INHIBITORY EFFECT OF N-ARACHIDONOYLSEROTONIN, A FAAH INHIBITOR, ON GASTRIC AND INTESTINAL MOTILITY IN MICE

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Recent evidence indicates that the endocannabinoid anandamide may regulate intestinal motility through activation of CB_1 receptors. Furthermore, inhibitors of anandamide inactivation via the putative "anandamide membrane transporter" were found to inhibit intestinal motility under certain pathological conditions. However, inhibitors of endocannabinoid enzymatic hydrolysis have never been tested on gastrointestinal motility. In the present study, we have evaluated the effect of *N*-arachidonoylserotonin (AA-5HT), a selective inhibitor of the fatty acid amide hydrolase (FAAH, i.e. the enzyme mostly responsible for the hydrolysis of anandamide), on gastric emptying and upper gastrointestinal transit in mice.

Gastric emptying was evaluated by measuring with a spectrometer the amount of phenol red (administered orally into a standard meal) recovered into the stomach; upper gastointestinal transit was evaluated by measuring the distance traversed by a marker (given orally) from the pylorus to caecum. Drugs were given intraperitoneally.

AA-5HT, at the dose of 10 mg/kg, significantly reduced both gastric emptying (% reduction: 33±5, n= 5-6 $P<0.05$) and upper gastrointestinal transit (% reduction: 37±4, n= 5-6, $P<0.05$). The inhibitory effect of AA-5HT was reduced by the CB_1 receptor antagonist SR141716A (0.2 mg/kg), but not by the CB_2 receptor antagonist SR144528 (1 mg/kg).

These preliminary data suggest that FAAH might be a physiological regulator of gastric and intestinal motility. The CB_1 receptor, but not the CB_2 receptor, might be involved in such regulation. FAAH inhibitors might be used as templates for the development of new therapeutic drugs useful for some gastrointestinal disorders.

CANNABINOID-MEDIATED INHIBITION OF NICOTINIC ACH CURRENTS IN MYENTERIC NEURONS

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Pre-synaptic CB_1 receptor stimulation is thought to inhibit gut motility by reducing excitatory neurotransmitter release in the myenteric plexus (Coutts and Pertwee, **1997**, Br. J. Pharmacol., 121, 1557-1566). Myenteric neurons grown in primary culture allow patch clamp recordings from individual cells to study effects of cannabinoids on ion conductance. Immunohistochemical data has previously validated these neurons as a model for those in situ, demonstrating that all CB_1 receptor-positive cells express the cholinergic marker, cholinacetyltransferase (Coutts et al., **2003**, Int. Symp. G.I. Motility, #567). Nicotinic ACh (nACh) receptors are expressed on these neurons and their electrophysiological and pharmacological properties have been described (Zhou et al., **2002**, J. Pharmacol. Exp. Ther., 302, 889-897). A direct interaction of cannabinoid ligands with ion channels has previously been reported (Barann et al., **2002**, Br. J. Pharmacol., 137, 589-596; Nicholson et al., **2003**, Brain Res., 978, 194-204), in particular the endogenous cannabinoid anandamide has been shown to inhibit nicotinic currents through a direct effect on the channel (Oz et al., **2003**, J. Pharmacol. Exp. Ther., 306, 1003-1010). The aim of the present study was to investigate the effects and site of action of cannabinoids on nACh currents induced in myenteric neurons in primary culture.

Myenteric cultures were prepared from ileal segments of guinea pigs by a technique adapted from that of Barajas-Lopez et al. (**1993**). Nicotinic currents were measured using whole-cell patch clamp at a holding potential of –60 mV. Currents are expressed as pA per pF membrane capacitance to correct for cell size. Data are presented as mean ±S.E.M. ($n \geq 8$).

Nicotine (1mM) produced a transient inward current with a peak amplitude of 29.5 ±5.2 pA/ pF, which was significantly reduced by pre-treatment with the cannabinoid agonist CP 55, 940 (1 μM; 12.9 ±3.9 pA/ pF and 10 μM; 3.9 ±2.0 pA/ pF). SR 141716 was used to ascertain if the CP 55,940-induced inhibition of nicotine currents were mediated by CB_1 receptor activation. Interestingly SR 141716 (300 nM) alone significantly reduced nicotine responses (10.6 ±2.4 pA/ pF) but showed no significant reduction of the CP 55,940 (1 μM)-evoked inhibition (20.6 ±5.6 pA/ pF). This suggests that the cannabinoid-mediated inhibition is not entirely dependent on CB_1 receptor activation. The endogenous cannabinoid anandamide (1 μM) also significantly reduced the nicotine (1 mM)-induced current to a peak amplitude of 7.2 ±2.7 pA/ pF.

The effects of SR 141716 on the anandamide-induced inhibition will be explored further, as will the effects of palmitoylethanolamide, a compound whose cannabimimetic actions are thought to be independent of the known cannabinoid receptors (Lambert and Di Marzo, **1999**, Curr. Med. Chem., 6, 757-773). The results suggest that cannabinoids interact with nACh currents through a CB_1 independent pathway in myenteric neurons.

REDUCED LIVER FIBROSIS IN MICE INVALIDATED FOR CB_1 RECEPTOR

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Cannabinoids are the active components of marijuana and act via two G protein-coupled receptors, CB_1 and CB_2. We have shown that CB_2 receptors are upregulated in hepatic myofibroblasts during chronic liver diseases. Here, we studied the regulation of CB_1 receptors expression during chronic liver diseases, and evaluated the consequences of their inactivation on liver fibrogenesis.

Immunochemistry showed a faint expression of CB_1 receptors in normal liver, contrasting with a marked upregulation in cirrhotic samples of various etiologies, predominating in nonparenchymal cells within and at the edge of fibrous septa. Double immunohistochemistry identified myofibroblasts as a source of CB_1 receptors, and accordingly, CB_1 receptors were also expressed in cultured human hepatic myofibroblasts. The role of CB_1 receptors in the progression of liver fibrosis was studied in CB_1 receptor knock-out mice (CB_1KO, n=15) and their wild type counterpart (WT, n=12) in a model of chronic tetrachloride intoxication. Fibrosis and necroinflammation were assessed by a METAVIR-derived score. CB_1KO mice showed reduced fibrosis compared to WT animals (2.59±0.13 vs 3.33±0.13, $p<0.05$). Accordingly, hepatic collagen was decreased by 40 % in CB_1KO mice as compared with WT animals (0.46±0.06 vs 0.73±0.11 mg hydroxyproline /mg tissue, $p<0.05$). The necroinflammatory score was similar in both groups.

In conclusion, CB_1 receptors are upregulated in hepatic myofibroblasts during chronic liver diseases, and that their inactivation reduces the progression of liver fibrosis, suggesting a profibrogenic role of CB_1 receptors. Therefore, inactivation or blockade of CB_1 receptors may constitute a novel therapeutic approach for the treatment of liver fibrosis.

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ATYPICAL CANNABINOIDS AND THEIR ROLE AS BLOOD PRESSURE REGULATORS

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Cannabinoids exert a wide range of biological activities including the well-known psychotropic and hypotensive effects. Most of the effects are known to be caused by the activation of two receptors, CB_1 and CB_2.

Numerous endogenous and plant derived cannabinoids are vascular modulators (Mechoulam et al., **1992**); most of the effects are mediated by CB_1 receptors (Kunos et al., **2000**). Attempts to separate the hypotensive action of Δ^9-THC from its psychotropic properties have achieved only partial success (Zaugg and Kyncl, **1982**).

Recently a new pharmacological target (Abn-CBD sensitive receptor) was tentatively shown to be present in the endothelium of peripheral blood vessels (Jarai et al., **1999**). This putative receptor induces hypotension when activated.

Cannabigerol (CBG) is a non-psychotropic cannabinoid found in the plant and is believed to be involved in the biosynthesis of other cannabinoids such as Cannabidiol (CBD) and THC. It has been reported to be an anti-glaucoma agent (Colasanti, **1978**). We synthesized the dimethyl heptyl (DMH) homolog of CBG. CBG-DMH does not bind either to CB_1 or CB_2.

Adult male Sabra rats weighing 225 to 250 gr. had their femoral vein cannulated for i.v. drug administration. Anestesia was induced by the i.p. injection of Pentobarbital sodium 6%, 60-mg/kg. The femoral artery was cannulated and a catheter (PE 10 cannulae) was connected to a pressure transducer for continuous monitoring of blood pressure with a physiograph (AcKnowledge program).After a 30-min. stabilization period, the animals received either vehicle or the drug (varying from 1 to 10-mg/kg i.v.) injected in bolus i.v. doses in volumes $\leq$ 500 µl. Twenty minutes later, a single dose of the compound tested (either agonist or antagonist) or vehicle was administered and the changes in blood pressure were monitored for 60 minutes.

CBG-DMH caused hypotension in rats in doses of 5mg/kg without causing change in the heart rate. The effect was antagonized by CBD in similar doses. The hypotensive activity of CBG-DMH leads the way to a new class of atypical cannabinoids with no psychotropic activity and with a mechanism of action differing from the anti-hypertensive drugs known to date.

THE VASOCONSTRICTOR U-46619 BUT NOT SEROTONIN INCREASES ENDOCANNABINOID CONTENT IN THE MIDDLE CEREBRAL ARTERY: EVIDENCE FOR FUNCTIONAL RELEVANCE

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Cerebral vascular smooth muscle cells (VSMC) express the cannabinoid receptor (CB_1) and CB_1 agonists produce vasodilation (Gebremedhin et al., Am. J. Physiol. **1999**, 276, H2085). The purpose of the current studies was to determine whether cerebral arteries have "endocannabinoid tone" either at rest or during vasoconstriction. Therefore, we used two vasoconstrictors, 5-hydroxytryptamine (5-HT; serotonin) and the thromboxane A_2 mimetic, U-46619, and investigated both endocannabinoid content and vessel reactivity in the presence and absence of the CB_1 receptor antagonist, SR141716, in rat middle cerebral artery (MCA). Liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry was used to measure the endocannabinoids, N-arachidonylethanolamine (AEA) and 2-arachidonylglycerol (2-AG) following treatment of MCA with 5-HT or U-46619. Both AEA and 2-AG were detected in non-treated MCA. Whereas 5-HT (0.1, 10 nM) decreased AEA and 2-AG MCA content in an endothelium-independent manner, U-46619 (10, 100 nM) increased AEA and 2-AG MCA content in an endothelium-independent manner. For vessel reactivity studies, MCA were placed in a heated, superfusion and perfusion chamber and cannulated with glass micropipettes. Vessel diameter of endothelium-denuded MCA was determined by video microscopy. Consistent with the results of our previous study (Gebremedhin et al., Am. J. Physiol. **1999**, 276, H2085), Win 55212-2 (10–1000 nM) produced a concentration-dependent dilation of endothelium-denuded MCA with an EC_{50} of 21 nM. Consistent with evidence that MCA exhibit a resting endocannabinoid content, SR141716 (10-1000 nM) produced a concentration-dependent contraction of endothelium-denuded MCA with an EC_{50} of 240 nM. The presence of SR141716 (100 nM) in the bath had no effect on the dose-response curve to 5-HT (0.1-1000 nM); but resulted in a significant leftward shift in the dose-response curve to U-46619 (10-300 nM). The EC_{50} (95% CI) for U-46619 before treatment with SR141716 (281 nM (152 nM, 524 nM)) was significantly different compared to the EC_{50} (95% CI) after treatment with SR141716 (48 nM (26 nM, 88 nM)). Taken together, these data suggest that U-46619 negatively regulates its vasoconstriction of MCA by increasing endocannabinoid production by non-endothelial cells. By contrast, 5-HT positively regulates its vasoconstriction of MCA by decreasing endocannabinoid production by non-endothelial cells.

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CANNABINOID MODULATION OF STRESS-INDUCED AMYGDALA ACTIVATION AND NEUROENDOCRINE RESPONSES

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The most common adverse effects of cannabis intoxication are anxiety and panic, reactions that are potentiated by environmental stress. Data suggest cannabinoid modulation of amygdalar activity contributes to this phenomenon. Using Fos as a marker, we tested the hypothesis that there is an interaction between mild, environmental stress and CB_1 cannabinoid receptor activity in the regulation of amygdalar neuronal activity. Within the central amygdala (CeA), no increases in Fos expression were induced by either 30 min of restraint or CB_1 receptor agonist treatment (Δ^9-tetrahydrocannabinol (THC; 2.5 mg/kg) or CP55940 (0.3 mg/kg)) alone. However, the combination of restraint and CB_1 agonist administration produced robust Fos induction in the CeA; evidence for a synergistic interaction between environmental stress and CB_1 receptor activation. In contrast, an inhibitor of endocannabinoid degradation and transport, AM404 (10 mg/kg), produced only an additive interaction with restraint. Mice treated with the selective AEA degradation inhibitor (URB 597 1 mg/kg) and mice lacking fatty acid amide hydrolase (FAAH) did not exhibit any differences in amygdalar activation in response to restraint. Interestingly, the CB_1 receptor antagonist SR141716 alone also increased Fos expression in the CeA, which, at 5 mg/kg was only additive with restraint stress. In the basolateral amygdala (BLA), restraint stress alone produced a low level of Fos induction which was unaffected by any of the drug treatments. These data are consistent with the hypothesis that disproportional or hyperactive CeA activity could contribute to the expression of anxiety precipitated by the combination of cannabis administration and environmental stress in humans, and suggest EC signaling exerts a tonic suppression over amygdala activity. In addition, these data suggest that selective inhibitors of FAAH could have a low propensity to worsen anxiety or induce panic, and are therefore attractive candidates for the treatment of anxiety disorders.

Cannabinoids can also have profound effects on neuroendocrine function, and recent data suggest that endogenous cannabinoid (EC) signaling modulates hypothalamic-pituitary-adrenocortical (HPA)-axis function. We explored the role of EC signaling in 1) the tonic modulation of HPA axis activity, and 2) the responsiveness of this system to physiological activation. Systemic administration of the CB_1 receptor antagonist SR141716 (0.2, 1, and 5 mg/kg i.p.) produced a small, dose-dependent increase in plasma corticosterone in male mice. At 5 mg/kg, however, there was no significant increase in the activity of neurons within the paraventricular nucleus of the hypothalamus (PVN), as measured by induction of Fos protein. Exposure of mice to 30 minutes of restraint increased plasma corticosterone, but was not sufficient to produce a statistically significant increase in Fos expression within the PVN. However, administration of SR141716 (5 mg/kg) prior to restraint resulted in a robust potentiation of restraint-induced corticosterone release and a robust induction of Fos protein within the PVN. In addition, a low dose of the CB_1 agonist CP55940 and the indirect agonists AM404 both decreased restraint-induced corticosterone release. These data indicate that EC signaling tonically inhibits HPA axis activity, and physiological activation of the HPA-axis, by restraint stress, results in recruitment of EC signaling, which serves as a negative feedback mechanism to attenuate stress-induced neuroendocrine activation. These data provide insight into the mechanisms subserving the anxiolytic effects of cannabinoid agonists and the role of EC signaling in the regulation of neuroendocrine function.

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THE ENDOCANNABINOID SYSTEM LOWERS THE STRESS RESPONSE

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The Endocannabinoid-CB_1 receptor system is involved in the stress response. However the nature of the association is unclear. Thus whereas cannabinoids have anxiolytic effects, anxiogenic activities have been shown after administration of exo-and endocannabinoids, accompanied by activation of the hypothalamic-pituitary-adrenal stress axis. Morover, it is not clear whether an enhanced stress response is maladaptive, or rather, an appropriate coping mechanism.

In the present series of experiments, we exposed wild type C57BL/6 as well as $CB_1^{-/-}$ receptor deficient mice* to two types of stress (bell noise or forced swimming) and measured three different parameters of stress. In addition we were interested in the stress response in developing normal as compared to $CB_1^{-/-}$ receptor knockout pups.

The effects of stress were assayed by the stress hormone corticosterone (CCS), ultrasonic vocalizations (USV's, which have been used to assess the stress response) and by motor performance/freezing in an open field. Pups were assessed for maternal separation stress-induced USV's between day 1 and 16 of age.

Male C57BL/6 and $CB_1^{-/-}$ receptor knockout mice were exposed to 4 min of (school) bell noise. Six min later, trunk blood was collected for analysis of corticosterone. In the second series of experiments, mice were exposed to similar bell noise stress, after which USV's and motor activity were recorded for one hour. In the last series experiments mice were forced to swim for 2 min, after which USV's and motor activity were recorded, again for one hour.

Results: Bell stress significantly increased corticosterone levels in wildtype mice. Baseline levels of $CB_1^{-/-}$ mice were as high as the stressed wildtypes, but the stress did not further increase the level of corticosterone. USV's in unstressed animals were higher in knockout mice. Bell stress increased USV's in wild types, but, in contrast, reduced the rate of USV's in the knockouts. Water stress, similarly to bell stress, increased USV's in wildtypes but did not elicit a response from the knockouts.

Bell noise did not affect motor activity in wild types but inhibited movements in the knockouts. The more severe forced swimming stress, inhibited movements in wild type mice, whereas it caused complete freezing of movements in the knockouts.

Finally, during postnatal development, $CB_1^{-/-}$ knockout pups, emitted fewer USV's compared to wild type pups. This was significant between days 6 and 10 and again on days 14-16.

These observations suggest that stress activates the endocrine system and ultrasonic communication in normal animals but "paralyzes" these responses in CB_1 receptor knockout mice. This is supported by the swim stress-induced "freezing" of motor movements in the CB_1 receptor knockout mice.

We conclude that CB_1 receptors are required to mount an effective stress response, while the severity of the stress stimulus determines the extent of the CB_1 receptor-mediated involvement.

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PHARMACOLOGICAL ENHANCEMENT OF CB_1 RECEPTOR SIGNALING ELICITS AN ANTIDEPRESSANT EFFECT IN THE RAT PORSOLT FORCED SWIM TEST

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Recent evidence has demonstrated that cannabinoid signaling becomes compromised during stress, and thus suggests that deficient endocannabinoid activity may play a functional role in the pathogenesis of depression. Enhancement of cannabinoid activity is known to result in mood elevation in humans, which leads to the suggestion that endocannabinoids may be a target for antidepressant pharmacotherapy. To assess whether enhanced CB_1 receptor activity may possess antidepressant properties we assessed various pharmacological manipulations on this system and examined how they affect behaviour in the rat Porsolt forced swim test, an animal model of screening for antidepressant efficacy. Results demonstrated that administration of AM404, which results in increased brain endocannabinoid content, caused a dose dependent decrease in immobility in the forced swim test, suggestive of its antidepressant properties. To futher assess the role of CB_1 activity in the forced swim test we used the selective CB_1 receptor agonist HU-210, which at doses of 5 and 25 ug/kg, elicited a reduction in immobility. Interestingly, administration of the CB_1 receptor antagonist AM 251 had no effect on immobility alone. Ultimately, this data suggests that enhancement of CB_1 receptor signaling results in antidepressant effects in the forced swim test, and suggests that future research should be geared at examining as to whether elevation of endogenous cannabinoids may be a suitable target for the pharmacotherapy of affective and stress related disorders.

SIGNIFICANT CORRELATIONS BETWEEN CANNABINOID AND SEROTONIN/GLUTAMATE RECEPTOR DENSITIES IN THE ANTERIOR CINGULATE CORTEX IN SCHIZOPHRENIA: A SITE OF FUNCTIONAL INTERACTIONS?

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We have recently reported changes in the cannabinoid system in the anterior cingulate cortex (ACC) of patients suffering schizophrenia. More specifically, using the selective CB_1 antagonist [^{3}H]SR141716A, and quantitative autoradiography on ACC sections we found a significant increase in [^{3}H]SR141716A specific binding to CB_1 receptors in the schizophrenia group as compared to the control group (mean ± S. E. M.: 46.15±6.22 versus 28.02± 4.20 fmoles/mg estimated tissue equivalents; p=0. 03). Using the same technique and consecutive ACC sections from the same schizophrenia cases used in the above study we have also found reduced density of serotonin 5HT2 receptors (targeted with [^{3}H]spiperone) and increased density of AMPA and MK801 receptors (targeted with [^{3}H]AMPA and [^{3}H]MK801 respectively) in the schizophrenia group as compared to the control group. CB_1 receptor binding was quantified across all layers of the ACC, 5HT2 binding across layers III-V and AMPA and MK803 binding across layers II-III. Most specifically, we found significant Spearman's nonparametric correlations between CB_1 and $5HT_2$ receptor binding (r=-0.762, p=0.004) and CB_1 and AMPA receptor binding (r=0.650, p=0.022). The topographic co-occurrence of CB_1 and other neurotransmitter receptor abnormalities in the ACC in schizophrenia may signify important functional interactions with underlying impaired neural circuits. Further investigation of these interactions would help not only to construct specific hypotheses regarding disturbed neural circuits in schizophrenia but may also assist in the designing of novel therapeutic interventions.

CLINICAL RESULTS WITH RIMONABANT IN OBESITY

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Rimonabant (SR 141716) is a cannabinoid CB_1 receptor antagonist showing high selectivity for the central CB_1 receptor compared to the peripheral cannabinoid CB_2 receptor in rat tissues and in CHO cells expressing human CB_1 and CB_2 receptors.

The compound also selectivity antagonises pharmacological responses elicited by the cannabinoid receptor agonists such as WIN 55212-2. A great deal of interest has recently been centred on the role of CB_1 receptors and eating behavior as they appear to be largely distributed in brain areas involved in the control of feeding behavior (i.e. lateral hypothalamus, limbic system) and additionally seem to be implicated in food intake control. Results show that endocannabinoids may tonically activate the CB_1 receptors to maintain food intake, and increase the incentive value of food as well as reinforcing the rewarding effects of nicotine involving the brain reward circuits. Further evidence shows that the CB_1 receptors may be involved in the motivational aspects of eating by enhancing the satisfaction derived from eating through activation of the meso-limbic dopaminergic system. All this evidence would seem to indicate that specific CB_1 antagonists like rimonabant should have some effect in body weight control and it has been shown that rimonabant reduces the appetite and rewarding properties of food and drink in numerous pharmacological models. Studies in a diet-induced obesity model in mice, widely used for research in the human obesity syndrome, have shown that the compound during a 5-week treatment induced a transient reduction in food intake with a marked but sustained reduction of body weight. Rimonabant has no effect in CB_1 receptor knockout mice, which confirmed the implication of CB_1 receptors in the activity of the compound. Previous Phase II Clinical studies have shown that it reduced hunger, caloric intake and body weight in obese patients. A Phase III study (1 year treatment) in obese patients with hyperlipidemia as a comorbidity has shown a very significant reduction in body weight which is maintained throughout the 52 weeks with a compensatory reduction in the waist circumference. Over 72% of patients at 1 year showed a weight loss of >5% with over 44% showing a weight loss of >10%. There was also an increase in HDL-cholesterol values, a reduction in triglyceride values and reductions in glucose and insulin values after an oral glucose tolerance test. The general tolerance of the compound was excellent. In addition, the compound is being evaluated in smoking cessation, and preliminary results from Phase III studies have shown an increased abstinence with the compound with a prevention of the secondary weight gain often seen in this situation.

INVESTIGATION OF THE POSSIBLE NON-MOTIVATIONAL FACTORS THAT COULD CONTRIBUTE TO THE SUPPRESSION OF FEEDING PRODUCED BY CB_1 ANTAGONISTS

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Previous work from our lab suggests that CB_1 inverse agonists such as AM251 have therapeutic promise as appetite suppressants. However, it is possible that other factors, such as the induction of behaviors incompatible with feeding, motor impairments, or the induction of food aversions, could contribute to the suppression of feeding produced by these drugs. We have previously reported that AM251 induces grooming. Further analysis of the behavioral satiety sequence indicates that while this behavior differs in some respects from normal grooming, eating bouts do not appear to be markedly disturbed by them. Using an FR5 operant schedule, we also examined alterations in food-reinforced behavior produced by AM251, and compared these effects with those produced by prefeeding and administration of the serotonergic appetite suppressant d-fenfluramine. Pre-feeding to reduce food motivation suppressed food reinforced lever pressing, with the predominant effect being a reduction in time spent responding. Pre-feeding did not substantially reduce high local rates of responding as measured by the interresponse time distribution. In contrast, both fenfluramine and AM 251 suppressed the relative number of fast interresponse times, as well as time spent responding. Finally, we examined the effects of AM251 on food handling and feeding rate. While there was a dose-related reduction in food intake, handling and feeding rate were minimally impaired. Thus far, it does not appear that the anorexia produced by AM251 results from severe impairments in motor control, though the extent to which subtle drug-induced changes in motor function affect food intake is unclear. Putative appetite suppressants such as AM251 (as well as d-fenfluramine) are distinct from prefeeding in terms of their effects on food-related behavior. Moreover, additional research needs to focus on the possibility that AM251 or other CB_1 antagonists or inverse agonists can induce food aversions that lead to the suppression of food intake.

Δ^9-THC BUT NOT WIN55,212-2 PRODUCES BIPHASIC EFFECTS ON THE GROWTH OF MULTIPLE HUMAN GLIOMA CELL LINES

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Activation of the two known cannabinoid receptors, CB_1 and CB_2, can produce apoptosis in multiple types of cancer cell lines and reduce the growth of tumors[1]. The result of the accumulation of these promising data has led to the acceptance of a clinical trial for the treatment of human brain cancer with a cannabinoid. Human gliomas are highly heterogeneous and vary in their response to therapeutic treatments. We wanted to determine how this heterogeneity would be reflected in the response of gliomas to different classes of cannabinoids. The effects of Δ^9-THC and WIN 55,212-2 (WIN) on cell growth were compared against five glioma cell lines: U87 MG, SF188, SF126, U251, and U373 MG.

All human cancer cell lines were treated for seven days with multiple concentrations of Δ^9-THC and WIN in 1% serum, 0.1% or serum free media. The serum concentrations were varied because previous studies, in a rat glioma cell line, demonstrated that the sensitivity to the antiproliferative effects of classical cannabinoids increases with decreasing serum concentrations[2]. At the end of the treatment periods, cell proliferation was measured using the MTT assay. Percent control was calculated as the absorbance of treated cells/control cells x 100. The corresponding EC_{50} and E_{max} values were calculated for each cell line. Both cannabinoids killed 100% of all the glioma cell lines by day seven but with varying potencies. In 1% serum the EC_{50} values of Δ^9-THC ranged from 3.1 μM to 6.8 μM. The EC_{50} values of WIN ranged from 0.86 μM to 1.9 μM. In 0.1% serum the potency of Δ^9-THC was increased approximately 1.9 to 6.8 fold. The EC_{50} values ranged from 0.84 μM to 1.1 μM. The potency of WIN remained the same in 0.1% serum compared to 1%. The EC_{50} of WIN ranged from 0.84 μM to 1.7 μM. The activities of both compounds in serum free media were similar to that observed in 0.1% serum. The EC_{50} of Δ^9-THC ranged from 0.82 μM to 1.1 μM. The EC_{50} of WIN was 0.93 μM to 2.1 μM. Overall, SF126 was most sensitive to the antiproliferative effects of both Δ^9-THC and WIN and U373-MG was the least sensitive. At 100 nM of Δ^9-THC but not WIN a small stimulation of cell growth was observed with the SF126 = 140 % (± 36), U373-MG = 121 % (± 3), and U251 = 139 % (± 35) cell lines.

Δ^9-THC and WIN can inhibit the growth of multiple human glioma cell lines. The potency of Δ^9-THC but not WIN is increased in 0.1% or serum free media. Both compounds are equally effective at inhibiting glioma cell growth under the reduced serum conditions. The data also show that certain classes of cannabinoids exert a biphasic response on the growth of multiple types of human gliomas. The most identifiable is an inhibition of cell growth produced at higher concentrations. However, in some glioma cell lines there was also a small stimulation of cell growth produced at low nanomolar concentrations of Δ^9-THC.

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ROLE OF ENDOCANNABINOIDS IN TUMOUR PROGRESSION: ANANDAMIDE INHIBITS CELL MIGRATION IN HUMAN BREAST CANCER CELLS

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The endogenous cannabinoid system regulates cell proliferation in human breast cancer cells. We have previously observed that anandamide inhibits the proliferation of human breast cancer cells (HBCCs) by blocking the G_0/G_1-S phase transition of the cell cycle through interference with CB_1 receptor-coupled signal transducing events (De Petrocellis et al., PNAS, **1998**). It appears that endocannabinoids can act as selective inhibitors of human breast cancer cell proliferation through a growth-factor-dependent mechanism (Melck et al., Endocrinology, **2000**). We have also shown that a metabolically stable anandamide analog (Met-F-AEA) stops the growth of K-ras-dependent tumors, induced and/or already established, in vivo and it inhibits metastatis in the Lewis lung carcinoma model, two effects that are mediated by CB_1 receptors (Bifulco et al. FASEB J. **2001**, Portella et al. FASEB J. **2003**). Therefore, we reasoned that endocannabinoids could induce a non-invasive phenotype in breast metastatic cells. In order to investigate this hypothesis we used: i. MDA-MB-231 cells, an highly invasive and metastatic human breast cancer cell line, characterized by constitutive activation of K-ras; ii. TSA-E1 cells, a murine breast cancer cell line.

Met-F-AEA strongly reduced proliferation of MDA-MB-231 and TSA-E1 breast cancer cells. The hypothesis that CB_1 receptor stimulation could interfere with metastatic processes was tested in a model of metastatic infiltration in vivo, the murine breast cancer cell line (TSA-E1) in singenic C57Bl/6 mice. Met-F-AEA significantly reduced the number and dimension of metastatic nodes in a way antagonized by SR141716A. Furthermore Met-F-AEA inhibited MDA-MB-231 and TSA-E1 cell migration, evaluated by an in vitro migration assay on collagen, and this effect was antagonized by SR141716A. In order to understand the molecular mechanism involved in these processes and clarify further the role of the endocannabinoid system in tumour progression, we analysed the activity of FAK and Src, two tyrosine kinases involved in the processes of cell migration and adhesion. We observed a decreased activity of both FAK and SRC after incubation with Met-F-AEA, in a way antagonized by SR141716A.

We propose that MET-F-AEA, by modulating FAK tyrosine phosphorylation, can inhibit tumor cell invasion and metastasis. These data suggest that CB_1 receptor activation can represent a therapeutic strategy to retard not only the growth of breast carcinomas but also to inhibit their metastatic diffusion in vivo.

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MECHANISM OF CANNABINOID-INDUCED APOPTOSIS OF LEUKAEMIA CELL LINES

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Although during the last few years research from several laboratories has ravelled the therapeutic potential of cannabinoids as antitumoral agents, the mechanisms involved in these antiproliferative actions have been only partially elucidated. Here, we employed the human leukaemic cell lines Jurkat and SupT1 -that had been previously shown to undergo apoptosis in response to cannabinoids- to gain insight into the intracellular pathways linking activation of the CB_2 receptor and apoptosis of tumoral cells.

Cells were incubated with cannabinoids in the presence or absence of several inhibitors and antagonists, and cell viability, apoptosis, ceramide levels and different enzyme activities were determined. Western blot analyses were performed to measure expression, distribution and phosphorylation of several proteins.

Challenge of Jurkat and SupT1 cells to different cannabinoid receptor agonists and antagonists show that apoptosis occurs via CB_2 receptor. Regarding intracellular signaling, data show that: (i) the pro-apoptotic effect of cannabinoids was prevented by pharmacological abrogation of the ceramide synthesis *de novo* pathway; (ii) cannabinoid treatment triggered a rapid accumulation of ceramide and a parallel stimulation of serine palmitoyltransferase activity; (iii) activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase was observed after cannabinoid challenge; (iv) cannabinoid treatment led to cytochrome c release from mitochondria and to activation of caspase 3.

Taken together, these data provide clues about the mechanism of cannabinoid pro-apoptotic action, and point to the involvement of *de novo*-synthesized ceramide in the process of CB_2-induced apoptosis of human leukaemia cells.

EFFECT OF PLANT CANNABINOIDS ON CANCER CELL PROLIFERATION

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Both plant cannabinoids and endocannabinoids are known to exert anti-proliferative and pro-apoptotic effects on various cancer cell lines by acting at both cannabinoid CB_1 and CB_2 receptors and non-cannabinoid receptors (e.g. vanilloid TRPV1 receptors) (Bifulco and Di Marzo, Nat. Med., **2002**; Guzman, Nature Cancer Rev, **2003**). Apart from Δ^9-tetrahydrocannabinol (THC), also the non-psychotropic cannabidiol (CBD), which is almost inactive at cannabinoid receptors, inhibits glioma growth both in vitro and in vivo through induction of apoptosis (Jacobsson et al., **2000**; Massi et al., **2003**). In the present study, several plant cannabinoids, including THC, CBD, and the non-psychotropic cannabigerol (CBG), cannabichromene (CBC), CBD-COOH and THC-COOH, were tested on the growth of several cancer cells in vitro, including: MCF-7 and MDA-MB-231 human breast carcinoma, DU-145 human prostate carcinoma, Caco-2 human colorectal carcinoma, AGS human gastric adenocarcinoma, C6 rat glioma cells and RBL-2H3 rat basophilic leukaemia cells. Cells were seeded in 6-well dishes at an initial density of 50,000 cell/well and then treated every day with increasing concentrations of each drug, drug combination or extract for four days. On the fifth day from seeding, live cells were counted with the crystal-violet assay. THC, CBD, CBG, CBC, CBD-COOH and THC-COOH were also tested on human TRPV1 over-expressed in HEK-293 cells.

Of all compounds tested, CBD was always the most potent inhibitor of the proliferation of human breast carcinoma, Caco-2 human colorectal carcinoma, C6 rat glioma cells and RBL-2H3 rat basophilic leukaemia cells, with IC_{50} values around 8 μM. In these cell lines, the CBD was always the most potent inhibitor of cell proliferation, CBG the second most potent, and CBD-COOH or THC the least potent. CBD-rich *Cannabis* extracts were significantly more potent than THC-rich extracts, and were slightly more potent than pure CBD (tested at concentrations identical to those present in the extracts) only for C6 and MCF-7 cells. Sub-effective concentrations (4-8 μM) of either CBD or THC were also tested in combination with each other or with each of the other four cannabinoids on MCF-7 cells. The effects of other cannabinoids were generally additive to those of THC, and occlusive to those of CBD. THC and CBD inhibited the growth of DU-145 human prostate carcinoma and AGS human gastric adenocarcinoma cells only at the highest concentration tested (25 μM).

Although a most thorough analysis of the mechanism of the anti-proliferative actions of non-psychotropic cannabinoids must be performed before drawing any definitive conclusion, our data suggest that these effects are due to possibly common and non-CB_1 non-CB_2 mediated mechanisms. Furthermore, we found that, unlike CBD (Bisogno et al., **2001**), CBG and CBC, which also exert potent anti-proliferative effects, do not activate TRPV1 receptors, thus ruling out the participation of these receptors in these effects of plant cannabinoids. Our data indicate that *Cannabis* extracts and non-psychotropic plant cannabinoids might be promising anti-proliferative agents for some types of cancer cells.

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MARIJUANA SMOKING AND HYPERMETHYLATION OF THE PROMOTER REGION OF THE O^6(6)-METHYLGUANINE DNA METHYLTRANSFERASE GENE (MGMT) DNA REPAIR GENE MGMT

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We have previously shown overexpression of molelcular markers of pre-tumor progression (EGFR and Ki-67) in endobronchial biopsies from 12 habitual smokers of marijuana alone (MS), compared to 28 nonsmokers (NS) and 14 tobacco-only smokers (TS), suggesting that marijuana (M) may exert a "field cancerization" effect on bronchial epithelium resulting in an increased risk for lung cancer (*Barsky et al. JNCI **1998**; 90:1198-1205*). Similar to the bronchial mucosa, the mucosa of the buccal cavity is exposed chronically to carcinogenic constituents in the mainstream smoke from habitually inhaled M and/or tobacco (T) (T) and thus comprises part of the cancerization field. We therefore extended studies involving genetic injury in the bronchial mucosa of MS to include upper airway cells obtained by swabs of the buccal mucosa. Buccal cells were obtained from 93 participants in a cohort study of the pulmonary effects of habitual use of M and 1,910 participants in an epidemiologic case-control study of M use and the risks of lung and other cancers. Thus far, analysis has been performed on DNA extracted from buccal cells from 138 of these subjects: 26 MS (20 current, 6 former); 39 TS (14 current, 25 former); 38 smokers of both M & T (MTS, 14 current both, 10 former both, 14 current one only); and 35 NS. A methylation-specific PCR technique was used to detect hypermethylation of the gene promoter region of the DNA repair gene *MGMT*, which leads to *MGMT* gene suppression and is a common event in the development of many cancers. Preliminary results showed that *MGMT* promoter hypermethylation was detectable in 37% of NS, 44% of TS, 42% of MS and 46% of MTS. The odds ratio was 1.31 (95% C.I.: 0.51,3.32) for current TS, 1.24 (0.44,3.50) for current MS and 1.523 (0.60,3.88) for current MTS. Multiple logistic regression adjusted for age, M joint-yrs, T pack-yrs and current vs. former use did not show obvious a significant effects of either T or M. These preliminary results suggest that marijuana and/or tobacco use may lead to hypermethylation of the *MGMT* gene in buccal cells that are exposed to the actively inhaled smoke. However, Since buccal cells that have already been collected from a much larger number of subjects, including smokers of one or both of these substances, we will be able to assay methylations of the *MGMT* gene and other tumor suppressor genes to have aachieve a much higher power to need to be analyzed for statistical confirmation of these preliminary observations. Future analysis will also need to account for possible gene-gene interactions involving leading to varying genetic susceptibility to neoplasia. For this purpose, we are genotyping polymorphisms of glutathione S-transferase (GST) genes in buccal cell samples, including GSTM1, GSTT1 and GSTP1, which could influence susceptibility to smoking-related respiratory cancer.

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CANNABINOID CB_2 RECEPTOR AND HUMAN OSTEOPOROSIS

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We previously reported progressive expression of CB_2, but not CB_1 mRNA during osteoblastic differentiation. In this study we report the presence of CB_2 receptors in trabecular and diaphyseal osteoblasts and osteoclasts. Analysis of the skeletal phenotype in sexually mature CB_2 deficient mice, which are otherwise normal, demonstrates a low bone mass (LBM) phenotype accompanied by increases in osteoblast activity and osteoclast number as well as cortical expansion. These features are reminiscent of post-menopausal bone loss in humans, which in addition to cortical expansion features increases in bone formation and resorption with a net negative balance. In line with the LBM phenotype in *CB_2* knockout mice we found that HU-308, a specific CB_2 agonist, stimulates dose dependently the number of preosteoblasts derived from primary cultures of bone marrow stromal cells. Using the same dose range, HU-308 also restrains osteoclast differentiation of bone marrow derived monocytes. These activities are not shared by noladin ether, a CB_1 specific ligand. Most importantly, HU-308 attenuates ovariectomy-induced bone loss mainly by inhibiting osteoclast number. These data assign for the first time a physiologic role to CB_2. The regulation of bone remodeling via functional expression of CB_2 in bone cells offers novel molecular and pharmacological targets for the treatment of osteoporosis.

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HUMAN CB_2 RECEPTOR IS ASSOCIATED WITH OSTEOPOROSIS

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Mice with a deletion in either the *CB_1* or *CB_2* gene (*Cnr1/2*) have a low bone mass (LBM) phenotype. To investigate whether the causative role of cannabinoid receptor dysfunction in decreasing bone mass also applies to the development of the complex inherited human osteoporosis, we performed genetic association studies in a case-control approach. The sample consisted of 163 osteoporotic women and 208 ethnically and age matched female controls with normal bone density. Human *CNR1* is located on the long arm of chromosome 6 (6q15). We typed four single nucleotide polymorphisms (SNPs) covering a region of about 20 kb and encompassing the single coding exon by using commercially available TaqMan®-SNP assays. The genotypic distributions of patients and controls did not deviate significantly ($p \geq 0.05$) from those expected from the Hardy-Weinberg equilibrium. As in *CNR1*, the coding sequence of *CNR2* consists of a single exon. The genotypic distributions of patients and controls were in Hardy-Weinberg equilibrium ($p \geq 0.30$). We typed SNPs covering a region of about 100 kb. For one of the silent polymorphisms in the *CNR2* we found a highly significant difference of allelic (p=0.00119) and genotypic (genotype 11 *vs.* 22; p=0.00057) distributions, strongly arguing for a causative involvement of this locus with human osteoporosis.

Our case-control study with its highly significant association of the human *CNR2* coding region together with the LBM of CB_2 knockout mice strongly suggests a causative role for CB_2 in regulating bone mass in humans.

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EVIDENCE FOR A REGULATORY ROLE OF ENDOCANNABINOID SYSTEM IN THE RAT PINEAL GLAND ACTIVITY

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Extensive studies demonstrated pineal gland activity exhibits a circadian rhythm. During the night norepinephrine (NE), released from sympathetic terminals, activates adrenoceptors on pinealocytes to stimulate N-Acetyl transferase (NAT), the key enzyme in melatonin (ME) biosynthesis. At present, considerable evidence suggests that NE is not the unique factor involved in such regulation. Along this line, results showing cannabinoid receptor agonists modulate NE release from postganglionic nerves have raised the hypothesis that cannabinoid system may be part of the molecular mechanism underlying ME synthesis. The present study has been performed to evaluate this assumption. Exposure of primary cultures of rat pineal to (+) WIN 55,212-2 (1-10μM) decreased, of 50% and 80% respectively, the isoproterenol-induced NAT/PBGD mRNA expression assessed by quantitative RT-PCR. Conversely the low affinity stereoisomer (-) WIN 55,212-2 did not exert any significant inhibition of NAT mRNA expression. These in vitro results paralleled the findings of the in vivo experiments in which WIN 55.212-2 (0.5-1-2 mg/Kg) antagonized the nocturnal increase of melatonin synthesis (from 986±47pg/gland to 390±38 pg/gland at 0.5mg/Kg, and to 278±24 g/gland at 1mg/Kg ($p<0.001$). In line with these data, HU-210 (0.25mg/Kg and 1 mg/Kg) respectively reduced the physiological increase of ME content occurring during darkness from 3808±37 pg/gland to 1684±23 pg/gland ($p<0.004$) and to 408±48 pg/Kg ($p< 0.003$), whereas also in vivo (-) WIN 55,212-2 did not modify pineal activity. Furthermore evidence has been provided, by western blotting, that CB_1 and CB_2 receptors are highly expressed in pinealocytes. In addition in the present study a CB_1 receptor antagonist (SR141716A) failed to antagonize at different doses both in vitro and in vivo the above reported decreasing effects, whereas a CB_2 receptor antagonist (SR144528) did, in a dose-dependent manner. Finally, the endocannabinoid content was detected (by GC-MS) in the pineal glands of rats sacrificed either during the day and the night. 2-AG fluctuated from 163±15.2 pmol/mg during the night, to 111±6.1 pmol/mg during the day, while anandamide did not show significant circadian changes. The data above reported, provide evidence that endocannabinoids system play a physiological role in regulating rat pineal activity during the night, and CB_2 receptor is the site implicated in this modulation.

CANNABINOIDS MODULATE THE LIGHT RESPONSE IN GOLDFISH CONES

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Psycophysical data suggest that cannabinoids enhance photosensitivity in human subjects. Previously it has been shown that goldfish cone photoreceptors: contain CB_1 receptors at the synaptic terminal, selectively accumulate ^{3}H-anandamide, contain fatty acid amide hydrolase-immunoreactivity, and voltage-gated calcium and potassium currents are modulated by CB_1 ligands (Yazulla et al **2000**, Vis Neurosci 17:391; Fan and Yazulla, **2003**, Vis Neurosci 20:177; Glaser et al., **2004**, Vis Neurosci, **In Press**). These data suggest a retinal mechanism for the psychophysical effects of cannabis. To this end we studied the effect of a cannabinoid agonist on cone light responses and adaptation. Whole-cell patch clamp recordings were made of cones in the isolated goldfish retina. The cells were stimulated with a spot of light of variable wavelength and intensities in combination with voltage- and current-clamp protocols. Pharmacological manipulation was performed with bath application of the cannabinoid agonist WIN 55212-2 (10 μM), as well as modulation of the extracellular calcium concentration. WIN had no effect on the absolute sensitivity of the cones or on the kinetics of the onset response. However, there were significant effects on the light offset response in that the offset response became faster and the depolarizing overshoot was enhanced. This was found under current-clamp as well as under voltage-clamp conditions, indicating that the effect of WIN was mediated directly or indirectly by modulation of the cGMP-gated channels in the outer segment of the cones rather than by voltage-dependent currents. The effects of WIN were absent in a low calcium medium, a condition that suppresses the calcium-dependent dark-adaptation pathway (Nakatani & Yau **1988**, J. Physiol. 395:731). Given that WIN accelerated the cone offset reponse, we used sinusoidal stimulation and found that WIN significantly increased the flicker fusion frequency of cones. In summary, cannabinoids speed up the dynamics of the calcium-dependent dark adaptation pathway in cone outer segments. The functional consequence of this effect is to shorten the recovery time to the offset of bright flashes, resulting in an increase in flicker response and perhaps accounting for an apparent increase in sensitivity.

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TISSUE DISTRIBUTION OF 2-AG, PEA, ANANDAMIDE AND PROSTAMIDES E_2 AND $F_{2\alpha}$ IN FAAH -/- AND FAAH +/+ MICE

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The prostamides (prostaglandin ethanolamides) have recently been described as pharmacologically unique. Their distributio was analyzed by isotope-dilution liquid chromatography-mass spectrometry and compared to that of anandamide, 2-AG, and PEA in various tissues from FAAH +/+ and FAAH -/- mice. A lipid with the same molecular ion and chromatographic retention time as prostamide $F_{2\alpha}$ was detected in every tissue examined (brain, lung, spleen, colon, heart and eye) from both FAAH +/+ and -/- mice (8-100 pmol/g wet tissue weight). Highest levels were found in the eye, lung and spleen. A component with the same molecular ion and chromatographic properties as prostamide E_2 was usually less abundant or below the detection level. In agreement with previous studies, comparison of 2-AG and anandamide levels revealed marked differences between tissues. In brain, colon, heart, lung and spleen, 2-AG levels (2-17 nmol/ g) were up to 250-fold greater than anandamide (25-155 pmol/ g tissue). However, as found in the human eye (see Poster communication by Matias et al. at this meeting), these very high levels of 2-AG were not detected in the mouse eye where they were in a similar range to anandamide (414±16 and 101± 3 pmol/g tissue, respectively). Levels of N-palmitoyl ethanolamide (PEA) in the eye were extraordinarily high (~ 6 nmol/g tissue), as abundant as in the colon and about 5 to 10-fold higher than in other tissues. In FAAH -/- mice, higher levels of either AEA or PEA, or both, were found in the brain, eye and spleen. In mice treated with LPS (2 mg/ml x 0.1 ml, subplantar) slightly higher levels of prostamides, and lower levels of PEA, were observed in FAAH -/- mouse brain and eyes.

EFFECTS OF ANANDAMIDE ON HUMAN NEUTROPHIL MIGRATION

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In HEK cells transfected with the CB_1 receptor, anandamide induces cell migration with an EC_{50} of ~40nM, an effect that is PTX sensitive and blocked by SR141716A. Neutrophils play a critical role in the development of inflammation. Cannabinoid CB_2 receptors are expressed on polymorphonuclear neutrophil cells (PMNs). The aim of this study was to investigate the effect of anandamide on the migration of human neutrophils.

Peripheral polymorphonuclear neutrophils were isolated from normal whole blood by centrifugation over Polymorphprep™. The isolated cells were resuspended at a concentration of $1x10^6$ $cellsml^{-1}$ in phosphate buffered saline containing $CaCl_2$ and $MgCl_2$. *In vitro* cell migration assays were performed using a modified 48-well Boyden Chamber. Incubation lasted 30 minutes in a 5% CO_2 atmosphere at 37°C. After incubation, the migrated adherent cells on the underside of the 3μm pore filter were stained using a Diff-Quik stain set. Each well was counted in ten non-overlapping fields (x40) using a light microscope.

First, we investigated whether anandamide (0.1nM – 1μM) induced migration of human neutrophils. In this series of experiments anandamide was placed in the lower wells of the Boyden chamber. N-formyl-methione-leucine-phenylalanine (fMLP), a well-established chemoattractant peptide was used at a concentration of 1μM as positive control. Anandamide had little effect on neutrophil migration; migration being 7.29 ± 6.87%, 14.47 ± 4.78, 10.86 ± 7.59 and 6.86 ± 3.50% of the fMLP control at 1nM, 10nM, 100nM and 1μM respectively.

Next we investigated whether anandamide inhibited neutrophil migration induced by fMLP (1 μM). Neutrophils were pre-incubated with anandamide (0.1nM - 100nM) or vehicle (0.01% DMSO) for 30 minutes at 37°C before being loaded into the wells of the upper chamber. The lower wells contained the corresponding concentration of anandamide and fMLP (1μM). Under these conditions, fMLP-induced neutrophil migration in the cells pre-exposed to vehicle was 2174 ± 597 cells/well (n = 6). The fMLP-induced migration was significantly attenuated by anandamide, the inhibition being 90.78 ± 20.43%, 62.31 ± 9.95%, 63.73 ± 7.47%, 63.22 ± 7.87% and 43.64 ± 8.02% with 100nM, 10nM, 1nM, 0.1nM and 0.01nM anandamide respectively.

These data suggest that anandamide is a potent inhibitor of human neutrophil migration. Experiments to identify the receptor(s) that underlie this effect are ongoing.

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MONOCYTES EXPRESS FUNCTIONAL CB_2 RECEPTORS AND THEIR DIFFERENTIATION INTO DENDRITIC CELLS IS MODULATED BY THC

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Cannabinoid receptors type 1 (CB_1) and 2 (CB_2) are present on human peripheral blood mononuclear cells and their expression pattern is altered *in vivo* by marijuana smoking, suggesting a role for both endocannabinoids and exogenous Δ^9-tetrahydrocannabinol (THC) as immune modulators. Monocytes play a central role in immune responses by differentiating into either macrophages or antigen-presenting dendritic cells (DC). Purified human monocytes from normal donors were evaluated for CB_1 and CB_2 mRNA by semi-quantitative RT-PCR. Monocytes expressed higher levels of receptor mRNA than did T cells, and CB_2 was expressed at 4 to 6 fold higher levels than CB_1. To confirm that CB_2 was functionally coupled to G_i proteins, THC was evaluated for its effects on forskolin-induced cAMP. THC (500 ng/ml = 1.59 uM) inhibited the generation of cAMP by 45% in monocytes and by 60% in CHO cells that were transduced to express human CB_2 (CHO-CB_2). The same effects were observed in response to a selective CB_2 agonist, JWH-015, and the inhibitory effects of both THC and JWH-015 were blocked by SR144258, a selective CB_2 antagonist. Addition of THC (250 to 1000 ng/ml) to human monocytes altered their capacity to differentiate into DC when cultured for 7 days *in vitro* with GM-CSF and IL-4. THC produced a dose-dependent decrease in the expression of MHC molecules (HLA-DR) and co-stimulatory molecules including CD40, CD80, and CD86. The differentiation from monocytes into DC was also associated with the down-regulation of mRNA encoding for CB_2, an effect that was blunted when cells were cultured in the presence of THC. DC prepared in the presence of THC produced normal amounts of IL-10, a cytokine associated with activation of T-helper type 2 (Th2) cells, but were impaired in their capacity to secrete IL-12, a cytokine associated with activation of T-helper type 1 (Th1) cells. Furthermore, exposure to THC during differentiation significantly impaired the ability of DC to stimulate T cells in a mixed leukocyte reaction. Both T cell proliferation and the production of interferon-gamma were inhibited. We conclude that functional CB_2 receptors are expressed by resting monocytes and that exposure to exogenous THC impairs their differentiation into functional antigen-presenting DC. The cannabinoid receptor system may play an important role in regulating antigen presentation and T cell immunity.

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INHIBITION OF MICROGLIAL PROLIFERATION BY THE PLANT-DERIVED CANNABINOIDS THC AND CBD

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As the innate immune cells of the central nervous system, microglia normally reside in a resting, ramified state in the adult brain. Upon neuronal injury, microglia can proliferate, and migrate to sites of damage. Furthermore, they become amoeboid and capable of producing inflammatory cytokines and reactive oxygen species. Sustained microglial activation has been implicated in the progression of such neurodegenerative diseases as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Using rat and murine microglial cell lines dependent on macrophage-colony stimulating factor (M-CSF) for growth, we demonstrate that the plant-derived cannabinoids Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are potent inhibitors of microglial proliferation. In RTMGL1 rat microglia, THC and CBD produce complete inhibition of proliferation at 1 μM and exhibit IC_{50} values of 146 and 171 nM, respectively. Similar anti-proliferative effects and comparable potencies were observed in EOC-2 and EOC-20 murine microglial cell lines.

Because the structure activity profile of cannabinoids in inhibiting proliferation is not consistent with the known affinities of the compounds for the cannabinoid receptors, this inhibition is unlikely to be mediated by either the CB_1 or CB_2 cannabinoid receptor. This is supported by the fact that moderate concentrations of SR141716 or SR144528 fail to reverse cannabinoid-induced blockade of proliferation. The decrease in microglial proliferation is also not mediated by the putative abnormal-cannabidiol (abn-CBD) receptor. However, the anti-proliferative effects of THC and CBD correlate with a sharp decrease in p38 mitogen-activated protein kinase (MAPK) activity, but not p42/p44 MAPK activity. Inhibition of p38 MAPK with the drug SB203580 mimics the decrease in proliferation seen with THC and CBD. This suggests that a blockade of the p38 MAPK pathway, or an upstream mediator, is responsible for the anti-proliferative effects of THC and CBD.

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DELTA-9-THC ENHANCES HIV REPLICATION IN A huPBL-SCID MOUSE MODEL

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Epidemiologic studies have suggested that marijuana may act as a potential cofactor in the development and progression of AIDS. To evaluate this interaction, we have developed a hybrid human mouse model in which human peripheral blood leukocytes (PBL) are implanted into severe combined immunodeficient mice (huPBL-SCID) and infected with an HIV reporter construct in the presence or absence of delta-9-THC, the principal psychoactive component of marijuana. In huPBL-SCID animals, co-administration of delta-9-THC and HIV resulted in a significantly higher percentage of HIV-infected human PBL when compared to control saline-treated animals (17% versus 7%). RNA PCR confirmed a 50- to 60-fold increase in HIV viral load. Despite a significant delta-9-THC-mediated effect on viral replication, we did not detect a decrease in the number of CD4 target cells or changes in the CD4:CD8 ratio in THC-treated animals when compared to saline controls. The latter observation may be due to the related finding that delta-9-THC, in the absence of HIV infection, affects T-cell-subset distribution in the huPBL-SCID mouse resulting in a significant decrease in CD4+ cells. In spite of this potential diminution in the number of HIV target cells, daily exposure to THC, however, resulted in a 2.2- to 2.7-fold increase in HIV-positive cells harvested from huPBL-SCID animals at 7 days post-infection. We have recently focused on the specific mechanisms by which THC promotes HIV infection in our mouse model. Following the co-administration of delta-9-THC and HIV, we observed upregulated expression of chemokine receptors CCR5 and CXCR4, HIV co-receptors that mediate viral entry. However, enhanced co-receptor expression precedes the boost in viral infection and subsequently wanes as HIV infection progresses, probably as a result of post-infection co-receptor down-modulation. We conclude that the huPBL-SCID model can be used to define and study the biological interaction between delta-9-THC and HIV, leading to a better understanding of how marijuana use may impact on AIDS pathogenesis. Moreover, preliminary data using this model suggests that marijuana has the potential to modulate known parameters of HIV infection, resulting in enhanced replication and spread of HIV *in vivo*.

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CHRONIC NICOTINE ALTERS CANNABINOID RECEPTOR DENSITY IN ADOLESCENT MALE BUT NOT FEMALE OR ADULT RATS

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Studies show that a significant number of youths use cigarettes, and more than half of the youths who smoke daily also use illicit drugs. Teens who smoke are also more likely to use marijuana than teens who don't smoke. We have shown previously that different behavioral adaptations occur in response to chronic treatment with nicotine in male and female adolescent and adult rats. For example, repeated nicotine administration produced sensitization to its locomotor-activating effects in male and female adult rats, and female adolescent rats. In contrast, no sensitization to the locomotor-activating effects was seen in male adolescent rats. In addition, pretreatment with nicotine produced cross-sensitization to a cocaine or amphetamine challenge in adolescent male rats, but not in adolescent female rats or in adult rats of either sex. Because of the increased use of marijuana by teenage smokers compared to non-smokers, and the literature suggesting that cannabinoid receptors might play a role in mediating the behavioral effects of stimulant drugs, this study was done to determine whether there are differential effects on cannabinoid receptors in adolescent male compared to female and adult rats after treatment with nicotine. Rats (postnatal days 31-38) were injected with nicotine or saline for 7 days and killed on day 8 for quantitative autoradiography studies of the CB_1 receptor using [^{3}H]CP55,940. Cannabinoid receptor density was increased overall in the medial prefrontal cortex in adolescent male rats pretreated with nicotine compared to vehicle controls. Subdividing the regions of the medial prefrontal cortex showed that there were significant increases in receptor density in both the cingulate cortex, area 3 (CG3) and CG1 regions. There were no differences, however, in the more dorsal and lateral regions of the frontal cortex, including the Fr2 region and the agranular insular cortex, in the nucleus accumbens core or shell, or in the caudate putamen in nicotine-pretreated adolescent male rats compared to their vehicle controls. In contrast to the adolescent male rats, there were no significant changes in cannabinoid receptor binding in any of the brain regions examined in adolescent female or adult male or female rats pretreated with nicotine, compared to their respective controls. The medial prefrontal cortex has been shown previously to be involved in stimulant reinforcement, thus it is possible that these changes contribute to the unique behavioral effects of chronic nicotine in the adolescent male rats compared to the adolescent female or the adult rats. We are currently examining additional brain regions, as well as the behavioral effects of cannabinoids subsequent to nicotine treatment. This information may help us to better understand the development of drug addiction in adolescents and lead to differential treatments or preventions specific for age and sex.

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MODULATION OF ALCOHOL-WITHDRAWAL SYMPTOMS BY CB_1 RECEPTOR ANTAGONIST SR141716A

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Previously, we have reported: (a) down-regulation of CB_1 receptors, (b) reduced net CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding, (c) increased AEA and 2-AG (two naturally occurring cannabimimetic brain constituents), and (d) inhibition of FAAH (the key enzyme that regulates the synthesis and breakdown of AEA) in chronic EtOH-exposed mouse synaptosomal membranes and cultured primary neuronal cultures. Because behavioral tolerance and dependence to EtOH produces long-term changes in the cannabinoid signaling system, we examined the effect of the CB_1 receptor antagonist, SR141716A, on behavioral dependence. Male Swiss-Webster mice were EtOH- dependent following the EtOH vapor inhalation procedure. The animals were administered with the antagonist SR141716A (3mg/kg) either 2h after cessation of EtOH exposure or once every 24h during a 72-hour chronic EtOH vapor exposure period. The mice were then tested for handling-induced convulsions 2,4,6, and 8h after withdrawal from EtOH. Animals exposed to chronic EtOH showed a significant increase in the handling-induced convulsions with increasing period of withdrawal from EtOH peaking at 8h after withdrawal. Whereas, animals treated with SR141716A 2h after withdrawal from EtOH exhibited severe withdrawal symptoms (precipitated withdrawal symptoms). Interestingly, mice co-treated with SR141716A showed significantly reduced withdrawal symptoms suggesting that the blockade of CB_1 receptors throughout the alcoholization period minimizes the alcohol withdrawal effect. On the contrary, antagonist given at the end of alcoholization period exacerbates the handling-induced convulsions. No significant effect on handling-induced convulsions was observed with the animals treated with SR141716A alone. The data suggest that the manipulation of CB_1 receptor function during chronic alcoholization may attenuate EtOH-induced withdrawal symptoms. Drugs targeted against endocannabinoid system may thus have clinical value in the treatment of chronic alcohol abuse.

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CANNABIS WITHDRAWAL AMONG NON-TREATMENT-SEEKING CANNABIS SMOKERS

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Cannabis is used by an estimated 147 million people worldwide, with about 10% of users becoming dependent. A withdrawal syndrome can be part of drug dependence, but relatively little is known about cannabis withdrawal. We studied cannabis withdrawal by retrospective self-report among a convenience sample of 78 non-treatment-seeking cannabis smokers (mean [SD] age 38.6 [10.6] years, 87% male, 69% white, 28% African-American, 3% other) who reported having made a "serious" (self-defined) quit attempt. Forty-five subjects were participating in a longitudinal lung health study in Los Angeles, CA; 33 subjects were participating in non-treatment residential studies at the NIDA IRP. All subjects were primary cannabis users with no other substance abuse or dependence, except tobacco in some cases. They began cannabis use at the age of 17.0 [3.9] years, had been using for 20.7 [7.8] years, and were currently averaging 2.5 [3.7] joints/day.

Subjects reported making 4.6 [13.0] serious quit attempts. 64% of subjects reported experiencing at least one withdrawal symptom; of those reporting symptoms, 72% reported $\geq$ 3 symptoms. The commonest withdrawal symptoms were craving for cannabis (63% of all subjects [92% of subjects reporting withdrawal]), irritability (42.5% [62%]), boredom (35.9% [56%]), anxiety (33.8% [50%]), improved memory (33.3% [48%]), and difficulty sleeping (32.4% [48%]). Physical symptoms were rare: nausea (4.8% [6%]), upset stomach (4.8% [6%]), tremor (3.2% [4%]). Factor analysis yielded two major factors accounting for 41% of the variance: a physical factor (including all physical symptoms) and psychological factor (anxiety, irritability, difficulty sleeping). All withdrawal symptoms tended to appear one day after quitting cannabis use. Psychological symptoms tended to last 3-4 weeks, while physical symptoms resolved within 1-2 weeks. Up to one-third of subjects did something to relieve psychological symptoms, most commonly smoking cannabis again. No subject reported physical symptoms troublesome enough to require self-medication.

These findings suggest that a clinically significant withdrawal syndrome does occur in non-treatment-seeking cannabis smokers, based on the co-occurrence of multiple symptoms following a consistent time course. This withdrawal syndrome is more psychological than physical, but can be troublesome enough to generate self-medication with cannabis.

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ANTAGONISM OF INHALED CANNABIS BY SINGLE AND MULTIPLE DOSES OF SR141716 (RIMONABANT) IN MALE SUBJECTS WITH A HISTORY OF CANNABIS USE

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Rimonabant is a potent and specific CB_1 cannabinoid receptor antagonist (CB_1A) that blocks the effects of tetrahydrocannabinol (THC) in vitro and in vivo. We previously reported blockade of smoked cannabis effects in human cannabis users by a single 90 mg oral dose of rimonabant. We now report results of a randomized, double-blind, placebo-controlled, double-dummy, parallel group clinical study comparing the effects of single and multiple doses of oral rimonabant on physiological and behavioral effects induced by cannabis. Subjects provided written informed consent. The NIDA Institutional Review Board approved the study. Male cannabis users (N=42) were randomly assigned to receive either 40 mg rimonabant once daily for 15 days, 14 daily doses of placebo and a single oral 90 mg dose of rimonabant on day 15, or placebo for 15 days. Subjects smoked one placebo or active marijuana (2.78% THC cigarette) 2 hours following rimonabant or placebo on days 8 and 15. Plasma samples were collected on days 8 and 15 for up to 24 hours. Concentrations of rimonabant were determined by LC/MS/MS with a limit of quantification (LOQ) of 1 ng/mL; THC concentrations by GC/MS with an LOQ of 0.5 ng/mL. The primary end-points for assessment of blockade were composite visual analog scales for "Drug High", "Stoned" and "Drug Strength" (VAS), marijuana-Scale (M-scale), and heart rate. Planned contrasts were conducted to compare placebo with the 40 and 90 mg groups, and also the 40 with the 90 mg group. The percent blockade of effect was determined for the 90 and 40 mg rimonabant doses with 95% confidence intervals calculated using Feiller's method. After active cannabis, the maximum increase in heart rate was significantly lower for both the single 90 mg group (64% blockade) and multiple 40 mg group (66%) compared to placebo ($p < 0.001$). Peak VAS composite scores also tended to be lower for the 90 mg (29% reduction) and the 40 mg (14% reduction) rimonabant groups compared to placebo. Peak M-Scale scores were reduced by 23% for both the 90 and 40 mg rimonabant groups; none of the reductions in psychological measures reached statistical significance. Following administration of 40 mg for 15 days or a single 90 mg dose, exposure to rimonabant was similar (C_{max} and $AUC_{0\text{-}24h}$) for both groups and for participants in the 40 mg group after 8 and 15 days. Peak heart rate increases after cannabis smoking were also significantly reduced in the 40 mg group on day 8. There were no significant differences in peak THC or $AUC_{0\text{-}2h}$ THC between the placebo group, 40 mg rimonabant group on days 8 and 15, or the 90 mg rimonabant group day 15, indicating that the blockade of smoked cannabis' effects was not due to a pharmacokinetic interaction between THC and rimonabant. In conclusion, this study further substantiates the role of CB_1-cannabinoid receptors in mediating the physiological and behavioral effects of smoked cannabis in humans.

MARIHUANA'S IMPACT ON COGNITIVE PERFORMANCE IN YOUNG ADULTS – ARE THERE DIFFERENTIAL FINDINGS WHEN PRE-DRUG ABILITITIES ARE TAKEN INTO ACCOUNT?

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A fundamental problem in determining marihuana's impact upon cognitive performance beyond the acute intoxication period arises when the premorbid status of the marihuana smokers is unknown. As described by workers in this arena, without this knowledge, observed drug effects within specific spheres of cognitive functioning may reflect preexisting differences. Attribution of cause and effect becomes quite problematic. In order to address this investigators have employed such strategies as statistically controlling for non-marihuana variables and/or matching regular users with non- and irregular users on such dimensions as cultural-economic background or education. There have also been attempts to estimate premorbid intellectual functioning by using standardized test scores derived from early school records or contemporary measures of general cognitive functioning (e.g. IQ or surrogates of IQ) that are assumed to be relatively resilient to brain damage that might result from marihuana use. Although an important step, the usefulness of these procedures in ascertaining pre-drug levels of performance in specific cognitive domains is problematic. As well as evaluating premorbid functioning, suggestions for facilitating the interpretation of findings emphasize that comparison groups be as similar as possible to drug-using groups.

The concerns described above can be addressed by utilizing a longitudinal, prospective design in which users and non-users, derived from the same population, have been 'followed' over a period of time commencing before the age of initiation of marihuana use. The Ottawa Prenatal Prospective Study (OPPS) provides such an opportunity. Participants, born to mothers recruited from a predominantly low-risk, middle class population, are presently young adults and have been assessed since birth. We have taken advantage of the pre-drug assessments available from these subjects by taking into account their abilities prior to the age of initiation of regular marihuana use when evaluating the contemporary performances of both regular users and comparison groups. These results have been described at earlier ICRS meetings.

The question arises as to how does the evaluation of premorbid functioning within broad and specific neurocognitive domains impact upon the findings. Inclusion of pre-drug abilities may have a variety of consequences. They may not alter the outcomes, they may serve to identify inappropriate attribution of drug effects or, in contrast, they may unmask previously statistically non-significant relationships. In the present report, we will describe how the results and interpretation within various domains assessed in the OPPS young adults are impacted by the inclusion of their premorbid performances.

Acknowledgements: The OPPS longitudinal study has been and continues to be supported by NIDA awards to PAF.

EFFECTS OF LONG-TERM CANNABIS CONSUMPTION WITH EARLY AGE OF ONSET ON OCULOMOTOR CONTROL AND VISUAL INFORMATION PROCESSING

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Research on effects of long term cannabis consumption on cognitive functions such as memory and attention has resulted in heterogeneous and sometimes contradictory results. As yet no clear answer can be given to the question of whether there are sustained adverse effects and which subsystems of information processing are affected. Ehrenreich et al. (*Psychopharmacology*, **1999**) studied a sample of chronic THC-users using a large battery of neuropsychological tasks. Their results indicated that long-lasting impairments were present exclusively in users with early onset of consumption. Interestingly, performance deficits were pronounced in a search task requiring sequential visual processing. Further experiments of our research group confirmed these results and found differences in eye movement patterns in a visual scanning task between cannabis users and normal controls (Huestegge et al., *ProgBrainRes*, **2002**). To directly address the question of adverse effects on visual processing and oculomotor control we compared the performance of 20 long-term cannabis users without acute intoxication with 20 control subjects in three well-established oculomotor paradigms. In the prosaccade task, participants execute single eye movements to targets in the periphery. In the antisaccade task, participants are asked to direct their eyes in the direction opposite to an upcoming stimulus. This involves endogenous suppression of a reflexive movement as well as a recoding of target coordinates. In the memory-guided saccade paradigm, participants have to remember the location of targets and subsequently direct their eyes to these locations. These paradigms allow us to assess temporal and spatial aspects of information processing on different levels of visuomotor control (Findlay & Walker, *BehavBrainSci*, **1999**). As a result, we found substantially prolonged saccadic response times for chronic cannabis users with early age of consumption onset in the pro- and antisaccade tasks. Furthermore, eye movements that are not visually guided (antisaccade and memory-guided saccade paradigm) were hypermetric in the THC-group compared with the control group. These results point to specific deficits in temporal and visuospatial processing that can be linked to specific frontal and parietal brain areas. In contrast, oculomotor parameters related to the basic brainstem circuitry appeared unaffected. Implications of these results for more complex visual-cognitive tasks like reading will be discussed.

FUNCTIONAL MAGNETIC RESONANCE IMAGING INDICES OF MEMORY FUNCTION IN LONG-TERM CANNABIS USERS

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The precise neurocognitive impairments associated with frequent (heavy) cannabis use have remained elusive. Nevertheless, growing evidence indicates that regular cannabis use may result in enduring cognitive impairments involving learning, memory, and executive functioning (e.g., attention and planning). Research suggests that the longer the duration of cannabis use, the more pronounced the cognitive impairment. Cannabis is thought to produce cognitive deficits via inhibitory changes in prefrontal, hippocampal and cerebellar brain regions. The cognitive deficits have generally been most conspicuous during tasks involving verbal learning and memory.

The present study examined differential neurocognitive effects of frequent and infrequent long-term use of cannabis using functional magnetic resonance imaging during performance of the Rey Auditory Verbal Learning Task (modified for visual presentation). This instrument was selected for its ability to provide multiple measures of learning and memory and its reliability in detecting impairments associated with cannabis use[1,2,3]. Thirty right-handed males were assigned on the basis of stringent exclusion criteria to one of three comparison groups: long-term heavy cannabis use, long-term light cannabis use, or non-user controls. Group differences in blood oxygen level dependent activation (BOLD) were investigated within the prefrontal cortex, hippocampus, and cerebellum. Additionally, participants completed the Cambridge Neuropsychological Test Automated Battery (CANTAB), and a comprehensive assessment of current psychological functioning.

Preliminary analyses indicate differences between groups in neuropsychological test performance with the worst performance in the long-term heavy user group. Accordingly, long-term heavy users showed the greatest alteration in BOLD activation, with lower activation in regions relevant to memory function.

These results are discussed in terms of the varying effects of frequency and duration of cannabis use on brain function and similarities to neurocognitive functioning in schizophrenia.

[1]Solowij et al (**2002**) JAMA, 287, 1123-1131; [2]Bolla et al (**2002**) Neurology, 59, 1337-1343; [3]Pope et al (**1996**) JAMA, 275, 521-7.

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TOLERANCE AND THC: CANNABIS BASED MEDICINE EXTRACTS MAINTAIN LONGTERM CLINICAL EFFICACY WITHOUT DOSAGE INCREASES

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Many medicines are known to induce tolerance: a diminution of pharmacological effects of a given dose over time. This presentation will review experimental and clinical work on tolerance and "reverse tolerance," then present new data from long-term safety studies (SAFEX) of patients with chronic neuropathic pain, and spasticity associated with multiple sclerosis (MS) employing Sativex®, a 1:1 tetrahydrocannabinol (THC):cannabidiol (CBD) cannabis based medicine extract (CBME) administered via the oro-mucosal route.

Experimental work in the 19th-20th centuries demonstrated the ability to expose animals to cannabis preparations for prolonged intervals without signs of developing tolerance. The concept of reverse tolerance was first claimed by Ludlow (New York: Harper; **1857**.), who noted smaller doses of Indian hemp extract were needed on subsequent trials to induce a comparable subjective intoxication. Whereas Abood noted a 27-fold behavioural tolerance in mice with chronic THC injection (*Pharmacol Biochem Behav* **1993**; 46:575-9.), no changes were noted in brain cannabinoid binding or mRNA levels. Rather, Pertwee judged cannabinoid tolerance under preclinical experimental conditions to be mainly pharmacodynamic in nature with changes of cannabinoid receptor density to differing degrees in various brain loci (*Cannabinoid Receptors.* London: Academic Press; **1995**). In humans, marked tolerance was observed to cannabis side effects in chronic administration: tachycardia, hypothermia, orthostatic hypotension, dry mouth, and ocular injection (Jones RT et al., *J Clin Pharmacol* **1981**; 21:143S-152S), while subjective tolerance to subjective effects was observed with high dose oral THC (Haney M et al., *Psychopharmacology* **1999**; 141(4):395-404.). Therapeutically, chronic cannabis smoking over two decades was observed to lead to no loss of analgesic efficacy, control of intraocular pressure or MS symptoms and signs in Compassionate Use Investigational New Drug patients (Russo EB et al., *Journal of Cannabis Therapeutics* **2002**; 2(1):3-57).

The use of CBME oro-mucosally for MS (Wade DT et al., *Clinical Rehabilitation* **2003**; 17:18-26) and neuropathic pain (Notcutt W et al., *Anaesthesia* **2004**: in press) has now been extended in SAFEX studies employing Sativex with over 600 patient-years of available results. Following the initial period of titration, the data presented will demonstrate a continuation of minimal subjective patient intoxication scores, with diminished adverse event profiles over time. Consistent maintenance of symptom control (pain, spasm, sleep, bladder disturbances) with stable or even diminishing CBME dosages was noted. Sativex® in chronic administration demonstrates a favourable side effect profile in comparison with standard medicines for neurogenic symptoms, with no tolerance developing to its clinical benefits.

RESEARCH MEDICAL GROUP DEVELOPS CLINICAL DATA COLLECTION METHODS FOR MEDICINAL CANNABIS PATIENTS

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Pre 1937 citations in the medical literature for cannabis treated conditions include 28 codeable diagnoses among them chronic pain, neurodegenerative diseases, seizure disorders, mental disorders, drug and alcohol dependence, cerebral palsy, migraine, colitis, gastritis, premenstrual syndrome, arthritis, (rheumatoid, degenerative and post-traumatic), nausea, anorexia and insomnia. Since **1996** these earlier cited conditions and over one hundred other ICD-9 codeable conditions have been encountered by California physicians in the evaluations of patients presenting for medicinal cannabis use. There are >30,000 patients followed by the physician members of the California Cannabis Research Medical Group, CCRMG. It has been the resolve of this group to develop a comprehensive health history questionnaire and data entry program to build a research database, further knowledge of cannabis therapeutics, and identify a patient population for future approved cannabis research.

Health history forms were collected from physicians who are evaluating patients with serious medical conditions for cannabis use. Information from ten intake forms were collated and edited into the present document, The Health History Questionnaire, HHQ. It was then circulated among the contributing physicians for further discussion. A few physicians considered the form to be excessively long and more subject to inaccurate responses. For other physicians the form was fully adopted or used to modify their existing documents. Subsequently, a data entry program was designed to mirror the HHQ content and assign an identity to each question to facilitate future modifications in the questionnaire. The HHQ and data entry software are now available for those who wish to use them.

Because there is no requirement in California to report cannabis approved patients, it is impossible to accurately determine the numbers of physicians and patients involved in this form of therapy. At this time it has been estimated that 4000 physicians have approved the use of cannabis to 60,000 patients in California. It should be noted that fewer than 20 physicians, willing to be publicly identified as cannabis consultants have approved about half of these patients. Physician education remains the principle deficiency in fostering a more broad based involvement in the medical community. Use of the HHQ and data entry software and subsequent voluntary sharing of information will create a database that will help to educate physicians in cannabis therapeutics and advance cannabinoid research.

USE OF PRODRUG AND CYCLODEXTRIN TECHNOLOGIES FOR THE FORMULATION AND DELIVERY OF CANNABINOIDS

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Over the last few years cannabinoids have been reported to take part in the regulation of various processes in the body, including cognition, memory, emesis, euphoria, sedation, appetite, intraocular pressure, blood pressure and pain. The pharmaceutical development and therapeutic use of cannabinoids are, however, often hindered due to their poor aqueous solubility/dissolution characteristics and/or significant first-pass metabolism. In addition, the low aqueous solubility of cannabinoids may hinder their use in various scientific experiments.

In the present study, we synthesized phosphate esters of AEA, *R*-methanandamide and noladin ether, and evaluated their potential as water-soluble prodrugs. We also developed novel cyclodextrin formulations of tetrahydrocannabinol (THC) for the sublingual delivery of THC, in order to improve the bioavailability of THC.

The aqueous solubilities of phosphate esters were increased by 16,500-40,000-fold compared to the parent compounds AEA, *R*-methanandamide and noladin ether at pH 7.4. Phosphate esters provided adequate stability towards chemical hydrolysis and were rapidly hydrolyzed to their respective parent compounds in alkaline phosphatase solution, liver homogenate and in cornea homogenate. The phosphate esters of *R*-methanandamide and noladin ether reduced intraocular pressure in rabbits after their topical administration as aqueous eyedrops. These results indicate that the phosphate esters of AEA, *R*-methanandamide and noladin ether are potential water-soluble prodrugs.

Various cyclodextrins (e.g., β-CD and RM-β-CD) significantly increased the aqueous solubility and dissolution rate of THC, thus making the development of a sublingual THC formulation possible. THC/RM-β-CD solution and THC/β-CD powder were administered sublingually to anaesthetised rabbits, and results were compared to oral administration of THC (ethanolic solution). All doses were equivalent to 250 μg/kg of THC. The *in vivo* studies showed that $AUC_{0-\infty}$ values after the sublingual administration of THC/RM-β-CD solution and THC/β-CD solid complex were ten times higher than the $AUC_{0-\infty}$ after oral administration of THC. These results indicate that THC forms complex with CDs, which can be utilized in the development of novel THC sublingual formulations.

In conclusion, prodrug and cyclodextrin technologies are potentially useful approaches to formulate and deliver cannabinoids that are poorly water-soluble.

BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF SR147778, A NEW POTENT AND SELECTIVE CB_1 ANTAGONIST

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Recent developments in cannabinoid research suggest that some pathophysiological states are related to endocannabinoid system dysfunction. The following experiments indicate that SR147778 is a new potent antagonist at the CB_1 receptor which, considering SR141716 as the reference CB_1 antagonist, may be considered as a novel class of therapeutic agents.

Methods: Affinity of SR147778 for both CB_1 and CB_2 receptors was determined either in rat brain and spleen membranes or in CHO cell membranes expressing human receptors. Receptor antagonism properties of SR147778 for CB_1 receptors were investigated in vitro and in vivo in mice (hypothermia, analgesia, gastrointestinal transit). Pharmacological experiments investigated the activity of the drug in rats (antagonism of the cannabinoid cue, of spontaneous sucrose drinking, of food intake in fasted and non fasted animals) and also focused on spontaneous alcohol intake in mice and schedule-induced polydipsia (SIP) for ethanol in rats.

Results: SR147778 displayed nanomolar affinity (K_i = 0.56 and 3.5 nM) for both the rat brain CB_1 and human CB_1 recombinant receptors, respectively. It had low affinity (K_i = 400 nM) for both the rat spleen CB_2 and human CB_2 recombinant receptors. At 1 μM it had no affinity for any of the 100 targets investigated. In mice, SR147778 po dose dependently reversed the hypothermia (ID_{50} = 0.4 mg/kg), analgesia (ID_{50} = 0.4 mg/kg), and at 10 mg/kg the blockade of gastrointestinal transit, all induced by WIN 55,212-2. In rats trained to discriminate WIN 55,212-2 (0.3 mg.kg sc), SR147778 ip antagonized the WIN stimulus (ID_{50}s 2.3 mg/kg,) and its generalization to CP 55,940 (ID_{50}s 3.6 mg/kg) and to Δ^9THC (ID_{50}s 3.8 mg/kg). In the same species, SR147778 (3 mg/kg) reduced 5% sucrose solution drinking and also decreased food intake in both fasted and non-deprived animals (1 and 3 mg/kg). In mice, alcohol (10%) consumption was significantly reduced using doses from 0.1 to 3.0 mg/kg, sc, and, in rats, SIP for ethanol (10%) was decreased with doses from 1 mg/kg to 10 mg/kg, ip, versus vehicle, when SIP for water was unchanged.

Conclusion: SR147778 selectively binds with high affinity to the CB_1 receptor. In vivo, it behaves as a CB_1 receptor antagonist, as it suppresses the classical pharmacological responses to cannabinoid agonists. SR147778 alone decreases food intake and overconsumption of palatable drinks, probably by decreasing the incentive value of sucrose and alcohol. These results raise the possibility that some pathophysiological disorders concerned with excessive “ingestive” behaviours are due to the hyperactivity of the endocannabinoid system and could be treated by drugs regulating this process.

RADIOLIGAND PROBES FOR CANNABINOID RESEARCH

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Radioactive analogs of cannabinoids are valuable tools for elucidating processes in the cannabinoid neurochemical system. They have been used to detail such fundamental characteristics as the existence of receptors and subtypes, binding affinity, tissue binding specificity, differential ligand docking sites, and ADME to name a few.

Enabling such studies involves the development of syntheses of these radioligands which are tailored to the intended use by the choice of the isotope (^{3}H, ^{14}C, ^{125}I), labeling site, specific activity, and chemistry. The design and synthesis of some key cannabinoid radioligands prepared by RTI International on the NIDA Drug Supply program will be presented as examples that highlight our approaches to these compounds. Included among these will be tritium labeled SR141716, SR144528, and noladin.

Established syntheses for noladin from arachidonic acid would not be a preferred approach for the synthesis of high specific activity noladin. This is because the use of the corresponding tritium labeled arachidonic acid would require transforming substantial levels of radioactivity on small scale through numerous steps which raises concerns of safety and feasibility. We will present our approach to the synthesis of high specific activity tritium labeled noladin that introduces tritium at the end of the synthesis.

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ARYL PYRAZOLE LIGANDS AS CB_1 CANNABINOID ANTAGONISTS

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The discovery of SR141716, a potent aryl pyrazole CB_1 cannabinoid receptor antagonist with nanomolar affinity, provides a unique chemical tool for further characterization of the cannabinoid pharmacophore in its relationship to the binding domain of cannabinoid antagonists. A series of amide and hydrazide analogs of SR141716 were synthesized, and their binding affinities at the CB_1 and CB_2 receptors were characterized with the prototypical cannabinoid ligands [^{3}H]CP 55940, [^{3}H]SR141716, and [^{3}H]WIN55212-2. Competitive binding assays were carried out utilizing whole rat brain membrane preparations and Chinese hamster ovary cells transfected with the human CB_1 receptor. Most of the SR141716 analogs displaced [^{3}H]CP 55940 and [^{3}H]SR141716 with the same affinity ratio as [^{3}H]WIN55212-2. However, for certain analogs, notable differences in the displacement curves were pronounced in the CHO-hCB_1, but not in the rat brain preparations. In GTP-γ-[^{35}S] assays using whole rat brain membrane preparations, most of the analogs exhibited inverse agonist activity with EC_{50} values ranging from 5.3–12000 nM. The antagonist profile in biological signal transduction pathways was further characterized by examining the adenylyl cyclase activity in mouse N18TG2 neuroblastoma and human SH-SY5Y neuroblastoma cells.

Acknowledgements: Supported by NIDA grants F32-DA-016502 (M.E.F.), R01-DA-11638 (B.F.T.), and R01-DA-03690, R01-DA-06312, and U24-DA-12385 (A.C.H.).

NEW STRUCTURE ACTIVITY RELATIONSHIP STUDIES ON CB_1 RECEPTOR LIGANDS

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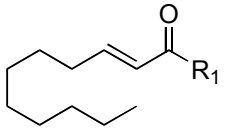
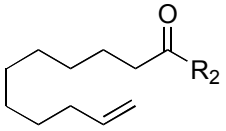
The aim of the work was to synthesize compounds that bind with high affinity to CB_1 receptors and to acquire new information on the Structure-Activity Relationships of CB_1 receptor agonists. The endocannabinoid anandamide shows some selectivity for CB_1 receptors: it contains a polar moiety (the ethanolamine "head") and a hydrophobic one (the arachidonate "tail"). The compounds that we have synthesized here are structurally related to anandamide and contain, on the ethanolamine "head", polar substituents such as 4-methoxyphenylamine, 4-amino-1-benzylpiperidine, 4-methoxy-benzylamine, and 3,4-dimethoxybenzylamine, or, instead of the arachidonate "tail", they exhibit a long hydrocarbon chain such as that of 2-*trans*-undecenoic acid and 10-undecenoic acid. With regard to the substitution of the ethanolamine "head", binding assays carried out with [^{3}H]SR141716A and rat brain membranes showed that the most active compound was the one containing the 4-amino-1-benzylpiperidine moiety (Ki=5.4 μM). On the other hand, the compounds with shorter chains in the place of arachidonate exhibited some binding activity only when chains with 11 carbon atoms and with the double bond in the α-position were introduced.

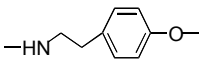
In conclusion, the new compounds synthesized showed moderate to low affinity for rat CB_1 receptors. They were also found to be inactive on FAAH, whereas their activity at CB_2 receptors is currently under investigation.



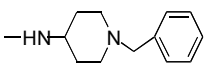


2-trans-undecenoic acid 10-undecenoic acid
some of the polar "heads" used:

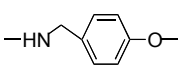
PP-1: R_1=



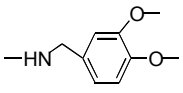
PP-4 : R_1=



PP-22: R_2=



PP-66: $R_{2=}$



SYNTHESIS AND PHARMACOLOGY OF PYRROLE-BASED CANNABINOIDS

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Since the discovery of the aminoalkylindole class of compounds as cannabinoid ligands by the Sterling-Winthrop group in **1991**, the range of compounds evaluated for receptor affinity has broadened dramatically. This has led to reevaluation of the possible causes of receptor interaction, resulting in at least two different pharmacophores suggesting alignment of the ligand with the CB_1 receptor. The presence of a naphthalene group in particular has been shown to increase receptor binding, possibly through the addition of lipophilic groups that aid in the migration into the cell membrane. 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 the 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. For aminoalkylindoles, there is also evidence that aromatic stacking may play a critical role in ligand affinity for the CB_1 receptor. (Bramblett and Reggio, **1995**, *Symposium on the Cannabinoids,* International Cannabinoid Research Society; Burlington, VT, **1995**, 16).

The use of indole as a cannabinoid nucleus has been well-explored by Huffman, *et al.,* the Sterling-Winthrop group, and others. (Huffman, J. W. *Curr. Med. Chem.* **1999**, *6*, 705.; Eissenstat, M. A. *et al. J. Med. Chem.* **1995**, 3094.) Evaluation of the current pharmacophores implies that the benzenoid moiety is unnecessary for receptor affinity. The elimination of the benzenoid moiety to provide 3-(1-naphthoyl)-*N*-pentylpyrrole results in a compound with significantly reduced affinity for the CB_1 receptor and decreased potency *in vivo* compared to the corresponding indoles. (Lainton, J. A. H. *et al. Tetrahedron Lett.* **1995**, *36*, 1401.) Functionalization of the pyrrole with a phenyl substituent to afford 2-phenyl-4-(1-naphthoyl)-*N*-pentylpyrrole gives a compound with significantly increased affinity over previous pyrrole series (K_i = 11 nM). To investigate the steric and electronic effects of the 2-substituent on the binding affinity of the ligand, a series of pyrroles with functionalized aryl substituents has been prepared and evaluated for pharmacological activity. The results of this study and the synthetic route to these compounds will be discussed.

Acknowledgements: This work was sponsored by the National Institute of Drug Abuse grants DA03590, DA03671 and F31 DA15579.

HYDANTOINS AND THIOHYDANTOINS AT THE CANNABINOID RECEPTORS

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INTRODUCTION:
We are currently involved in the synthesis and pharmacological evaluation of hydantoins and thiohydantoins derivatives as CB_1 cannabinoid receptor ligands. The first reports have concerned the evaluation of N_3-alkyl-5,5'-diphenylimidazolidinediones as cannabinoid ligands as well as the characterization of their antagonist properties (Kanyonyo *et al*., Bioorg. Med. Chem. Lett., **1999**, 9, 2233; Ooms *et al.*, J. Med. Chem., **2002**, 45, 1748). Recently, we have described an easy microwave-assisted synthesis of these compounds that has allowed us to further increment our library of compounds (Muccioli *et al*., Tetrahedron, **2003**, 59, 1301). In order to study the bioisosteric replacement of oxygen by sulfur atom, new N_3-alkyl- and N_3-alkylaryl-5,5'-diphenyl-2-thioxo-imidazolidin-4-ones, or 5,5'-diphenylthiohydantoins were synthesized, and evaluated for their affinity for the cannabinoid receptors, in comparison with the N_3-alkyl-5,5'-diphenylhydantoins.

METHODS AND RESULTS:
Using the previously described microwave-assisted method, 28 new N_3-alkyl-5,5'-diphenyl-thiohydantoins (**DML30-DML57**) were synthesized. Briefly, this method consist in applying to a mixture of benzil and alkylthiourea in DMSO/OH^- several microwave pulses. These thiohydantoins derivatives differ in the N_3-alkyl chain and in the substitution in position 4 of the phenyls. The yields vary from 75% to 30% depending on the nature of the phenyls substitution, the lower yields obtained when the substituent is a bromine.

DML30-DML57 were first screened at 10 μM to estimate their affinity for the cannabinoid receptors, using CHO cells expressing selectively either the hCB_1 (against [^{3}H]-SR141716A) or the hCB_2 receptor (against [^{3}H]-CP-55,940). The results are similar to those that have been obtained with the hydantoins: a bromine or a chlorine in position 4 on the phenyls enhances the affinity, and an alkyl chain not longer than four carbons or an alkylaryl such as a benzyl are the best substituents for the nitrogen in position 3. Moreover, very low displacements of the radioligand bound to the CB_2 receptor have been observed. Nine compounds were selected, among those showing the highest displacement, and their inhibition constant ([^{3}H]-SR141716A 1nM, and Kd = 13.9 nM) determined. The best results have been obtained for the 3-benzyl-5,5'-bis-(4-bromophenyl)-2-thioxo-imidazolidin-4-one (**DML56**) and the 3-allyl-5,5'-bis-(4-bromophenyl)-2-thioxo-imidazolidin-4-one (**DML55**) with pKi values of 6.03 and 6.06 respectively. When are compared the pKi values of a substituted thiohydantoin to the corresponding hydantoin the ΔpKi is 0.6 in favor of the thiohydantoins.

CONCLUSIONS:
We have reported here the synthesis of new N_3-alkyl- and N_3-alkylaryl-5,5'-diphenyl-2-thioxo-imidazolidin-4-ones showing affinity and selectivity, for the hCB_1 cannabinoid receptor expressed in CHO cells. Moreover, some of these compounds have been compared to the corresponding hydantoin and have shown a greater affinity. Other investigations are currently undertaken in order to further enhance the affinity of these original compounds at the cannabinoid receptors.

SYNTHESIS AND CB_1-RECEPTOR ACTIVITIES FOR REVERSED AMIDES OF ARACHIDONOYL ETHANOL AMIDE

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Retroanandamide (Fig. 1.; **1a**) and six analogues (Fig. 1.; **1b-d, 2a-c**) were synthesized, and the ability of these compounds to activate the CB_1 receptor was determined by [^{35}S]GTPγS binding assay using rat cerebellar membranes. The aim of the study was to develop potent CB_1 receptor agonists with improved enzymatic stability, and therefore longer duration of action, compared to the endocannabinoid arachidonoyl ethanol amide (AEA). In addition, the effect of the carbonyl carbon position on the CB_1 receptor activity was explored by synthesizing retroanandamide analogues having different chain lengths (**1a-c** versus **2a-c**).

Arachidonyl amine, a key intermediate for the synthesis of compounds **1a-d**, and retroanandamide **1a** were synthesized as previously described.[1] Compounds **1b-c** were prepared from arachidonyl amine and appropriate acid halides. The synthesis of compound **1d** was obtained by coupling 2-phenyl-5-carboxy-1,3-dioxane to arachidonyl amine with *N*'-(3-dimethylamino-propyl)-*N*-ethylcarbodiimide and 4-dimethylaminopyridine as coupling reagents. The desired product was obtained by removing the benzylidene acetal protective group with conc. HCl/ MeOH. Compounds **2a-c** were prepared by a method that allows one-pot conversion of *t*-butyl carbamates to amides. It is based on cleavage of the *t*-butoxy-carbonyl group with hydrogen iodide generated *in situ* by reacting acid halide with methanol, and on the acylation of the intermediate amine with excess acid halide in the presence of diisopropylethylamine. A key intermediate, *N*-*t*-butyl norarachidonyl carbamate, was synthesized from arachidonic acid and *t*-butanol by a modified Curtius reaction. Compound **2a** was synthesized by attaching *t*-butyldiphenylsilyl chloride protected 3-hydroxypropionyl chloride to the carbamate, and compounds **2b-c** were prepared similarly from appropriate acid halides.

All the synthesized compounds showed dose-dependent CB_1 activity. For example, the potency values for the compounds **1b** (E_{max} = 295 ± 2 % basal, -logEC_{50} = 5.6 ± 0.2) and **2b** (E_{max} = 274 ± 9 % basal, -logEC_{50} = 5.7 ± 0.0) were higher than those of the reference compound AEA (E_{max} = 380 ± 6 % basal, -logEC_{50} = 4.8 ± 0.0). These results indicate that shortening the chain length by one carbon does not have a significant effect on the CB_1 activity.

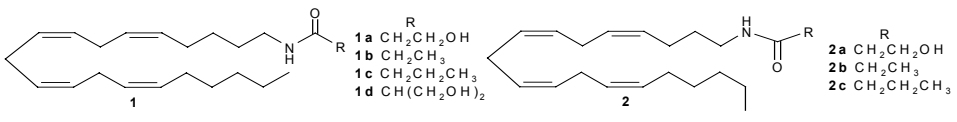

Figure 1. Chemical structures of reversed amides **1a-d, 2a-c**.

1. Lin, S.; Khanolkar, A.D.; et al. *J Med Chem* **1998**, *41*, 5353-5361.

PHARMACOLOGICAL DIFFERENCES BETWEEN CANNABIDIOL, ABNORMAL-CANNABIDIOL AND THE NEUTRAL CB_1 ANTAGONIST, O-2654, IN TWO ISOLATED NERVE-SMOOTH MUSCLE PREPARATIONS

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Cannabidiol (CBD) antagonizes *R*-(+)-WIN55212 (WIN), in the mouse isolated vas deferens (MVD) in a manner that appears to be competitive and yet not to involve direct competition for cannabinoid receptors [1]. In contrast, 6"-azidohex-2"-yne-cannabidiol (O-2654), does seem to antagonize WIN by competing for CB_1 receptors [2]. However, O-2654 resembles CBD in its ability to oppose α_1-adrenoceptor-mediated contractions of the MVD. In the present investigation we have further characterized the pharmacological properties of O-2654 and CBD and extended our research to include abnormal-CBD (abn-CBD). Experiments were performed with the MVD and with the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum (MPLM). O-2654, CBD or DMSO were added 30 or 90 min before WIN or non-cannabinoids. Drugs were dissolved in a 50% aqueous solution of DMSO (v/v) (WIN), or in DMSO (other cannabinoids) or saline. Other experimental details are given elsewhere [1, 2, 3]. Values have been expressed as means ± s.e. or with 95% confidence limits.

In the MVD, O-2654 (1 and 10 μM) differed from CBD and abn-CBD in opposing the contractile effect of the P2X receptor agonist, β,γ-methylene-ATP, in an insurmountable manner ($P<0.05$; ANOVA and Dunnett's test; n=7 to 12). In contrast, phenylephrine-induced contractions were attenuated by all three cannabinoids at 10 μM. The pEC_{50} of abn-CBD against phenylephrine (5.1 ± 0.07) approximates to pEC_{50} values of abn-CBD for antagonism of phenylephrine at the putative abn-CBD receptor [4]. 10 μM abn-CBD did not oppose WIN-induced inhibition of electrically-evoked contractions of the MVD or augment twitch amplitude when administered alone. In the MPLM, contractions induced by the muscarinic receptor agonist, methylcholine, were opposed by 10 μM O-2654 and CBD, which each produced a dextral shift in the log concentration-response curve of methylcholine. No such antagonism was induced by 10 μM abn-CBD. At 10 μM, O-2654 but not CBD also opposed the ability of WIN to inhibit electrically-evoked contractions of the MPLM. O-2654 behaved as a surmountable competitive antagonist with a K_B value of 709 nM (259 and 5117 nM; n=11). Accordingly, it resembles SR141716A which we have shown previously to be about 5 times less potent against WIN in MPLM than MVD. Unlike SR141716A at 0.01, 0.04 or 0.1 μM [3], O-2654 (10 μM) did not affect the amplitude of electrically-evoked contractions of the MPLM.

In conclusion, as in the MVD [2], O-2654 behaved as a neutral CB_1 receptor antagonist in the MPLM. CBD did not antagonize WIN in the MPLM, lending additional support to our hypothesis that its ability to antagonize WIN in the MVD does not involve competition for CB_1 receptors. Further experiments are required to establish the mechanisms by which (a) CBD antagonizes WIN and phenylephrine in the MVD and methylcholine in the MPLM and (b) O-2654 antagonizes phenylephrine and β,γ-methylene-ATP in the MVD and methylcholine in the MPLM.

Acknowledgements: Supported by NIDA and GW Pharmaceuticals

Pertwee, R.G. *et al.* (**2002**) *Eur. J. Pharmacol.* 456**,** 99-106.
Thomas, A. *et al.* (**2004**) *Eur. J. Pharmacol.* In press.
Pertwee, R.G. *et al.* (**1996**) *Br. J. Pharmacol.* 118**,** 2199-2205.
Offertáler, L. *et al.* (**2003**) *Mol. Pharmacol.* 63, 699-705.

DEOXY ANALOGS OF CP-47,497 AND CP-55,940 AS POTENTIAL CB_2 SELECTIVE LIGANDS

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Although the benzopyran ring system of THC was considered essential for cannabinoid activity, non-traditional cannabinoids, lacking the benzopyran ring system, such as CP-47,497, or CP-55,940, were developed by Pfizer. These compounds have high affinity for the CB_1 and CB_2 receptors and are potent cannabinoids *in vivo*.

1-Deoxy-3-(1',1'-dimethylbutyl)-Δ^8-THC (JWH-133) is a highly selective CB_2 agonist with K_i = 3.4 nM at the CB_2 receptor and K_I = 677 nM at CB_1 (Huffman, J.W.; *et. al. Bioorg. Chem.* **1999**, *7*, 2905). In an effort to develop new CB_2 selective ligands, the synthesis of a series of deoxy analogs of the Pfizer bicyclic non-traditional cannabinoids was initiated.

3-(4-[1,1-Dimethylalkyl]phenyl)-1-cyclohexanols, deoxy analogs of CP-47,497, have been synthesized. These compounds were prepared by coupling an aryllithium with 3-ethoxy-2-cyclohexen-1-one followed by dissolving metal reduction of the initially formed cyclohexenone. Stereoselective reduction of the carbonyl group provides the CP-47,497 analogs. In all cases both epimeric alcohols were prepared. With the exception of commercially available 4-*tert*-butylbromobenzene the aryl bromide starting materials were prepared from the corresponding phenol by a new procedure developed in our laboratory.

3-(4-[1,1-Dimethylalkyl]phenyl)-4-(3-propanol)-1-cyclohexanols, deoxy analogs of CP-55,940, have been synthesized. These compounds were prepared via conjugate addition of an aryl Grignard reagent to an enone, followed by stereoselective reduction of the ketone, and hydroboration-oxidation to form the propanol chain (Johnson, M.R.; *et. al. U.S. Patent No.: US 4,371,720*, **1981**).

Preliminary results indicate that the lower members of both homologous series have little affinity for either the CB_1 or CB_2 receptor. Increased chain length (up to 1,1-dimethyl heptyl) has increased the affinity for both receptors with the CB_2 receptor affinities being slightly higher than that of CB_1 but still very weak. The synthesis and receptor affinities of these CP-47,497 and CP-59,940 analogs will be discussed.

Acknowledgements: The work at Clemson University was supported by grants DA03590 and DA15340, and that at Virginia Commonwealth University by grant DA03671, all from the National Institute on Drug Abuse.

SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF *N*-ACYLETHANOLAMINES OXYGEN HOMOLOGATED AT THE AMIDE BOND

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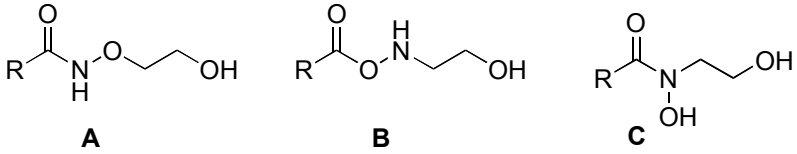
N-Acylethanolamines are an important class of endolipids that function as chemical messengers in the central nervous system. These compounds are exemplified by the endocannabinoid anandamide (arachidonylethanolamide, AEA), the PPAR-γ activator oleylethanolamide (OEA), and the neuroprotective agent palmitylethanolamide (PEA). Despite their chemical similarity, these compounds can show antagonistic activity at a functional level [AEA and PEA on neuroprotection (Leon et al., WO-03006007, **2003**), AEA and OEA on appetite (Rodriguez de Fonseca et al., Nature **2001**, 414,209)], and interference with their production and/or degradation has therapeutic potential for the management of pathological conditions that are top priorities in biomedical research (pain, anxiety, sleep and eating disorders). These compounds are rapidly inactivated by hydrolysis from a serine hydrolase widespread in the central nervous system (FAAH). S/A relationships within *N*-acylethanolamines have mainly been investigated in AEA, and have highlighted the critical role of the amide bond for bioactivity. Isosteric modification of this group was pursued with the aim of increasing metabolic stability, but was disappointing in term of activity. We have reasoned that oxygen homologation of the amide bond, while maintaining the basic topology of this group, has marked effect on its electronic properties. This could in principle provide the opportunity to dissect receptor interaction, a non-covalent event depending essentially on the spatial relationship between the heteroatoms of the polar head of ethanolamides, and the capacity of these compounds to act as substrates for FAAH inactivation, a dynamic event depending on the electronic properties of the substrate.

In principle, three distinct types of oxygen homologation of an amide bond are possible, resulting in *N*- or *O*-acylhydroxylamides and *N*-hydroxyamides, respectively (**A**-**C**). The chemistry of these functional groups has been so far poorly investigated. From a synthetic standpoint, while hydroxylamine would be a suitable and common starting material, the lability of the polyunsaturated olefin system of arachidonic acid greatly restricts the conditions available for its *N*-or *O*-alkylation. Therefore, soft alkylation procedures like the Mitsunobu reaction have played a critical role in our implementation of this project, whose chemical and pharmacological progress will be presented.



Acknowledgements: This work was supported by MIUR

INHIBITORS OF ANANDAMIDE UPTAKE LACKING ARACHIDONYL CHAIN

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We sought to discover small molecule inhibitors of cellular anandamide uptake to aid the pharmacological characterization of anandamide uptake and inactivation, and to evaluate its therapeutic potential. Structure-based computational screening was performed to identify candidates in a corporate compound collection that might substitute for the arachidonamide unsaturated carbon moiety and inhibit anandamide uptake. The arachidonyl chain has a high degree of conformational freedom, which hindered attempts to model its bioactive conformation. We used the X-ray crystal structure of arachidonic acid bound to cyclooxygenase-2 (COX-2) to model a potential arachidonyl-favoring binding site by computational docking of candidate ligands. This site, therefore, functioned as a virtual filter for the entire compound database. The top thousand compounds, ranked according to their docked interaction energy, were manually inspected using 3-dimensional computer graphics for close spatial overlap of the compound with the arachidonyl chain of the COX-2 ligand. This filtering process retrieved Anandamide itself. 96 compounds selected by this filtering process were screened for inhibition of radiolabeled anandamide uptake into human monocytes (U937 cells). This screening identified an inhibitor of anandamide uptake with IC_{50} = 5 μM. Two 96-compound solid phase combinatorial libraries were synthesized to probe the structure-activity around this screening hit, and several inhibitors with 100 nM IC_{50} were identified. One compound, SEP-0200228 (4-allyloxy-benzyl)-butyl-(3-phenyl-allyl)-amine, inhibited anandamide uptake in human monocytes (IC_{50} = 90 nM) and in synaptosomes 20-fold more potently than the arachidonamide inhibitor AM404. Inhibitors in this novel class, although lacking an arachidonyl chain, nevertheless have a degree of conformational flexibility, contain unsaturated carbon chains, and likely manage to prevent anandamide from associating with its putative transporter binding site.

SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF TRIFLUOROMETHYLKETONE COMPOUNDS DERIVED FROM IBUPROFEN

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Introduction- Anandamide, one of the major endocannabinoids, acts via cannabinoid and vanilloid receptors to produce analgesia. Oleamide, a sleeping inducer, acts also on the nociception and palmitoylethanolamide exhibits anti-inflammatory and analgesic properties without directly interacting with cannabinoid receptors. All of these lipids are cleaved by Fatty Acid Amide Hydrolase (FAAH). The inhibition of the FAAH would induce an increase of endogenous rates of these compounds and thus potentiate their effects. The nonsteroidal anti-inflammatory drug ibuprofen inhibits the FAAH with an IC_{50} of 270 µM [1]. The inhibitory potency is not negligible and would like contributing with the inhibition of the cyclooxygenase (COX) to their action mechanism. So, a combined FAAH/COX_2 inhibitor may be a useful therapeutic agent.

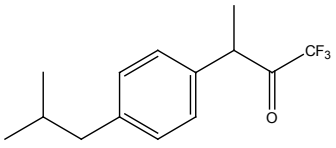
Aim – The aim of our study was to synthesize trifluoromethylketone inhibitors derived from ibuprofen and to evaluate the inhibition potential towards FAAH and COX. Finally, their affinity for the cannabinoid receptors has been determined.

Results

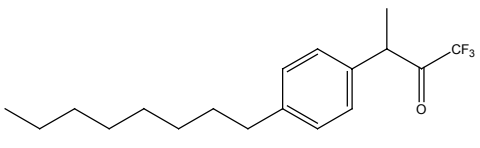
Chemistry: The trifluoromethylketones (TFMK) were synthesized from the corresponding acid chloride by reaction with trifuoroacetic anhydride and pyridine at –60°C, under N_2[2]. The acids were obtained from phenylalkanes by acylation of Friedel-Craft and Willgerodt Kindler reaction.

Pharmacology: The inhibition of FAAH was determined by monitoring the hydrolysis of [^{3}H]-anandamide on rat brain homogenates. The inhibition of COX_1 and COX_2 was determined by evaluating respectively the TXB_2 and PGE_2 content by E.I.A. in fresh blood. The affinity for the cannabinoid receptors was determined by the specific binding displacement of [^{3}H]-CP 55-940 on homogenates from CHO cells expressing either hCB_1 or hCB_2 receptors.

The derivative TFMK of ibuprofen (CL10) inhibited the FAAH with IC_{50} value of 2,86 µM. The potencies of a series of compounds related in structure to ibuprofen were determined and the most potent was 1,1,1-trifluorométhyl-3-(4-octyl-phényl)-butan-2-one (CL59) with an IC_{50} value of 1,89 µM.



CL10



CL59

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ACYL CHAIN REQUIREMENTS FOR ENDOCANNABINOID INTERACTION WITH FAAH

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The termination of anandamide (AEA, 20:4 $\Delta^{5,8,11,14}$) signaling relies primarily on chemical transformation via fatty acid amide hydrolase (FAAH). FAAH is integrated into cell membranes and AEA's direct access to the enzyme active site is via the bilayer (Bracey et al., *Science* **2002**, *298*, 1793). Although AEA interacts with both the CB_1 receptor and with FAAH, endocannabinoid SAR studies have shown some divergences in SAR requirements for interaction with each of these targets (Reggio and Traore, *Chem. Phys. Lipids,* **2000***,108*,15). FAAH shows a preference for C_{18}-C_{20} fatty acid acyl chains, while the CB_1 receptor appears to require and tolerate longer acyl chains (C_{20} or C_{22}). CB_1 requires ligands with a minimum of five saturated carbons at the end of the acyl chain, while ethanolamides with fewer saturated carbons (i.e. less than five) at the end of the acyl chain can compete with AEA for FAAH. CB_1 requires more unsaturation (three *cis* double bonds minimum), whereas FAAH can tolerate a minimum of two *cis* double bonds. We have previously explored the molecular basis underlying the acyl chain SAR requirements for AEA interaction with CB_1 (Barnett-Norris et al. *J. Med. Chem.* **2002,** *45*, 3649**).** The recently published crystal structure of FAAH now makes possible an exploration of the molecular basis for the acyl chain SAR requirements for FAAH.

In order to probe the acyl chain conformational requirements for hydrolysis at FAAH (F. Desarnaud et al. *J.Biol.Chem.* **1995,** *270*, 6030), we performed conformational analyses of the following ethanolamides using the biased Monte Carlo/simulated annealing technique, Conformational Memories (CM), which employs the Generalized Born/surface area continuum solvation model for chloroform : 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). Our CM results indicated that each analog has both an extended and a curved/U-shaped family of conformers, with the degree of curvature in the curved/U-shaped family increasing with the degree of unsaturation. The ability of FAAH to accommodate each of these analogs was examined in docking studies using the crystal structure of FAAH (Bracey et al., *Science* **2002**, *298*, 1793). Each analog headgroup was modeled as its tetrahedral intermediate in the FAAH active site in order to assure proper placement of the head group. In the FAAH crystal structure, the tunnel leading from the surface of FAAH containing the buried arachidonyl chain of the co-crystallized arachidonyl inhibitor, MAP, is lined with aromatic and aliphatic amino acids. Each analog acyl chain was adjusted to fit in this tunnel using interactive computer graphics and the resultant complex was energy minimized using Amber. The existence of possible interactions between acyl chain double bonds and aromatic residues that line the tunnel (F194, F381, F432) were assessed. The final docked conformation of each analog was compared to CM results to examine whether this conformer belonged to one of the low free energy conformational families for that analog identified by CM. Results of docking studies will be presented at the meeting.

Acknowledgements: Support: NIDA Grants DA03934 and DA000489 to PR

RELEASE OF INTRACELLULAR CALCIUM BY STIMULATION OF PLC/IP_3-PATHWAY LEADS TO ANANDAMIDE FORMATION IN SENSORY NEURONS

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Transient Receptor Potential Vanilloid type 1 (TRPV1) channels in sensory neurons of rat dorsal root ganglia (DRG) are involved in pain transmission. ATP may induce TRPV1-mediated inflammatory hyperalgesia through activation of the $G_{q/11}$-coupled P_2Y receptors and subsequent PKC-mediated phosphorylation of TRPV1. Since, DRG neurons are also able to synthesize anandamide in high amounts, we have tested whether anandamide can contribute to ATP-induced activation of TRPV1 in rat DRG neurons.

In the absence of extracellular Ca^{2+}, ATP mobilized intracellular Ca^{2+} and significantly enhanced intracellular anandamide levels, as measured by isotope-dilution liquid chromatography-mass spectrometry. Anandamide levels were even further increased by blocking its transport out of the neurons with VDM11. Upon reconstitution of Ca^{2+}, the subsequent Ca^{2+}-entry induced by ATP could be significantly attenuated by capsazepine and enhanced by VDM11. This latter effect was also blocked by capsazepine. When DRG neurons were pre-treated with U73122, the enhanced Ca^{2+}-influx by both ATP and VDM11 were inhibited. Accordingly, anandamide levels were reduced upon U73122-treatment. The role in anandamide formation of Ca^{2+} released from the endoplasmic reticulum (ER) was confirmed by the finding that thapsigargin, a Ca^{2+}-ATPase inhibitor that blocks the re-uptake of Ca^{2+} in the ER thus provoking Ca^{2+} store depletion, also caused formation of intracellular anandamide in DRG neurons.

These data indicate that intracellular anandamide produced via activation of the PLC/IP_3-pathway and subsequent mobilization of intracellular Ca^{2+}, may mediate TRPV1-induced Ca^{2+} entry in sensory neurons. These results may have implications for inflammatory pain.

LIPID ANALYSIS OF MAMMALIAN CELLS STABLY OVEREXPRESSING *N*-ACYLPHOSPHATIDYLETHANOLAMINE-HYDROLYZING PHOSPHOLIPASE D (NAPE-PLD)

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In animal tissues, *N*-acylethanolamines (NAEs) including anandamide are principally formed from their corresponding *N*-acyl-phosphatidylethanolamines (NAPEs) by a specific phosphodiesterase of the phospholipase D type (NAPE-PLD). Recently we cloned cDNAs of NAPE-PLD from mouse, rat, and human (Okamoto *et al*., J. Biol. Chem. **2004**, *279*, 5298). However, it remained unclear whether NAPE-PLD acts towards membrane NAPEs in living cells. We stably transfected two mammalian cell lines (HEK293 and CHO-K1) with mouse NAPE-PLD cDNA. The homogenates of the NAPE-PLD-overexpressing HEK293 and CHO-K1 cells showed specific NAPE-PLD activities of 1-2 nmol/min/mg protein with *N*-[^{14}C]palmitoyl-PE as substrate. The control cells transfected with the insert-free vector showed specific activities of 0.01-0.02 nmol/min/mg protein. To examine the endogenous levels of NAPEs and NAEs in these cells, total lipids were extracted from the cell pellets by the Folch procedure. NAPEs and NAEs were then purified, and subjected to gas chromatography-mass spectrometry. NAPEs and NAEs with different *N*-acyl groups were separately quantified. The results revealed that overexpression of NAPE-PLD caused a decrease in the total amount of NAPEs and an increase in the total amount of NAEs, suggesting that recombinant NAPE-PLD is active in the cells and utilizes endogenous membrane-bound NAPE as substrate. We also found that NAPE-PLD did not show selectivity for *N*-acyl species of the endogenous NAPEs. Anandamide represented only 1-2 % of the total NAEs with the NAPE-PLD-overexpressing cells. In conclusion, our results suggest that NAPE-PLD is actually responsible for the formation of NAEs including anandamide in living cells.

BIOSYNTHESIS OF ANANDAMIDE AND *N*-PALMITOYLETHANOLAMINE BY SEQUENTIAL ACTIONS OF PHOSPHOLIPASE A_2 AND LYSOPHOSPHOLIPASE D

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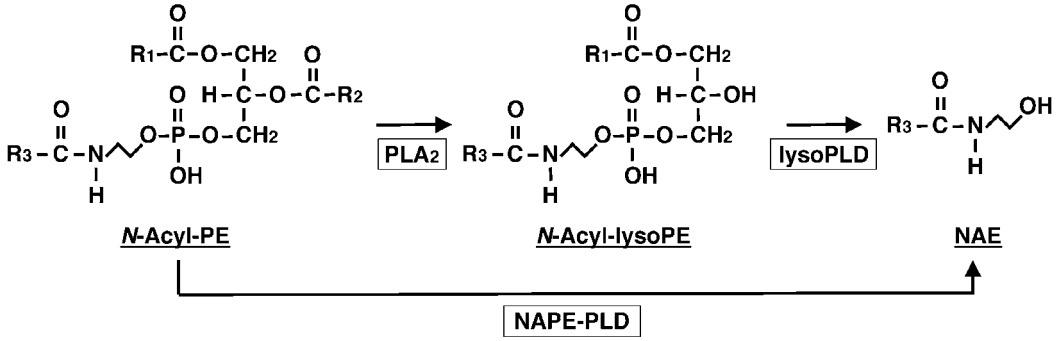
We recently cloned cDNAs of *N*-acyl-phosphatidylethanolamine (*N*-acyl-PE)-hydrolyzing phospholipase (PL) D (abbreviated to NAPE-PLD), which releases anandamide and other long-chain *N*-acylethanolamines (NAEs), from mouse, rat and human (Okamoto *et al*. **2004**, J. Biol. Chem. 279, 5298). Alternatively, the possible presence of a two-step pathway from *N*-acyl-PE has been suggested (Schmid *et al.* **1990**, Prog. Lipid Res., 29, 1), that contains (1) PLA_1/A_2 enzyme(s) hydrolyzing *N*-acyl-PE to *N*-acyl-lysoPE and (2) lysophospholipase D (lysoPLD) enzyme(s) releasing NAEs from *N*-acyl-lysoPE. We actually detected the PLA_1/A_2 activity for *N*-palmitoyl-PE in various rat tissues with the highest activity in the stomach, and identified the stomach enzyme as group IB secretory PLA_2 ($sPLA_2$). In addition, recombinant group IB, IIA, and V of $sPLA_2$s overexpressed in HEK293 cells were also active with *N*-palmitoyl-PE, while group X $sPLA_2$ and cytosolic $PLA_2\alpha$ were inactive. As for the lysoPLD releasing *N*-palmitoylethanolamine from *N*-palmitoyl-lysoPE, we observed the activity in various rat tissues, with higher activities in the brain and testis. Notably, this distribution was different from that of NAPE-PLD which shows a low activity for *N*-acyl-lysoPE. The brain lysoPLD was dose-dependently inhibited by methyl arachidonyl fluorophosphonate, whereas NAPE-PLD was insensitive to this compound. Moreover, the enzyme was poorly solubilized from the membrane in the presence of octyl glucoside in contrast to NAPE-PLD. Thus, the occurrence of lysoPLD distinct from NAPE-PLD was suggested. Coexistence of $sPLA_2$-IB and lysoPLD actually caused the generation of *N*-palmitoylethanolamine from *N*-palmitoyl-PE. Anandamide precursors also acted as substrates of these enzymes. Based on these results, $sPLA_2$ and lysoPLD may constitute another pathway for the formation of anandamide and other NAEs.



SEX DIFFERENCES IN LEVELS OF ENDOCANNABINOIDS ANANDAMIDE(AEA), 2-ARACHIDONYLGLYCEROL(2-AG) AND N-ARACHIDONYLGLYCINE(NAGly) IN THE HYPOTHALAMUS, PITUITARY AND CEREBELLUM

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Sex differences are often observed in the behavioral and therapeutic effects of exogenously delivered cannabinoids. These maybe attributed to differences in cannabinoid metabolism between males and females; however, there is evidence that the endogenous cannabinoid system may express central nervous system sex differences. Previous studies showed sex differences in levels of CB_1 receptor-mRNA transcripts in different areas of the brain and in levels of endocannabinoids in the anterior pituitary of rodents.

The purpose of the current study was to compare the levels of the endocannabinoids anandamide (AEA), 2-arachidonylglycerol (2-AG) and *N*-arachidonylglycine (NAGly) in the hypothalamus, pituitary and cerebellum between females in two phases of the ovarian cycle, estrus and diestrus, and males. In this report, we present findings from tandem mass spectrometric analyses of brain tissue collected from 21 Sprague-Dawley rats (n=7 per group).

2-AG levels in whole pituitary were significantly lower in diestrus females than estrus females (P=0.01) whereas there were no significant differences in 2-AG levels between diestrus or estrus females and males. In the cerebellum an inverse pattern of 2-AG levels was observed (P=0.03) where the levels of 2-AG were significantly higher in diestrus compared to estrus, with the levels in males not differing from either female group. There were no significant differences among any group in 2-AG levels in the hypothalamus. AEA levels were equivalent among groups in both hypothalamus and pituitary. AEA levels in cerebellum were significantly lower in estrus females compared with diestrus females (P=0.014) and males (P=0.006). Levels of NAGly were equivalent among groups in hypothalamus, pituitary and cerebellum.

These findings support differences in endocannabinoid levels between sexes and within females across the ovarian cycle.

ENZYMES FOR 2-AG BIOSYNTHESIS AND METABOLISM IN CELL LINES, AND THEIR PHARMACOLOGICAL INHIBITION

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Enzymes for the biosynthesis and degradation of the endocannabinoid 2-arachidonoyl glycerol (2-AG) have been cloned and are the sn-1-selective-diacylglycerol lipases α and β (DAGLαand β) (Bisogno et al., **2003**) and the monoacylglycerol lipase (MAGL) (Karlsson et al., **1997**; **2001**; Ho et al., **2002**; Dinh et al., **2002**), respectively. We have screened several cell lines for the presence of these enzymes, in order to use them for the development of inhibitors of 2-AG biosynthesis and metabolism. We used semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the enzymes, and specific enzyme assays to confirm their presence in the cells with the most abundant mRNA transcripts. We screened mouse neuroblastoma N18TG2 cells, rat basophilic leukaemia RBL-2H3 cells, rat glioma C6 cells, human embryonic kidney HEK-293 cells, human colorectal carcinoma Caco-2 cells at different stages of differentiation, human breast carcinoma MCF-7 cells, primate COS cells, etc.. DAGL and MAGL activity were assessed by using sn-1-[^{3}H]-oleoyl-2-arachidonoyl-glycerol and 2-[^{3}H]-arachidonoylglycerol as substrates, respectively.

All cells screened expressed high levels of at least one of the two DAGL isoforms as well as of MAGL, and there was a correlation between high mRNA levels and enzymatic activity, and between the capability of cells to produce 2-AG on stimulation with ionomycin and DAGL mRNA expression. Cytosolic + microsomial fractions from COS cell homogenates were used to screen substances for MAGL inhibitory activity, whereas 10,000 x g membranes from COS cells over-expressing DAGLαand β, or from N18TG2, RBL-2H3 and C6 cells, were used to search for DAGL inhibitors. The molecules screened were: 1) non-selective Ser hydrolase inhibitors; 2) 2-AG analogs; 3) previously described lipase inhibitors; 4) fatty acid amide hydrolase (FAAH) inhibitors; and 5) inhibitors of the endocannabinoid membrane transporter (EMT).

Among the substances tested on MAGL, apart from the non-selective methyl-arachidonoylfluorophosphonate (MAFP, IC_{50}=0.3 μM) and bromophenacyl-bromide (BPB, IC_{50}=1 μM), arachidonoyl ethylene glycol was the most potent and selective inhibitor (IC_{50}=25 μM), with no activity on DAGLα and inhibition of FAAH only at > 25 μM concentrations. Among the substances tested on DAGLα, apart from the non-selective MAFP (IC_{50}=0.2 μM), the most potent compound was the lipase inhibitor tetrahydrolipstatin (THL, orlistat®), which exhibited IC_{50} values (60-250 nM) much lower than those previously reported for other lipases. Interestingly, the EMT inhibitor, VDM-11, and the FAAH inhibitor N-arachidonoyl-serotonin, exerted little, if any, inhibitory activity on either MAGL or DAGL enzymes.

When tested in intact N18TG2, RBL-2H3 and C6 cells against ionomycin-induced 2-AG formation, THL and MAFP exerted opposite effects, with the former compound causing a significant inhibition, and MAFP causing instead an enhancement of 2-AG levels. This suggested that MAFP inhibitory action on MAGL prevails on that on DAGL in intact cells, and that THL can be used as a useful and relatively selective pharmacological tool to investigate 2-AG biological role in vitro.

INTERACTION OF ANALOGUES OF 2-ARACHIDONOYLGLYCEROL WITH FATTY ACID AMIDE HYDROLASE AND MONOGLYCEROL LIPASE

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It is now established that whilst fatty acid amide hyrolase (FAAH) is responsible for the metabolism of anandamide, both this enzyme and monoacylglycerol lipase (MAG lipase) can metabolise 2-arachidonoylglycerol (2-AG). The role of FAAH is becom-ing more clear due to the development both of selective inhibitors and genetically modified animals, whereas the role of MAG lipase is hampered by the lack of such experimental tools. In consequence, the present study was aimed at determining whether analogues of 2-AG selective for MAG lipase could be identified. Membrane and high-speed supernatant fractions from rat brain were assayed for FAAH and MAG lipase activities at an assay pH of 7.2 using 2 μM [^{3}H]AEA and [^{3}H]2-oleoyl-glycerol (2-OG) as substrates, respectively, essentially as described by Dinh *et al.*, (*PNAS* 99 [2002] 10819-24). IC_{50} values were determined assuming a max inhibition of 100%. The pH optimum and sensitivity of MAG lipase to inhibition by arachidon-oyl trifluoromethyl ketone [arachidonoyl-TMK] and palmitoylethanolamide differed from that of FAAH, consistent with the study of Dinh *et al.* (*ibid.)*. Oleoyl- and palmitoyl-TMK also inhibited MAG lipase (IC_{50} values of 2.3 and 7.8 μM *vs.* 2.9 μM for arachidonoyl-TMK), but were more potent towards FAAH (IC_{50} values 0.076 and 0.073 μM *vs.* 0.55 μM). 2-AG inhibited 2-OG hydrolysis with an IC_{50} value of 24 μM, whereas the compound was less potent as an inhibitor of AEA hydrolysis (IC_{50} >100 μM). 1-AG behaved very similarly to 2-AG. In contrast, 2-arachidonoyl serinol (where the ester linkage between the arachidonoyl side chain and the glycerol head group is replaced by an amide linkage) interacted less potently with MAG lipase (IC_{50} 73 μM) and showed no selectivity *vs* FAAH (IC_{50} 78 μM). Palmitoylserinol had little effect upon either MAG lipase or FAAH, producing ~20% inhibition at 100 μM. Replacement of the -CH_2 group with -NH at position 1 of the side chain of 1-AG, to produce an urea linkage instead of the ester linkage, gave an analogue (O-1502) of 1-AG which inhibited FAAH with an IC_{50} value of 19 μM, but only produced 41±3% inhibition of MAG lipase at 100 μM. Noladin ether (i.e. where the ester linkage of 2-AG is replaced with an ether linkage) inhibited MAG lipase less potently than FAAH (IC_{50} values of 36 and 3.0 μM, respectively). Insertion of a methyl group at position 1 of the side chain of 1-AG produced a compound (O-1428) which retained its affinity for MAG lipase (IC_{50} 17 μM) but was more potent than 1-AG towards FAAH (IC_{50} value 33 μM). This compound interacted more weakly with CB_1 receptors expressed in CHO cells (K_i value 1.8 μM) than the corresponding 1-AG (K_i value 0.24 μM). Insertion of two methyl groups at position 16 of the arachidonoyl side chain of 2-AG together with a hydroxy group at position 20 produced a compound (O-2204) that showed no selectivity for FAAH (IC_{50} 35 μM) vs MAG lipase (IC_{50} 32 μM). In conclusion, the present data show that MAG lipase and FAAH have very different inhibitor specificities, but that no selective MAG lipase inhibitor was identified.

LEPTIN AND PROGESTERONE REGULATE DIFFERENTLY THE FAAH PROMOTER IN HUMAN IMMUNE CELLS AND NEURONAL CELLS

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We have shown that in human T lymphocytes leptin stimulates activity and expression of fatty acid amide hydrolase (FAAH), through STAT3 and its CRE (cAMP response element)-like transcriptional target in the FAAH promoter. Furthermore we have seen that also progesterone, alone or synergistically with leptin, up-regulates the FAAH gene in human T-cells, through the Ikaros transcription factor. Here, we have found that in immortalized human lymphoma U937 cells, the stimulation of FAAH by leptin (up to ~300% of the controls) involves binding to a leptin receptor (K_d = 2.0 ± 0.1 nM, B_{max} = 382 ± 5 fmol.mg protein^{-1}, apparent molecular mass of ~110 kDa), and that by progesterone involves an intracellular progesterone receptor of ~120 kDa. Mutation of the CRE-like element or the Ikaros binding site in the FAAH promoter prevented the effect of leptin or progesterone respectively, in transient expression assays and in electrophoretic mobility shift and supershift assays. Interestingly, also human neuroblastoma CHP100 cells have a leptin receptor of ~110 kDa (K_d = 2.2 ± 0.2 nM, B_{max} = 339 ± 8 fmol.mg protein^{-1}), a progesterone receptor of ~120 kDa, STAT3 and Ikaros, yet they did not show any activation of FAAH through STAT3 or Ikaros upon treatment with leptin or progesterone, respectively. These data, taken together, suggest a never seen cell-specific regulation of FAAH gene in the neuroimmune system.

ANANDAMIDE HYDROLASE IS ACTIVATED IN INTACT UTERUS THROUGH A LIPID RELEASED BY MOUSE BLASTOCYSTS

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Anandamide (N-arachidonoylethanolamine, AEA) is a major endocannabinoid, known to impair mouse pregnancy and embryo development and to induce apoptosis in blastocysts. Here we show that mouse blastocysts rapidly (within 30 min of culture) release a soluble compound, that increases by ~2.5-fold the activity of AEA hydrolase (fatty acid amide hydrolase, FAAH) present in the mouse uterus, without affecting gene expression at the translational level. This "FAAH activator" was produced by both trophoblast and inner cell mass cells, and its initial biochemical characterization showed that it was fully neutralized by adding lipase to the blastocyst-conditioned medium (BCM), and was potentiated by adding trypsin to BCM. Other proteases, phospholipases A_2, C or D, DNAse I or RNAse A were ineffective. BCM did not affect the AEA-synthesizing phospholipase D, the AEA-binding cannabinoid receptors, or the selective AEA membrane transporter in mouse uterus. The FAAH activator was absent in uterine fluid from pregnant mice and could not be identified with any factor known to be released by blastocysts. In fact, platelet-activating factor inhibited non competitively FAAH in mouse uterus extracts, but not in intact uterine horns, whereas leukotriene B_4 or prostaglandins E_2 and $F_{2\alpha}$ had no effect. Overall, it can be suggested that blastocysts may protect themselves against the noxious effects of uterine endocannabinoids by locally releasing a lipid able to cross the cell membranes and to activate FAAH. The precise molecular identity of this activator, the first ever reported for FAAH, remains to be elucidated.

EXPRESSION AND CHARACTERIZATION OF THE RAT FATTY ACID AMIDE HYDROLASE FUSED TO THE MALTOSE BINDING PROTEIN

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The Fatty Acid Amide Hydrolase represents an attractive target for medicinal chemistry developments. It is an enzyme of 579 amino acids, widely distributed in the body and responsible of the hydrolysis of lipid substrates including endocannabinoids. Its recent crystallization gives some insight into its structure (Bracey, **2002**). The FAAH seems to be organized in a dimmer that would be anchored to the membrane through two α helices. The structure also reveals the existence of two channels that give the active site a direct access to the membrane and to the cytosol. This structure could be the basis for the design of novel inhibitors using a rational approach.

Our laboratory is involved in the development of novel Fatty Acid Amide Hydrolase inhibitors (Vandevoorde, **2003**). In a first step toward the utilization of a new source of enzyme in our inhibitors screening assay and with the aim of setting up kinetics studies, the FAAH has been expressed as a fusion to the Maltose Binding Protein from *Escherichia Coli*. This strategy has been widely used to improve the solubility of several target proteins (Smyth, **2003**).

Rat FAAH cDNA has been introduced into the pMALcRI vector. Expression was carried out in *E. Coli* in the presence of IPTG (0.3 mM) for 2 hours at 37°C. No detergent was needed to recover the hydrolyzing activity. The resulting protein was characterized in order to assess its functionality. The assay relies on the hydrolysis of radiolabelled AEA. The [^{3}H]-ethanolamine product is separated from the labelled substrate using a solution of chloroform/methanol (1/1) and radioactivity in the aqueous phase is quantified.

As a result the MBP-FAAH fusion was shown to retain the kinetics parameters (Km) of rat brain FAAH. Moreover, the protein possesses the same inhibition profile towards several inhibitors as for the FAAH present in rat brain homogenates. Two of those compounds were the well known ATFMK and PMSF; the others were CL9, L29 and L38, original compounds synthesised in our lab. The mode of inhibition of CL9 compound was also determined using the MBP-FAAH fusion. Otherwise, the similarity between the recombinant protein and the enzyme present in rat brain homogenates further confirms the selectivity of the widely used radioactivity-based activity assay toward the FAAH over other enzymes present in the endocannabinoid system.

In conclusion, Maltose Binding Protein can be used as a mean to easily express the Fatty Acid Amide Hydrolase, a membrane bound enzyme, as a functional fusion protein that can be used in kinetics studies.

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INFLUENCE OF FATTY ACID AMIDE HYDROLASE (FAAH) IN ANANDAMIDE UPTAKE: COMPARISON BETWEEN ANANDAMIDE TRANSPORT IN FAAH KNOCKOUT AND WILD TYPE NEURONS

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It is generally accepted that the inactivation of anandamide (AEA) is a two step process, composed of an uptake phenomenon followed by intracellular hydrolysis catalyzed by the enzyme fatty acid amide hydrolase (FAAH). However, the indirect pharmacological evidences available regarding the existence of such a transporter protein responsible for the uptake of anandamide are rather controversial, and it has been suggested that the uptake phenomenon could be a simple diffusion process driven by FAAH metabolism. In this work we have studied the contribution of FAAH to the AEA uptake using the selective uptake inhibitor UCM707 together with preparations from FAAH knockout (KO) mice, in an attempt to separate both contributions and measure uptake inhibition in FAAH KO preparations. Our results indicate that i) special care must be taken to avoid the presence of artifacts when studying the cellular uptake of highly lipophilic molecules such as AEA, ii) there is a partial contribution of FAAH hydrolysis to the AEA uptake, especially at the longest times, and iii) there exists a mechanism responsible for the uptake of AEA which is independent from FAAH, which is still present and active in FAAH KO neurons and which can be inhibited by UCM707. Collectively, these results support the notion of the existence of another entity different from FAAH which accounts, if not completely at least in an important extent, for the transport of AEA.

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ANANDAMIDE TRANSPORT IS INDEPENDENT OF FAAH ACTIVITY AND IS BLOCKED BY THE HYDROLYSIS RESISTANT INHIBITOR AM1172

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The endogenous cannabinoid anandamide is removed from the synaptic space by a high-affinity transport system, present in neurons and astrocytes, which is inhibited by N-(4-hydroxyphenyl)-arachidonamide (AM404). After internalization, anandamide is hydrolyzed by fatty-acid amide hydrolase (FAAH), an intracellular membrane-bound enzyme that also cleaves AM404. Based on kinetic evidence, it has been recently suggested that anandamide internalization may be mediated by passive diffusion driven by FAAH activity. To test this possibility, in the present study we have investigated anandamide internalization in wild-type and FAAH-deficient ($FAAH^{-/-}$) mice. Cortical neurons from either mouse strain internalized [^{3}H]anandamide through a similar mechanism - i.e., via a rapid, temperature-sensitive and saturable process, which was blocked by AM404. Moreover, systemic administration of AM404 to either wild-type or $FAAH^{-/-}$ mice enhanced the hypothermic effects of exogenous anandamide, a response that was prevented by the CB_1 cannabinoid antagonist rimonabant (SR141716A). The results indicate that anandamide internalization in mouse brain neurons is independent of FAAH activity. In further support of this conclusion, the compound AM1172, a novel inhibitor of anandamide transport, blocked [^{3}H]anandamide internalization in rodent cortical neurons and human astrocytoma cells without acting as a FAAH substrate or inhibitor. AM1172 may serve as a prototype for novel anandamide transport inhibitors with increased metabolic stability and target selectivity.

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EFFECTS OF "AEA TRANSPORT INHIBITORS" AND FAAH INHIBITORS IN FAAH KNOCKOUT MICE

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Anandamide (AEA) is an endogenous ligand that binds to cannabinoid receptors and it is inactivated by intracellular metabolism by the fatty acid amide hydrolase, FAAH, after its uptake into cells by a process that depends on a concentration gradient. Both FAAH inhibitors and inhibitors of AEA uptake have been described and shown to exhibit cannabimimetic activity in rats and mice. However, their mode of action remains to be further elucidated. For example, the selectivity of FAAH inhibitors in vivo remains unknown and it is possible that some of the cannabimimetic behavioral effects may be due to off-target reactions at sites other than FAAH. Likewise, uptake inhibitors may in fact be acting, in some cases as FAAH inhibitors. To determine the selectivity of FAAH inhibitors and putative uptake inhibitors, we have undertaken experiments using FAAH WT and FAAH knockout (KO) mice (Cravatt et al., PNAS, 98: 9371-9376, **2001**).

Mice received the drugs by I.P injection and were evaluated for their ability to modulate hypothermia, and antinociception. Hypothermia and antinociception were determined by measuring the rectal temperature and tail-flick reaction time to a heat stimulus respectively before vehicle or drug administrations as described elsewhere (Martin et al., JPET. 294: 1209-1218, **2000**).

UCM-707, AM404 and 0-1887 were found to inhibit FAAH with IC_{50}s of 40μM, 6μM, and 15nM, respectively. High and low doses of these inhibitors were studied alone and in conjunction with varying does of AEA to determine their effects upon hypothermia and antinociception in WT and KO mice. When AEA (high does, 25 to 50mg/kg) was administered I.P. alone, it had an effect upon antinociception in WT and KO mice. However, the effect was more dramatic in KO mice, particularly with antinociception. When the inhibitors were administered I.P. alone, they had little or no effect on antinociception and rectal temperature suggesting that I.V. or I.T. or I.C.V. routes may be necessary under the conditions used in our assays to see the tonic effects of AEA. However, when AEA (high dose) and inhibitors were co-administered I.P., hypothermic and antinociceptive effects were observed, but in general the co-administered drug effect was no greater than that observed with the high doses of AEA alone. However, with 0-1887 (2.5mg/kg), the co-administration route showed a more pronounced effect in the WT animals suggesting that the effect may be due mainly to FAAH rather than off-target reactions. These results suggest that in order to perfect this physiological assay for determination of inhibitor selectivity, AEA levels, inhibitor levels and the routes of administration need to be optimized.

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IN VIVO [^{3}H]-ANANDAMIDE ACCUMULATION IN WILD-TYPE AND FATTY ACID AMIDE HYDROLASE KNOCKOUT MOUSE BRAINS

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Arachidonoyl ethanolamide (anandamide) is a putative endocannabinoid that is inactivated by a process of intracellular accumulation followed by hydrolysis by fatty acid amide hydrolase (FAAH) to form arachidonic acid and ethanolamine. FAAH knockout mice are unable to metabolize exogenous anandamide, suggesting that FAAH is the principle enzyme responsible for anandamide metabolism (Proc Natl Acad Sci U S A **2001** 98:9371-6).

Prior studies concluded that anandamide accumulation in cultured cells is a process that is ATP and sodium independent, and is therefore driven by a concentration gradient (J Pharmacol Exp Ther **2000** 292:3 960-7; J Neurochem **1997** 69: 631-638). Anandamide uptake has been linked with FAAH activity. A comparison of steady-state anandamide accumulation in cell lines that contain active FAAH to cells lacking hydrolysis activity showed that FAAH was able to maintain the concentration gradient that drives the continual intracellular accumulation of anandamide and its metabolites (J Biol Chem **2001** 276:6967-6073; Mol Pharm **2001** 59:1369-1375).

There have been few studies examining the accumulation pattern of anandamide in intact brain. In the only published report to date, rat brain slices incubated in media containing radiolabeled anandamide showed a specific accumulation pattern that was significantly reduced by AM404 (Giuffrida et al., J Pharm Exp Ther **2001** 298: 7–14). In this study, we examined the effects of FAAH activity upon [^{3}H]-anandamide accumulation in the mouse brain. Wild type and FAAH knockout mice were intravenously injected with ^{3}H-anandamide. After 30 minutes, the mice were sacrificed and their brains fixed and processed for imaging with a beta imager (Biospace, France) to determine the distribution of accumulated tritium in the sections. Relative to the FAAH knockout mice, wild type brains had a slightly larger overall accumulation of [^{3}H]-anandamide and/or [^{3}H]-anandamide metabolites in the brain. Additionally, a heterogeneous distribution of the tritium accumulation was observed in the wild type brains, with the highest levels of tritium accumulation found in the cortex. By contrast, FAAH knockout mice brains showed a fairly uniform distribution of accumulated tritium over the brain sections, with no indication of greater uptake in the cortex.

In conclusion, these data suggest that FAAH activity plays a role in the accumulation of exogenously administered anandamide in the mouse brain.

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IDENTIFICATION OF A NOVEL HUMAN SPLICE VARIANT OF THE HUMAN CB_1 RECEPTOR

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Cannabinoid ligands are implicated in many physiological processes. To date two receptors have been identified as being responsible for mediating the actions for these ligands. However a growing body of evidence exists that suggests the presence of additional receptors. A splice variant of the human CB_1 receptor called CB_1a has previously been described but shown to be human specific. It was during the process of cloning this receptor that we identified from a foetal brain cDNA library a new variant that we now call CB_1b. This novel CB_1b variant was identified and cloned by PCR using the protocol initially used to clone the CB_1a variant described by Shire et al (**1995**). Characterising these two splice variants in our experimental system using [^{3}H]-CP55940 radioligand binding and GTPγS assays demonstrates that they both have altered ligand binding and activation properties as compared to the full length CB_1 receptor. Tissue distribution of CB_1a and CB_1b was performed using PCR on tissue-derived cDNA's. This expression analysis demonstrates that these are expressed in very low levels in a variety of tissues. Whilst our findings demonstrate a unique pharmacology for these variants, it remains to be seen what functional role these receptor variants may have.

Shire, D., C. Carillon, et al. (**1995**). "An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. Journal of Biological Chemistry 270(8): 3726-31.

EXPRESSION ANALYSIS OF CB_1 RECEPTORS USING CONDITIONAL CB_1 MUTANT MICE

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The expression pattern of CB_1 receptors in the mammalian brain has been extensively characterized. In cortical areas, CB_1 is expressed predominantly in GABAergic interneurons. However, evidence exists pointing to the presence of CB_1 also in other neuronal populations, including glutamatergic principal neurons. We recently generated conditional mutant mice that lack expression of the CB_1 gene in all principal forebrain neurons but not in GABAergic interneurons ($CB_1^{f/f;CaMKII\alpha Cre}$ mice), and showed that these mice are more prone to kainic acid-induced seizures than control littermates (Marsicano et al., *Science* 302:84, **2003**). These results prompted us to investigate in more details the expression of CB_1 receptor in the mouse brain.

By *in situ* hybridization, we analyzed the coexpression of CB_1 mRNA with markers of glutamatergic neurons and found that high numbers of glutamatergic cells contain CB_1 mRNA in cortical areas. By immunohistochemistry, using a N-terminus CB_1 antiserum kindly provided by Prof. Ken Mackie (Seattle, U.S.A.), we studied the expression of CB_1 protein in wild-type mice and in conditional mutant mice lacking CB_1 in different neuronal subpopulations, namely principal neurons of the forebrain ($CB_1^{f/f;CaMKII\alpha Cre}$ mice) or GABAergic neurons ($CB_1^{f/f;dlx5/6Cre}$ mice).

In the hippocampus, the expression pattern of the CB_1 protein was similar as previously described, with an intense meshwork of CB_1-positive fibers surrounding the cell bodies of principal cells in the CA1, CA3 and dentate gyrus regions. As expected, in $CB_1^{f/f;CaMKII\square Cre}$ mice, the expression of CB_1 protein was similar to wild-type mice, indicating that most of the protein detected by the CB_1 antibody is present on GABAergic interneurons. However, interestingly, in $CB_1^{f/f;dlx5/6Cre}$ mice, which lack expression of CB_1 in GABAergic neurons, the staining of fibers was not abolished. These results indicate that not all CB_1-positive fibers stained by the CB_1 antiserum belong to GABAergic neurons. In order to investigate which neuronal population(s) express CB_1, double immunohistochemical studies are being performed, combining the CB_1 antiserum with specific antibodies against markers of glutamatergic, cholinergic or serotonergic neurons. These results show that CB_1 receptors are not solely expressed by GABAergic interneurons in the hippocampus.

EXPRESSION OF FUNCTIONAL CB_1 CANNABINOID RECEPTORS IN P19 MOUSE EMBRYONAL CARCINOMA CELLS: EFFECT OF DIFFERENTIATION INTO A NEURONAL PHENOTYPE

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The study *in vitro* of the effects of cannabinoids upon neuronal function and survival require the use of primary cultures, usually obtained from embryonic rodents. In the present study, we have investigated an alternative approach, not requiring the use of embryos. P19 embryonic carcinoma (EC) cells are pluripotent and can be induced to differentiate into neurons, glial and fibroblast-like cells upon cellular aggregation and retinoic acid (RA) treatment. The differentiated P19 neurons express several different neurotransmitter receptors, including ionotrophic glutamate receptors, $GABA_A$ receptors, muscarinic acetylcholine receptors and opioid receptors, and serve as a model system for differentiation and maturation of CNS neurons. In the present study, we have investigated whether the differentiated neurons express cannabinoid receptors. P19 cells were cultured for 4 days as aggregates in the presence of 1 μM RA and plated on poly-D-lysine-coated 6-well culture plates or T-75 culture flasks in defined B27-supplemented Neurobasal medium. The differentiated neurons (cultured for 7 to 11 days) were able actively to accumulate GABA and expressed NMDA receptor immunoreactivity in Western blots, whereas the undifferentiated P19 cells did not. Both undifferentiated and differentiated P19 cells were positive, by using reverse transcriptase-polymerase chain reaction (RT-PCR), for CB_1 (but not CB_2) mRNA and the CB_1 mRNA level was increased in RA-treated P19 neurons cultured for seven days under serum-free conditions. Western blotting, using a polyclonal CB_1 receptor antibody, revealed a strong immunoreactive band at approx. 62 kDa in membranes from P19 neurons and mouse brain, but not in untreated P19 cells. Despite the lack of CB_2 mRNA in the P19 cells, Western blotting using a polyclonal CB_2 receptor antibody showed expression of several immunoreactive proteins on membranes from both untreated P19 cells and RA-treated P19 neurons. The potent cannabinoid receptor agonists CP 55,940 and HU-210 produced concentration-dependent inhibition of forskolin-induced (5 μM) cAMP production in both P19 cells (16% at 1 μM CP 55,940) and P19-derived neurons (29% at 1 μM CP 55,940 and 34% at 1 μM HU-210), which could be blocked by the CB_1-selective receptor antagonist AM251, but not by the CB_2-selective antagonist AM630. Furthermore, AM251 *per se* or in combination with AM630 enhanced forskolin-stimulated cAMP production in RA-treated neurons (about 40% by 1 μM). In conclusion, the protocol used provides neuronally differentiated P19 cells expressing functional CB_1 receptors and may be a useful model for the mechanistic study of CB_1 receptor-mediated events in cultured neurons.

CHARACTERIZATION OF A PUTATIVE SALT BRIDGE IN CANNABINOID RECEPTORS

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In G-Protein Coupled Receptors (GPCRs), there is a highly conserved triplet of amino acids, Asp-Arg-Tyr (DRY) at the junction of transmembrane helix 3 (TMH 3) and the second intracellular loop. The highly conserved R3.50 in the DRY motif is crucial for the interaction of receptor with G proteins. It has been suggested that ionic interactions between Asp/Glu3.49, Arg3.50, and Asp/Glu6.30 (the salt bridge) may constitute a common switch governing the activation of many rhodopsin-like GPCRs. In the inactive state, the D/E3.49-R3.50-D/E6.30 triad constitutes an ionic lock that maintains GPCRs in their inactive state and the release of this interaction could be a key step in receptor activation.

Unlike in other GPCRs, our previous studies have shown that D3.49 of the CB_2 receptor does not seem to be important in keeping the receptor in an inactive state. In the current study, a charge-neutralizing mutation at position 6.30 (D6.30N) was made in CB_2 to test the hypothesis that D6.30 may form a salt bridge with R3.50, thus keeping the receptor in an inactive state. The CB_2D6.30N mutant receptor was stably transfected into HEK293 cells. Ligand binding and cAMP accumulation assays were used to examine the effects of the mutation.

The CB_2D6.30N mutant receptor retained its ability to bind cannabinoid ligand CP55940. In addition, CP55940 was able to inhibit forskolin-stimulated cAMP accumulation in cells expressing CB_2D6.30N mutant receptors. To study the possible constitutive activation of the CB_2D6.30N mutant receptor, cells expressing this mutant receptor were assayed for cAMP accumulation in response to various concentrations of forskolin, in the absence of cannabinoid ligands. The level of forskolin-stimulated cAMP accumulation was lower in cells expressing the CB_2D6.30N than in mock-transfected HEK293 cells. Treatment with the CB_2 inverse agonist SR144528 returned the cAMP level of CB_2D6.30N expressing cells to that of mock-transfected cells.

These data demonstrate that the charge-neutralizing D6.30N mutation caused a constitutive activation of the CB_2 receptor. Therefore D6.30 seems to participate in a salt bridge between R3.50 and D6.30 in the CB_2 receptor, thus serving as an ionic lock of receptor activation.

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IDENTIFICATION OF RESIDUES IN THE CANNABINOD CB_2 RECEPTOR INVOLVED IN LIGAND BINDING

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Cannabinoid $(CB)_2$ receptor-selective agonists have been shown to have a range of effects that make them promising candidates for the treatment of pain, including inhibition of acute nociceptive, inflammatory and neuropathic pain. The peripheral distribution of CB_2 should avoid the psychoactive effects typically associated with CB_1 receptor agonists in the CNS. However our understanding of their mode of action has been limited by the absence of an experimental receptor structure. Site directed mutageneis and modelling studies have revealed that ligand binding regions lie within residues located within transmembrane helices 3-4-5 of both CB_1 and CB_2, based largely upon work using the synthetic agonists CP-55940 (Feng *et al*, **2003**; McAllister *et al*, **2002**), and WIN 55,212-2 (Chin et al, **1999**; Song et al, **1999**), as well as the CB_2 selective antagonist SR144528 (Gouldson et al, **2000**).

Early receptor models were developed prior to the publication of the rhodopsin crystal structure (Palczewski et al, **2000**). We have developed an alternative receptor model, using rhodopsin as the template, and docked key compounds into this model accounting for the experimental data above. This has revealed two possible ligand binding modes: Mode #1, identified by docking a CB_2-selective agonist, in which the proposed binding pocket lies high within the 7TM bundle with the ligand lying roughly perpendicular to the membrane, and Mode #2, identified by docking the alkylaminoindole WIN 55,212-2, which suggests a partially overlapping but not identical ligand binding pocket roughly parallel to the membrane.

Using these two models 14 residues hypothesised to be involved in ligand binding were mutated to alanine with a view to evaluating the predicted ligand binding modes and the structural model of CB_2. The majority of mutated residues were found in transmembrane helices 3 and 5, with a small number found in helices 2, 7, and ECL2. Preliminary phenotyping of these muatants in a functional assay format suggests that we have identified residues that are involved in binding some compounds but not others. This approach will begin to map possible ligand binding sites in CB_2, and may enable the design of potent CB_2-selective agonists.

LIGAND INDUCED ACTIVATION OF G-PROTEINS IS REGULATED BY LEUCINE OF THE CB_1 HELIX 8 DOMAIN

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The peptide of CB_1 401-417 can directly activate $G\alpha_o$ and $G\alpha i_3$, but not $G\alpha i_1/_2$ proteins (Mukhopadhyay and Howlett, **2001**). Modeling studies have identified the 404-414 segment of CB_1 receptor to be an intracellular helical extension of the TMH7 domain (called here Helix 8 (Hx 8)) (Bramblett et al., **1995**). The existence of a Hx8 domain was supported with the crystallization of bovine rhodopsin (Rho) (Palczewski et al, **2000**). In Rho, F7.60 is inserted into a groove formed by two beta branchers, T1.53/V1.56, stabilizing the association of Hx8 with the TMH1 domain. In CB_1, residue 7.60 is a Leu(404) and there is no clear insertion of any aromatic ring on CB_1 HX8 Hx8 into a groove on TMH1. It is possible that this difference may make CB_1 Hx8 more mobile. We tested the hypothesis that activation of CB_1 releases Hx8 such that it can interact with G proteins, and that differences in this association may contribute to constitutive activity. We hypothesized that replacing the L7.60 in WT CB_1 with a bulkier Ile residue would make the Hx8 more mobile, while replacing L7.60 with a Phe (as in Rho) may make the Hx8 less mobile than the wild-type human CB_1 receptor (WT hCB_1). Thus, CB_1 receptor constitutive activity would be increased or decreased, respectively.

Site-directed mutagenesis was performed and HEK293 cells were transfected with WT hCB_1, L7.60I or L7.60F mutant receptors. Stably expressing cell lines were generated and screened for cell surface receptor labeling with CB_1 receptor antibody. Saturation binding analysis with [^{3}H]CP-55,940 and [^{3}H]SR141617A showed that ligand affinity and receptor levels of the mutant receptors were similar to that of the WT hCB_1. [^{35}S]GTPγS binding analysis was performed with structurally different cannabinoid agonists. Although a slight increase in basal levels of [^{35}S]GTPγS binding in the L7.60I and a slight decrease for the L7.60F 60I mutant were noted, these did not reach the level of statistical significance. Both mutations were found to reduce the maximal stimulation (Emax). The L7.60F 60I mutation significantly reduced the Emax for CP-55,940>HU-210>WIN55,212-2. In addition, in the L7.60F mutation, SR141716A was unable to reduce [^{35}S]GTPγS binding.

These results suggest that L7.60 in the Hx8 domain may not significantly contribute to the constitutive activity of the hCB_1 receptor in this model system. However, these results suggest that this residue may be involved in ligand-specific differential coupling to G proteins in this system. .

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STERIC TRIGGER AS A MECHANISM FOR CB_1 CANNABINOID RECEPTOR ACTIVATION

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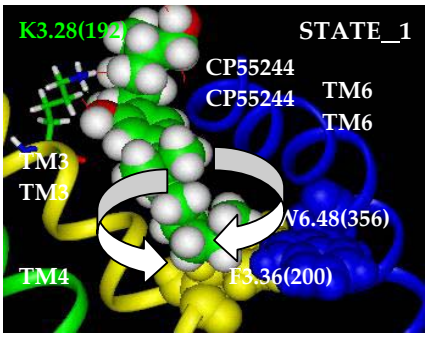
To determine the moiety that behaves as the steric trigger to activate the CB_1 cannabinoid receptor, conformational properties of the non-classical cannabinoid CP55244, one of the most potent CB_1 receptor agonists, were characterized by conformational analysis, rotational barrier calculations, and molecular dynamics (MD) simulations. It was shown from the present MD simulations that φ1 and φ4 of CP55244 showed the most dramatic change when compared with the ground state receptor-bound conformation, indicating that rotation around these torsion angles are responsible for releasing the ligand strain energy. Multiple stages would be involved in the ligand conformational change: starting from the ligand conformation (**STATE_1**: φ1 = 116°, φ4 = 171°) as found in the receptor-bound state [Shim et al., *Biopolymers (Peptide Science)* **2003**, *71*, 169-189], the ligand evolved through three low energy conformations, **STATE_2** with φ1 = 56° and φ4 = 61°, **STATE_3** with φ1 = -56°, φ4 = -61° and **STATE_4** with φ1 = 124°, φ4 = -61°. The conversion of the C3 side chain of CP55244 to **STATE_2** and **STATE_4** showed a highly unfavorable steric interaction with transmembrane (TM) helix 3 (TM3) of the receptor, while conversion to **STATE_3** showed highly unfavorable steric interaction with TM6 of the receptor. As a molecular mechanism for the ligand-induced CB_1 receptor conformational change, we propose that the C3 side chain serves as the steric trigger, while the ACD-ring moiety of CP55244 serves as the plug. Steric clash with helices within the binding pocket would induce micro-conformational adaptation within the protein (Kenakin and Onaran, *Trends. Pharmacol. Sci*. **2002**, *23*, 275-280). This mechanism would suggest that rotational flexibility in a ligand may be as important a determinant of agonist activity as the pharmacophoric elements that can be identified.



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MUTAGENESIS OF AROMATIC MICRODOMAINS AT HUMAN CB_2 CANNABINOID RECEPTOR

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Previous studies have shown that aromatic residues in the transmembrane helix (TMH) 3-4-5-6 regions of cannabinoid receptors (CBs) play important roles for the binding of structurally diverse classes of cannabinoid ligands. In a recent modeling and mutagenesis study on CB_1, F3.36, W5.43 and W6.48 have been found to be involved in the binding of SR141716A and WIN55,212-2, but not CP55,940 and anandamide. Since F3.36, W5.43 and W6.48 are also present in CB_2, the current study was carried out to evaluate the roles of these three aromatic residues in ligand binding to CB_2.

Mutations F3.36A, W5.43A and W6.48A, which destroy aromaticity, and mutations F3.36Y, W5.43F and W6.48F, which retain aromaticity, were made in CB_2. These mutant CB_2 receptors were stably transfected into the HEK293 cells and the effects of these mutations were examined with Western blot and ligand binding assays.

Western blot analysis demonstrated that all the six mutants had expression levels similar to that of the wild type CB_2. In ligand binding studies, at position 3.36, both the CB_2F3.36A and the CB_2F3.36Y mutant receptors were able to bind [^{3}H]CP55,940 with high affinities, with K_d values similar to that of wild-type CB_2. In addition, both CB_2F3.36A and CB_2F3.36Y mutant receptors bound to HU210, WIN55,212-2, anandamide and SR144528 with affinities similar to those of the wild-type CB_2, except that F3.36A mutation increased the affinity of SR144528. At position 5.43, the CB_2W5.43F mutation caused no change in the binding affinities of CP55,940, HU210, WIN55,212-2, anandamide and SR144528. In contrast, the CB_2W5.43A mutation completely eliminated ligand binding. These results indicate that the aromaticity at position 5.43 is critical for ligand binding. At position 6.48, both the CB_2W6.48A and the CB_2W6.48F mutant receptors completely lost their ligand binding ability, indicating that a tryptophan at this position is crucial for ligand binding.

Previous studies have shown that in CB_1, the affinities of CP55,940 and anandamide were unaffected by the F3.36A, W5.43A, or W6.48A mutations, whereas the affinities of WIN55212-2 and SR141716A were decreased by these mutations. The findings in the current study demonstrate that roles of F3.36, W5.43 and W6.48 in CB_2 are different from those of these three residues in CB_1. These data indicate a divergence between the aromatic microdomains in CB_2 and CB_1.

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PHOSPHORYLATION AND POSSIBLE REGULATION OF THE CB_1 RECEPTOR

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Tyrosine phosphorylation is a widespread mechanism involved in signal transduction and regulation of protein function. Despite this, only a little is known about the effects of phosphorylation on the functional activities of G protein coupled receptors (GPCRs) (Krupnick and Benovic, **1998**; Hall et al., **1999**). A few studies, mainly concerned with the β-adrenergic receptor, have shown that phosphorylation on critical tyrosines ablates certain functions such as the receptor's role in regulating cAMP levels (Garcia et al., **1998**). However, tyrosine phosphorylation has not been previously reported for cannabinoid receptors (CBR). We have performed preliminary kinase assays that indicate the CB_1R isolated from N18TG2 murine neuroblastoma cell-membranes is a substrate for PKC *in vitro.* Immunoprecipitation of CB_1R from N18TG2 cells as well as SHSY5Y human neuroblastoma cells, and Western blotting with a phosphotyrosine-specific antibody, suggests that the CB_1R is increasingly phosphorylated *in vivo,* in response to the addition of agonists. Homology models of the CB_1 receptor (based on the structure of rhodopsin; Shim et al., **2003**) suggest multiple Tyr phosphorylation sites that could be phosophorylated *in vivo* and might play a role in interactions with regulatory proteins as well as effectors such as the different G proteins. Continuing work is aimed at optimizing conditions for tyrosine phosphorylation of the CB_1R, phosphoamino acid analysis to determine the sites of phosphorylation within the CB_1R, and what effects changes in tyrosine phosphorylation levels have on CB_1R function.

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EFFECTS OF CHRONIC Δ^9-THC ADMINSTRATION ON INHIBITORY REGULATION OF ADENYLYL CYCLASE IN MOUSE CEREBELLUM

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Tolerance to the motor incoordinating effects of Δ^9-tetrahydrocannabinol (THC) is thought to result from adaptation of CB_1 receptor signaling in the cerebellum. Effects of chronic THC administration on cerebellar CB_1 receptors include receptor downregulation and desensitization of G-protein activation. Although cross-tolerance to the motor incoordinating effects of cannabinoid and adenosine A_1 agonists has been reported (DeSanty and Dar **2001**, Brain Res., 905,178), previous studies have found that desensitization of cerebellar CB_1 receptor-mediated G-protein activation was homologous (Sim et al. **1996**, J. Neurosci.; 16, 8057; Breivogel et al. **1999**, J. Neurochem. 73, 2447). However, few studies have examined the effects of chronic THC on CB_1 receptor-mediated inhibition of adenylyl cyclase. Thus, the present study examined the effects of chronic THC administration on G-protein activation and adenylyl cyclase inhibition by CB_1 and A_1 receptors in mouse cerebellum. In addition, $GABA_B$ receptor signaling was also examined because these receptors are co-localized with CB_1 and A_1 receptors on cerebellar granule cells. Mice were injected twice daily with escalating doses of THC or vehicle over a 14 day period. G-protein activation was measured by agonist-stimulated [^{35}S]GTPγS binding and adenylyl cyclase inhibition was determined by measuring the effects of agonists on forskolin-stimulated conversion of [α^{32}P]ATP to cAMP, in membranes prepared from dissected cerebella. Preliminary experiments in cerebella from naïve mice showed that maximally-effective concentrations of CB_1 and A_1 agonists produced less than additive stimulation of [^{35}S]GTPγS binding and inhibition of adenylyl cyclase activity, indicating that CB_1 and A_1 receptors converge on overlapping populations of G-proteins and adenylyl cyclase. CB_1 and $GABA_B$ receptors converged at the level of adenylyl cyclase only. Following chronic THC treatment, the potency of WIN 55,212-2 and the maximal ability of CP 55,940 to stimulate cerebellar [^{35}S]GTPγS binding were decreased relative to vehicle-treated mice. However, there was no effect of THC on the concentration-effect curves for G-protein activation by the A_1 agonist, (-)-N6-(2-phenylisopropyl)adenosine (PIA) or the $GABA_B$ agonist baclofen. At the level of adenylyl cyclase regulation, the inhibitory potency of WIN 55,212-2 and maximal inhibition by CP 55,940 or THC were decreased in cerebella from THC-treated compared to vehicle-treated mice. Interestingly, decreases in the maximal inhibition of cerebellar adenylyl cyclase activity by PIA and baclofen were also observed in THC-treated mice, in contrast to results observed at the level of G-protein activation. There was no significant effect of chronic THC on basal, forskolin- or G_s-stimulated adenylyl cyclase activity measured in the absence of inhibitory agonists. These results indicate that chronic THC administration produces a heterologous desensitization of receptor-mediated inhibition of cerebellar adenylyl cyclase at a level of signal transduction that is downstream of the receptor-G-protein interface.

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MOLECULAR MECHANISMS INVOLVED IN THE ASYMMETRIC INTERACTION BETWEEN CANNABINOID AND OPIOID SYSTEMS

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It is widely known that opioids and cannabinoids share several pharmacological effects, although they activate different receptors. Both drugs display analgesic properties and are used for chronic or persistent pain, but the analgesic benefit/disadvantage of cannabinoids and opioids combination have not been well explored. The central issue in this work was to study the mechanism of cross-modulation between cannabinoid and opioid systems during chronic exposure to both drugs. Specifically, we injected a synthetic cannabinoid CP55940 at two different doses (0.2-0.4 mg/kg i.p.) in rats made tolerant to morphine (5 mg/kg, s.c. twice a day for 4.5 days) and we measured its analgesic effect. The low dose of CP55940 that *per se* did not induce analgesia in naïve animals, produced a significant degree of antinociception in chronic morphine rats, while the high dose of synthetic cannabinoid displayed similar analgesic effect either in naïve as in morphine treated rats. To identify the mechanism of this enhanced analgesic response we checked the expression and functional activity of CB_1 cannabinoid receptors and its ability to modulate cAMP cascade. Autoradiographic binding studies revealed a slight but significant reduction in cannabinoid receptor level in the hippocampus and cerebellum of morphine tolerant rats while CP55940-stimulated [^{35}S]GTPγS binding showed a strong decrease in receptor/G protein coupling in the limbic areas. Taken together these results seem suggest that alteration in CB_1 receptor functionality can not be considered the cellular basis of the enhanced analgesic response to cannabinoids present in morphine tolerant rats. To better clarify this interaction we surveyed the responsiveness of the cAMP system to cannabinoid in two different cerebral areas, namely the caudate putamen and dorsal mesencephalon. These areas are involved in the functional control of antinociception and contain CB_1 and μ opioid receptor. *In vivo* chronic treatment with morphine sensitizes the *in vitro* efficacy of CP55940 to inhibit forskolin- stimulated cAMP production in both cerebral areas considered. In a second step, we administered morphine (5 mg/kg s.c.) in rats chronically treated with CP-55,940 (0.4 mg/kg i.p. twice a day for 6.5 days) and we observed its analgesic response. Morphine challenge did not produce any analgesic response in rats pre-exposed to cannabinoid agonist suggesting that cross-tolerance was present between these drugs. When we analysed μ opioid receptor binding we observed a significant increase in the lateral thalamus and PAG accompanied with an increase in DAMGO–stimulated-[^{35}S]GTPγS binding in the nucleus accumbens. Once again, alteration in μ opioid receptor functionality can not be considered the cellular basis of the cross-tolerance observed between cannabinoids and opioids. We measured cAMP production in the caudate putamen and dorsal mesencephalon of CP55940 treated animals to verify the involvement of this system in the opioid and cannabinoid interaction. *In vivo* chronic treatment with CP55940 desentizes the *in vitro* efficacy of DAMGO to inhibit forskolin-stimulated cAMP production. Concluding, different long term interactions between opiate and cannabinoids can take place depending on the experimental set up. We suggest that the cellular mechanism of opioids and cannabinoids cross-talk could be differently impinged cAMP cascade.

PRIMARY NEURONS FROM RAT EMBRYONIC CNS: AN USEFUL MODEL FOR STUDYING CANNABINOID RECEPTOR ACTIVATION AT SINGLE-CELL LEVEL

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CB_1 receptor probably is the most abundant G-protein-coupled receptor in mammalian brain, and its presence in several CNS areas (e.g.: neocortex, hippocampus, basal ganglia, cerebellum, brainstem) accounts for most of the behavioural effects of cannabinoid drugs. Acute and chronic exposure to cannabinoids, either alone or with other drugs, such as opiates, exert complex and locally diversified actions. Moreover, unexpected long-term effects may arise and the actions of selective CB_1 receptor antagonists may reverse at high doses. This points to the importance of setting up an experimental model to dissect the cellular aspects of cannabinoid receptor activation. Primary cultures of CNS neurons constitute such a model; in particular, calcium currents modulation is an efficient indicator to monitor the activation of the cannabinoid receptor transduction pathway in these cells. We examined whether CB_1 cannabinoid agonist CP 55940 (100 nM) and antagonist SR 141716A (300 nM) modulate calcium currents in hippocampal and cortical neurons from rat embryonic CNS. In whole cell patch-clamp recordings, voltage-dependent calcium channel currents were inhibited by exposure to CP 55940, by 30-50%. The fractional inhibition was not substantially affected by channel block by means of various drugs, consistent with the hypothesis that cannabinoid agonists interfere with calcium channels phosphorylation, rather than directly with channel molecule. In neurons exposed for 18 hours to pertussis toxin (PTX), the inhibitory action of CP 55940 on calcium currents was lost; in hippocampal neurons the effect was clearly reverted. The increase of calcium currents in these experimental conditions was antagonized by SR 141716A, suggesting a specific CB_1 receptor involvement. In cortical neurons the inhibitory effects of CP 55940 on calcium currents, though comparable to what observed hippocampal neurons, were not abolished by PTX pre-treatment. In these neurons SR 141716A inhibited calcium currents by itself as well as in association with CP 55940, suggesting that, at the used concentration, this compound may behave as an agonist. These observations support the proposed view that CB_1 receptor activation may involve transduction pathways alternative to G_i, and that reversal of the effect may arise due to coupling with G_s, if G_i becomes unavailable – e.g. due to α subunit ribosylation.

These results are in general agreement with behavioural and biochemical observations in the literature. Still, it is difficult to fit them into a precise and coherent picture. The availability of an adequate cellular model may help shed some light on the question.

STUDIES OF [^{3}H]CP55940 DISSOCIATION FROM MOUSE BRAIN MEMBRANES

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When an allosteric modulator binds to its site on the receptor protein, the resulting conformational change yields a protein with different properties. Allosteric modulators will cause a change in the association and/or dissociation characteristics of the orthosteric ligand at its receptor. A change in the dissociation characteristics of an orthosteric ligand in the presence of a second agent is most likely to occur as a consequence of a conformational change in the receptor. Consequently, the dissociation kinetic assay has become a standard technique for the detection of allosteric modulation. The aim of this study was to search for allosteric interactions between CB_1 receptor ligands by using a radioligand binding assay to investigate the kinetics of dissociation of these ligands from CB_1 receptors in the brain.

We utilised the 'isotopic dilution' method to measure the dissociation rate constant for [^{3}H]CP55940 from brain membranes. [^{3}H]CP55940 (0.7nM) was incubated with mouse brain membranes (100µg) for 60 minutes at 25°C in Tris (50mM) containing 1mg/ml BSA. Dissociation was initiated by the addition of 1µM CP55940 in the presence and absence of test compounds. Dissociation times of 0.5 to 120 minutes were used. To determine the non-specific binding, some experiments were also performed in the presence of 1µM CP55940. The dissociation curve for [^{3}H]CP55940 was best-fit to a biphasic exponential ($P<0.01$, Prism 4), the dissociation rate constants (k_{-1}) for the two "agonist dissociation states" being 0.02399 ± 0.0139 min^{-1} ($t_{1/2}$ = 28.9 min) and 0.2416 ± 0.1087 min^{-1} ($t_{1/2}$ = 2.87 min). These constitute a slowly dissociating component, that accounts for 39.62 ± 8.35% of the specific binding and that presumably reflects binding to higher-affinity coupled receptors, and a rapidly dissociating component that presumably reflects binding to lower-affinity uncoupled receptors.

In the presence of the inverse agonists, SR141716A, AM251 and AM281 (10µM), the dissociation curve of [^{3}H]CP55940 was best-fit to a monophasic exponential (Prism 4) and the lower affinity rapidly-dissociating component was lost. In the presence of these compounds the dissociation rate constant did not differ from that of the slowly dissociating component for [^{3}H]CP55940 alone. These data are in line with the hypothesis that inverse agonists act to promote the formation of a G-protein-coupled inactive GDP-bound CB_1 receptor. In presence of the putative neutral antagonists, VCHSR, O-2050 and O-2654, [^{3}H]CP55940 was best-fit to a biphasic exponential and neither the dissociation rate constants nor the proportion of specific binding in each state were altered. Finally, we investigated a novel compound, Org-27569. In the presence of Org-27569 (1µM) the dissociation curve of [^{3}H]CP55940 was best-fit to a monophasic exponential. In addition, the dissociation rate constant of 0.04454 ± 0.00951 min^{-1} ($t_{1/2}$ = 15.56 min) was significantly higher ($P<0.05$, ANOVA) than the k_{-1} (slow) for the control. The compound may therefore act to modulate the CB_1 receptor allosterically. Data that we have obtained with the mouse isolated vas deferens support this interpretation.

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OPTIMISED METHODOLOGY TO DETECT ENDOCANNABINOID SIGNALLING IN RAT BRAIN BY [^{35}S]GTPΓS BINDING TECHNIQUES

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The evaluation of true intrinsic activity of major endocannabinoids, 2-arachidonylglycerol (2-AG) and arachidonylethanol amide (AEA), to stimulate cannabinoid CB_1 receptor-mediated G protein activity in native brain tissue has been a problematic issue, mainly by the following reasons. Firstly, elimination of the high basal G protein activity originating from tonic adenosine A_1 receptor activity has been commonly ignored and secondly, endocannabinoids are rapidly degraded during assays by either fatty acid amide hydrolase (FAAH; 2-AG and AEA) or monoacylglycerol lipase (MGL; 2-AG). For these reasons, we have focused on the optimisation of [^{35}S]GTPγS binding methodology in this field. In agreement with recent findings, the tonic A_1 activity is preventable with specific A_1 antagonist DPCPX but against expectations, only partially with the adenosine degrading enzyme adenosine deaminase. Comparably, the role of CB_1 receptors in basal G protein activity was examined by determining effects of selective CB_1 antagonists, SR141716 and AM251, on basal [^{35}S]GTPγS binding. In striking contrast to previous conclusions with native brain tissues that the inhibition of basal G protein activity by SR141716 (>10^{-6}M) would be an indication of constitutively active CB_1 receptors, we found that the inhibition of basal is evident only when the tonic adenosine A_1 receptor signalling is not eliminated with DPCPX. Moreover, both CB_1 antagonists (10^{-5}M) right-shifted A_1 agonist dose-response curves without affecting maximal responses. These results indicate that CB_1 receptors are not constitutively active in native brain tissues and that SR141716 and AM251, at the micromolar concentrations, block A_1 receptor activity. In degradation studies, we have previously reported that the membrane preparation we use shows no significant enzymatic activity towards AEA or noladin ether (the third putative endocannabinoid) but 2-AG is degraded substantially during [^{35}S]GTPγS binding assay. This enzymatic degradation has been partially inhibited by PMSF. However, our recent results show that pretreatment of membranes with methyl arachidonoyl fluorophosphonate (MAFP) fully inhibits this degradation and concomitantly, enhances potency of 2-AG to activate G proteins. Furthermore, MAFP showed no significant antagonist nor agonist properties at cerebellar membranes. Finally, we tested the ability of 2-AG to stimulate CB_1 receptors in rat brain autoradiographic sections under such optimised conditions. Consistently, pre-treatment of brain sections with DPCPX and enzyme inhibitors (particularly PMSF) enabled the detection of 2-AG signalling also in [^{35}S]GTPγS autoradiography. In summary, we have established an optimised methodology with improved signal-to-noise ratio to assess CB_1-mediated G protein activity in rat cerebellar membranes and rat brain sections under conditions where enzymatic degradation of endocannabinoids and basal adenosinergic tone is fully eliminated.

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ARACHIDONOYL-SERINE, AN ENDOCANNABINOID-LIKE BIOACTIVE CONSTITUENT OF RAT BRAIN

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Abnormal-cannabidiol (abn-CBD) is known to cause in rats reduction of blood pressure, which returns to normal values by cannabidiol (CBD) administration. We assumed that CBD mimics the effects of a natural brain constituent. We report now that we have identified in rat brain such a constituent, arachidonoyl-serine (ARA-S), an endocannabinoid-like compound. Like CBD, ARA-S does not bind to the CB_1 receptor. Adult male Sabra rats weighing 225 to 250 gr. had their femoral vein cannulated for i.v. drug administration. Anesthesia was induced by i.p. injection of Pentobarbital sodium 6%, 60-mg/kg. The femoral artery was cannulated and the catheter (PE 10 cannulae) was attached to a pressure transducer for continuous monitoring of blood pressure with a physiograph (AcKnowledge program).

After a 30-min. stabilization period, the animals received either vehicle (saline, ethanol, emulphor 18:1:1) or Abn-CBD (10-mg/kg) administered i.v. in bolus doses in volumes of 0.5 ml. Abn-CBD induced a drop of the blood pressure from 120/80 mmHg to 80/40 mmHg. Administration of ARA-S (5 mg/kg in the above described solvent mixture) led to blood pressure recovery (120/66 mmHg) after 20 min. Changes in the blood pressure were monitored for 60 min. When both drugs were administered in an alternate order no reduction in blood pressure by abn-CBD was observed up to 30 minutes following ARA-S administration. ARA-S also inhibits nitric oxide (NO) and reactive oxygen intermediates (ROI) formation by a macrophage cell line (RAW 264.7 up to 65% and 75% respectively. These effects parallel actions by CBD. ARA-S was isolated from rat brain following procedures previously reported by us for the isolation of anandamide. The bioassay employed was the above described blood pressure lowering. ARA-S was synthesized in 2 steps. Condensation of arachidonic acid with N-hydroxysuccinimide in the presence of the condensing agent DCC yielded the N-hydroxysuccinimide ester of the fatty acid, which on reaction with serine gave the desired compound. The synthetic compound and the natural product had identical GC-MS and NMR spectra.

We plan to compare the activities of CBD and ARA-S in various additional bioassays.

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CANNABINOMIMETIC MOLECULES NEGATIVELY MODULATE IgE-MEDIATED CELL ACTIVATION IN VITRO

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Immunological activation of mast cells is long known to result in their degranulation and extracellular release of various preformed mediators, such as β-hexosaminidase. While studies in animal models of inflammation support the hypothesis that endogenously-produced cannabinoids may negatively modulate tissue inflammation by decreasing mast cell activation, in vitro studies conducted utilizing exogenously- added cannabinoids or endocannabinoids have, to date, provided contradictory results. By using RBL-2H3 cells, we here report that: pretreatment with synthetic cannabinoid receptor agonists, such as Win 55.212-2 and CP 55940, inhibits the anti-DNP IgE-DNP-HSA-induced β-hexosaminidase release; the inhibitory effects are concentration-dependant with approx. 50 % maximal inhibition occurring at concentrations of 3- 10 μM and, interestingly, evident solely following activation (i.e. no effect on basal release); the inhibitory effect of Win 55.212-2 is stereoselective, and pertussis toxin sensitive, suggesting the involvement of a G protein-coupled receptor; the inhibitory effect of Win 55.212-2 is not counteracted by the CB_2 receptor antagonist AM630, a result in line with the finding that the selective CB_2 agonist JWH-133 does not exert any significant inhibitory effect; similarly to Win 55.212-2, saturated (palmitoylethanolamide and steraoylethanolamide) but not unsaturated (anandamide) N-acyl-amides inhibit the anti-DNP IgE-DNP-HSA-induced β-hexosaminidase release; the inhibitory effects of both the synthetic cannabinoids or the saturated N-acyl-amides on β-hexosaminidase release are associated with inhibition of the anti-DNP IgE-DNP-HSA-induced intracellular calcium rise.

The reported data clearly demonstrate the ability of cannabinomimetic molecules (synthetic or endogenously-produced) to negatively modulate the immunological IgE-induced activation of mast cells via non-CB_2 receptor-mediated mechanisms. In addition, our results, together with other evidences (herein included the demonstration of the absence of CB_1 receptor expression in the RBL-2H3 cells available in our laboratory) raise the possibility of the existence of a yet uncharacterized cannabinoid receptor expressed on mast cells. Identification of the receptor or specific binding sites involved has, given its (their) role in the cannabinoid/endocannabinoid mediated control of inflammatory reactions, the potential of leading to novel therapeutic targets useful in the treatment of allergic inflammatory responses.

STEAROYLETHANOLAMIDE DOWN REGULATES ALLERGIC INFLAMMATORY REACTIONS IN VIVO WITH A MECHANISM DIFFERENT FROM SYNTHETIC CANNABINOID AGONISTS

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As cannabinoids (CB) or endocannabinoids have been reported to exert anti-inflammatory actions in vivo, we here examined whether a cannabinoid/endocannabinoid-based pharmacology may be of therapeutic utility in allergy. To this end, we here evaluated whether synthetic CB receptor agonists and/or long chain unsaturated or saturated N-acyl-amides (e.g. anandamide and stearoylethanolamide), i.p. administered, were capable of reducing the oedema and/or extravasation induced in the mouse ear pinna by locally administered anti-DNP IgE followed by systemic administration of DNP-HSA (i.e. type I passive cutaneous anaphylaxis, PCA).

Results show that, while the selective CB_2 agonist JWH-133 is without effect, non-selective CB_1 and CB_2 receptor agonists (e.g. Win 55.212-2, CP 55940 and HU-210) significantly reduce the development of PCA-induced oedema and extravasation in the mouse ear pinna. In addition, while the endocannabinoid anandamide is also ineffective, the N-acyl-amide stearoylethanolamide (SEA) is as effective as Win 55.212-2 in reducing the local inflammation. If, on one hand, the inefficacy of JWH-133 is suggestive of the involvement of non-CB_2 related mechanisms, additional results showing that Win 55.212-2-induced hypothermia does not account for its anti-inflammatory effects excludes the involvement of the central CB_1 receptors. Indeed, neither CB_1 nor CB_2 receptor antagonists counteracted the anti-inflammatory effects of Win 55.212-2 or SEA indicating that neither of these receptors are involved. Furthermore, while capsazepine, a VR1 receptor antagonist, did not modify the pharmacological efficacy of Win 55.212-2, it interestingly counteracted that induced by SEA.

The above, together with other evidences, support the hypothesis that synthetic non-selective CBs exert anti-allergic actions in vivo via a mechanism not involving known CB nor VR1 receptors. In addition, our results show, for the first time, that systemically-administered SEA down regulates allergic inflammatory reactions in vivo via mechanisms, at least in part, involving VR1 receptors. Hence, although both non-selective CBs and N-acyl-amides, such as SEA, behave as anti-allergic compounds, the underlying mechanisms are nevertheless clearly distinguishable. Further comprehension of these mechanisms together with in-depth analyses of their structure-activity relationships has the potential of leading to novel therapeutic agents useful in the treatment of allergic inflammatory responses.

THE ENDOVANILLOID/ENDOCANNABINOID *N*-ARACHIDONOYL- DOPAMINE INHIBITS HIV-1 REPLICATION IN NEURONAL AND NON-NEURONAL CELLS

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The central nervous system (CNS) is a major target for HIV-1 infection. This retrovirus enters the CNS early after primary systemic infection and persists there for life. About 30% of the AIDS patients develop HIV-1-induced encephalopathy also called HIV-associated dementia. However, the mechanism of HIV-1 induced neuropathogenesis is an unresolved mystery. HIV-1 is found in the brain in infiltrating macrophages, microglia cells, astrocytes, oligodendrocytes, and in brain microvascular endothelial cells. How the virus penetrates through the blood-brain barrier is not completely understood but local productive HIV-1 replication is though to induce brain damage either by a cytophatic effect or by the release of viral proteins with neurotoxic activities. Thus, the identification of endogenous compounds able to prevent HIV-1 induced brain damage would be of outstanding interest to develop new therapies for AIDS-associated dementia. Endogenous *N*-acyl dopamines like *N*-arachydonoyldopamine (NADA) and *N*-oleoyldopamine (OLDA) have been recently identified as a new class of brain neurotransmitters sharing endocannabinoid and endovanilloid biological activities. We have recently shown that NADA is a potent inhibitor of the transcription factor NF-κB by targeting the phosphorylation of the subunit p65 (Sancho et. al., **2004**. J. Immunol. 172:2341-51). Since NF-κB is a cellular factor that plays a major role in HIV-1 replication we have investigated the effects of several lipid mediators in an "in vitro" model of HIV-1 infection, and we have found that NADA> OLDA> *N*-palmitoyldopamine > *N*-stearoyldopamine inhibit either VSV-pseudotyped or gp160-enveloped recombinant HIV-1 infection in several T cell lines and in the astrocyte cell line U373-MG (with an IC50 $\leq$ 2.5 μM). These recombinant viruses express the luciferase gene as a marker of viral replication. By the contrary no HIV-1 inhibitory effects were found using anandamide and its palmitoyl, oleoyl and stearoyl congeners. The molecular mechanism by which NADA inhibits HIV-1 replication will be discussed.

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THE TAMING OF CAPSAICIN. SYNTHESIS AND VANILLOID ANTAGONISTIC ACTIVITY OF ISOSTERIC AND REGIOISOMERIC HALONONIVAMIDES AND THEIR PRODUCTS OF HALOGEN-CARBON EXCHANGE

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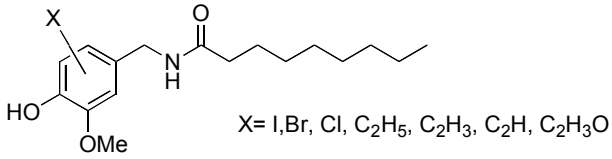
Capsaicin (CPS) is the archetypal obnoxious compound, as testified by its controversial use as an anti-riot agent (Olajos et al, J. Appl. Toxicol. **2001**, 21, 355) and the sensory properties of hot pepper, its only natural source. The offensive properties of CPS are mediated by the interaction with a specific receptor (TRPV1), an integrator of physical (heat) and chemical (acidity) painful stimuli, whose manipulation has obvious clinical implications. TRPV1 is also activated by endocannabinoids such as anandamide and N-arachidonoyldopamine, and has been suggested to act as an ionotropic receptor for these fatty acid amides, with functions often opposed to those of their metabotropic receptors, the cannabinoid receptors (Di Marzo et al., Curr. Opin. Neurobiol., **2002**, 12, 372). Functional inactivation of TRPV1 can be achieved by desensibilisation, a prolonged refractory status mediated by phosphorylation, or by blockage with antagonists. Several classes of structurally unrelated TRPV1 antagonists have recently emerged from random screening of libraries of synthetic small molecules (Appendino et al, Exp. Opinion Pharmac. Pat. **2003**, 13, 1825). Surprisingly, natural products lagged behind synthetic compounds in providing TRPV1 antagonist leads, despite the wealth of activators that have emerged from the treasure trove of plant extracts. While the defence role of secondary metabolites might provide an evolutionary explanation for this observation, the serendipitous discovery that iodination of the ultrapotent agonist resiniferatoxin (RTX) reverts its vanilloid activity (Wahl et al, Mol. Pharmacol. **2001**, 59, 9) was seminal in providing new opportunities of exploitation for the pool of natural TRPV1-agonists. Modulation of activity by halogenation of aromatic amino acids has been reported in the realm of opioids (Guerrini et al, J. Med. Chem. **2001**, 44, 3956), but the effect of iodination on the activity of RTX is nevertheless quite remarkable, and has prompted us to carry out a systematic investigation on the aromatic halogenation of nonivamide (1), the so called "synthetic capsaicin" and an archetypal capsaicinoid. While resiniferonoids are essentially expensive rarities, nonivamide is commercially available, providing the opportunity for extensive investigations. To better understanding the role of iodine in the reversal of activity, we have synthesized all three possible iodo-nonivamides to assess the effect of iodine location on the activity. The active iodo-nonivamides were next isosterically modified to compare the reversal potential of iodine, bromine and chlorine. Finally, the most active compound emerging from this study was subjected to halogen-carbon exchange to assess the potential of carbon – halogen exchange as a vanillloid reversal manoeuvre. The results were that: TRPV1 antagonist activity depends on the site of halogenation (6>5>>>2); Iodine is more efficient than chlorine or bromide in reverting the agonistic activity; Iodine-carbon exchange via palladium chemistry decreases the antagonist activity.



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STEREOSPECIFIC HIGH-AFFINITY ANTAGONISTS OF THE VANILLOID RECEPTOR 1

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The vanilloid receptor (VR1) is a member of the transient receptor potential (TRP) superfamily. VR1 is activated by protons, heat, natural ligands such as capsaicin (CAP), resiniferatoxin (RTX), and endogenous substances such as anandamide and the lipoxygenase product 12-HPETE. Since VR1 functions as a non-selective cation channel with high Ca^{2+} permeability, its activation by these agents leads to an increase in intracellular Ca^{2+} that results in excitation of primary sensory neurons and ultimately the central perception of pain. The involvement of this receptor in both pathological and physiological conditions suggests that the blocking of this receptor activation, by desensitization or antagonism, would have considerable therapeutic utility. VR1 antagonists have attracted much attention as promising drug candidates to inhibit the transmission of painful signals from the periphery to the CNS and to block other pathological states associated with this receptor. The therapeutical advantage of VR1 antagonism over agonism is that it lacks the initial excitatory effect preceding the desensitization. The initial acute pain associated with capsaicin treatment has proven to be the limiting toxicity.

We have previously reported that isosteric replacement of the phenolic hydroxyl group in potent vanilloid receptor agonists with the alkylsulfonamido group provided a series of compounds which are effective antagonists to the action of capsaicin on rat VR1 heterologously expressed in Chinese hamster ovary (CHO) cells. As a lead compound, N-[2-(3,4-dimethylbenzyl)-3-pivaloyloxypropyl]-N'-[3-fluoro-4-(methyl sulfonylamino) benzyl] thiourea showed a high binding affinity with a K_i = 54 nM for the inhibition of [^{3}H]RTX binding and potent antagonism with an IC_{50} = 7.8 nM for the inhibition of $^{45}Ca^{2+}$ uptake in response to capsaicin (Lee, J. et al., J. Med. Chem. **2003**, 46, 3116). Recently, we have investigated a series of α-substituted analogues of potent antagonists and their optically pure isomers. The SAR result indicated that they interacted to the receptor with high-affinity in a stereospecific manner and antagonized the effect of capsaicin efficiently. Among them, N-[2(R)-(4-t-butylbenzyl)-3-pivaloyloxypropyl] 2(S)-[3-fluoro-4-(methylsulfonylamino) phenyl] propionate (MK-271) showed high binding affinity with a K_i = 4.24 nM and excellent antagonism with an IC_{50} = 0.58 nM, which were 300-fold in binding affinity and 900-fold in antagonism more potent than those of capsazepine (K_i = 1,300 nM, IC_{50} = 520 nM), respectively.

EXPRESSION OF VR1 RECEPTOR IN PROSTATE CELLS. INCREASE OF INTRACELLULAR CALCIUM CONCENTRATION EITHER BY CANNABINOID THAN VANILLOID AGONISTS

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Several endogenouos ligands for cannabinoid receptors have been identified to date. Some of them, i.e. arachidonoyl ethanolamide (anandamide) and methanandamide (MET), was also activate vanilloid receptors, although the last shows lesser efficacy.

Vanilloid receptor-1 (VR1) is a non-selective cation channel, that is present in various brain nuclei, as well as, in non-neuronal tissues and they have been shown to be involved in pain perception. There are several reported chemical classes of agonists at vanilloid receptors. These include capsainicoids (i.e.capsaicin, the pungent ingredient in hot peppers), resiniferanoids (i.e. resiniferatoxin, RTX) and endocannabinoids that are structurally related to capsaicin (CAP), like anandamide and methanandamide (Smart D et al., Br J Pharmacol (**2000**) 129: 227-30).

In the present work we show that VR1 vanilloid receptor is expressed in human prostate gland as well as the prostate tumor cells PC-3 and LNCaP. The functionality of the vanilloid receptors expressed in prostate cells has been analized.

Cells were treated with different concentration of VR1 agonists and then, the increase in intracellular calcium concentration (Ca^{2+}) using the Ca^{2+}-sensitive fluorescent probe FURA-2 changes in fluorescence excitation (λ_{EX}= 380nm , λ_{EM}=340nm) was monitored by fluorescence microscopy, before and after the addition of various agonists.

Results obtained indicate that capsaicin, resiniferatoxin and methanandamide evoked an increase in fluorescence intensity, that correlated with an increase in intracellular Ca^{2+} concentration. The maximal effect produced by capsaicin and RTX was observed at 10 nM concentration whereas the maximal effect induced by MET was observed at 0.1 μM.

Capsaicin, RTX and MET induced an increase in intracellular Ca^{2+} concentration that was blocked by pre-treatment with 1μM capsazepine, a VR1 antagonist.

These results indicate that the vanilloid receptors espressed in prostate cells were functionally active and that the cannabinoid methanandamide induced an intracellular calcium concentration increase, acting through vanilloid receptors.

CANNABINOID AND VANILLOID RECEPTORS ARE ACTIVATED BY N-ARACHIDONYL-DOPAMINE IN RAT MIDBRAIN DOPAMINERGIC NEURONS.

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We have previously shown that vanilloid receptors (TRPV1s) are modulated by the endocannabinoid anandamide and are tonically active in the substantia nigra. Recent studies have identified another endogenous anandamide analog, the N-arachidonyl-dopamine (NADA) which is more potent at TRPV1 than anandamide, but still active on CB_1 receptors. Since both receptors are expressed in the midbrain, in this study we attempted to discriminate the physiological response of TRPV1 and CB_1R to the NADA by means of whole-cell patch clamp recordings. In the presence of a selective CB_1R antagonist AM 281, bath application of NADA induced a significant increase of spontaneous excitatory postsynaptic currents (sEPSCs) on dopaminergic neurons, via a presynaptic mechanism. This effect were caused by stimulation of TRPV1s since it was counteracted by the antagonist iodoresineferatoxin (IRTX).

Conversely, in the presence of IRTX, stimulation of CB_1R by NADA significantly reduced the frequency of spontaneous glutamatergic and GABAergic synaptic events without affecting their amplitude, suggesting a presynaptic site of action. These effects were blocked by AM281. Interestingly, when we bath applied NADA together with the OMDM-2, a new and selective inhibitor of the cellular uptake of the endocannabinoid anandamide, both sEPSCs and spontaneous inhibitory postsynaptic currents (sIPSCs) frequency were reduced, suggesting a CB_1R-mediated effects. In addition, the activation of TRPV1 by NADA were still observed in the presence of ETYA, an inhibitor of lipoxygenase and cyclo-oxygenase enzymes. Therefore, the NADA mediated effects were not due to the lipoxygenase metabolites, that work as vanilloid receptor agonists.

Altogether these results showed that both VR1 and CB_1R are presinaptically activated by endogenous substances and modulate in opposite way the glutamatergic transmission. Moreover CB_1R stimulation selectively inhibit GABAergic transmission onto DA neurons.
Our study suggests that modulation of CB_1R and VR1 might differently take part in physiological and pathological conditions such as reward, excitotoxicity or voluntary motor control .

ENTOURAGE EFFECTS OF N-PALMITOYLDOPAMINE AND N-STEAROYLDOPAMINE ON N-ARACHIDONOYLDOPAMINE ACTIVITY

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N-arachidonoyldopamine (NADA), N-oleoyldopamine (OLDA), N-stearoyldopamine (STEARDA), and N-palmitoyldopamine (PALDA) are a series of endogenous compounds that belong to a family of lipids called the N-acyldopamines (Bisogno et al., **2000**; Huang et al., **2002**; Chu et al., **2003**). In addition to exhibiting activity at cannabinoid CB_1 receptors and capsaicin receptors (TRPV1) in vitro, NADA and OLDA induce significant hyperalgesia when administered in the periphery. In contrast, STEARDA and PALDA exhibit little to no activity at these receptors. Previous studies demonstrating the enhancement of anandamide activity at TRPV1 and CB_1 receptors by saturated N-acylethanolamines that do not directly activate these receptors led us to investigate the possible entourage properties of saturated N-acyldopamines on NADA and OLDA activity (De Petrocellis et al., **2002**; Smart et al., **2002**).

Following the determination of baseline withdrawal latencies of the rat hind paw from a radiant heat source, drug or vehicle was injected subcutaneously into the plantar (i.pl.) surface of the left paw of each animal (n=6-15 per dose). Doses of NADA and OLDA were chosen based on their inability to induce thermal hyperalgesia when administered alone. The administration of 50 µl of vehicle (2:3:5:90 DMSO/ethanol/emulphor/saline), NADA (0.5 µg), or OLDA (0.1 µg) alone induced a mean decrease in withdrawal latency of –0.16 ± 0.67 ($F_{1, 14} = 0.02$, NS), –0.90 ± 0.58 ($F_{1, 12} = 3.27$, NS), and –1.13 ± 0.54 sec ($F_{1, 14} = 0.03$, NS), respectively. While administration of STEARDA (5 µg) alone induced a mean deviation from baseline of –1.85 ± 0.52 sec ($F_{1, 16} = 8.29$, $p<0.05$), administration of NADA (0.5 µg) with STEARDA (5 µg) and OLDA (0.1 µg) with STEARDA (5 µg) induced a mean deviation of –4.86 ± 0.70 ($F_{1, 16} = 16.72$, $p<0.01$) and –3.06 ± 0.52 sec ($F_{1, 16} = 9.34$, $p<0.01$), respectively. When pre-incubated for 5 min with TRPV1-tranfected HEK293 cells, PALDA and STEARDA (0.1-10 µM) dose-dependently enhanced NADA's TRPV1-mediated effect on intracellular Ca^{2+}, lowering the EC_{50} of NADA from ~90 to ~30 nM. The effect on intracellular Ca^{2+} by N-arachidonoylethanolamine (anandamide, 50 nM) was also enhanced dose-dependently by PALDA and STEARDA. Furthermore, PALDA and STEARDA acted in synergy with low pH (6.0-6.7) to enhance intracellular Ca^{2+} via TRPV1. These data suggest that while the hyperalgesia induced by administration of STEARDA with OLDA may be additive, the hyperalgesia induced by STEARDA with NADA and the enhanced intracellular Ca^{2+} induced by PALDA or STEARDA with NADA, anandamide, or low pH at TRPV1 may be entourage effects where PALDA and STEARDA act as an endogenous potentiators of TRPV1 activation.

VASODILATION PRODUCED BY METHANANDAMIDE AND WIN 55,212-2 IN RAT AORTA: ARE CB_2 AND VR_1 RECEPTORS INVOLVED?

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The vasodilation produced by cannabinoids varies among different species and vascular beds. The possible mechanisms implicated have not been established. Different studies have proposed either intra or extracellular mechanisms, including effects mediated by endothelium-derived factors (Hillard, C. J Pharmacol Exp Ther **2000**, 294, 27), and CB and VR_1 vanilloid receptors (Zygmunt et al., Nature **1999**, 400, 452; Ralevic et al., Life Sciences **2002**, 71, 2577). Previous studies in our laboratory have shown that vasodilation produced by methanandamide and WIN 55,212-2 has an endothelium-dependent component. The aim of our study was investigate the possible endothelial mechanisms implicated in the vasodilation caused by these cannabinoids in isolated rat aorta.

Male Wistar rats (250-300 g body weight) were used. Intact aorta rings were prepared and suspended in a 5 ml jacketed glass organ bath, containing Krebs-Henseleit buffer at 37ºC continuously bubbled with 95% O_2 and 5% CO_2. Tissues were equilibrated for 90 minutes (resting tension of 2 g). An isometric force transducer were used for tension measurements. Cumulative concentration-response curves of cannabinoids were constructed (10^{-9}M – 10^{-4}M) in Phenyleprine (Phe) 1 μM-precontracted arteries, in presence or absence of antagonists. Relaxant responses were expressed as the percentage of reversal of the contraction elicited by Phe. Only one antagonist was used in each experiment. Drugs: Phe, L-NAME, pertussis toxin, methanandamide, WIN55,212-2, SR141716A, SR144528, capsazepine. Data are given as the mean ± s.e.m for 8-12 rings. A two-way ANOVA (Bonferroni/Dunn post-hoc test) was used (* $P \leq 0.05$; *** $P \leq 0.01$).

Treatment	Methanandamide (10^{-4}M)	Win 55,212-2 (10^{-4}M)
Control	71.04 ± 4.7	52.87 ± 6.2
SR141716A 1μM, 15min	31.85 ± 4.3 ***	44.51 ± 3.19
SR144528 1μM, 15min	42.09 ± 5 ***	52.26 ± 4
SR141716A + SR144528 1μM, 15 min	21.93 ± 1.10 ***	40.48 ± 2 *
CAPSAZEPINE 0.1μM, 30min	31.23 ± 4.5 ***	26.07 ± 4.3 ***
L-NAME 10μM, 30min	11.56 ± 0.9 ***	10.1 ± 2.2 ***
PERTUSSIS TOXIN 300ng/ml, 3h	19.48 ± 3.5 ***	25.78 ± 1.9 ***

Our results show that CB_1, CB_2 (or a possible unidentified CB_x?) and vanilloid receptors could be implicated in the vasorelaxation produced by methanandamide. The vasorelaxation produced by WIN 55,212-2 involved, among others, VR_1 receptor. Both cannabinoids elicited vasorelaxation via L-arg/NO pathway.

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TISSUE SPECIFIC ENDOCANNABINOID DYSREGULATION IN A MOUSE MODEL OF HYPERDOPAMINERGIA: POSSIBLE THERAPEUTIC IMPLICATIONS FOR THE VR1 RECEPTOR

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There is continuously increasing evidence for a dynamic multi-level dopaminergic (DAergic) and endocannabinoid interaction critically implicated in neurophysiological, endocrine and metabolic responses, notwithstanding the fact that contradictory hypotheses on the pathogenic or protective role of endocannabinoid signalling in DA-related pathologies, such as psychosis and attention deficit/hyperactivity disorder (ADHD), have been advanced. Mice invalidated for the dopamine transporter, DAT knockout (KO), are hyperdopaminergic, hyperactive and display perturbed sensorimotor gating, habituation and cognitive performances. They thus constitute a valuable model to study neurobiological alterations due to hyperdopaminergia that could be relevant to schizophrenia and ADHD. Strikingly, biochemical studies exploring this animal model have been limited to DAergic -and to a much lesser extent to serotonergic/glutamatergic- systems. Here we (i) measured tissue levels of the endocannabinoid anandamide in different brain regions (cortex, hippocampus, striatum and cerebellum) in DAT wild-type (WT), heterozygous (HZ), and KO mice and (ii) evaluated the ability of endocannabinoid ligands to normalize behavioral deficits, namely spontaneous hyperlocomotion in DAT KOs. Adult male and female DAT WT, HZ and KO mice (C57Bl6XDBA2 hybrids) were used. Anandamide contents were measured with mass spectrometry. Locomotor activity was measured in activity monitors. We show that in DAT KO mice anandamide levels were markedly reduced, specifically in the striatum, the dopamine terminal region, which has been associated with motor, appetitive, affective and gating control. Furthermore we show that the anandamide uptake inhibitor AM404, but not the selective CB_1 receptor antagonist AM251, reduced spontaneous hyperlocomotion in DAT KOs. AM404 was effective in reducing locomotor activity in DAT KOs at doses (0.3, 1 and 3 mg/kg) that did not affect locomotion in DAT WT animals. The hypolocomotor effect of AM404 (3 mg/kg) in DAT KOs was not modified by simultaneous administration of AM251 (3 mg/kg), suggesting that it is not CB_1 mediated. Interestingly, this AM404-induced hypolocomotion was significantly attenuated by coadministration of the vanilloid receptor type 1 (VR1) antagonist capsazepine (5 mg/kg) that by itself did not affect hyperlocomotion in DAT KO mice. We conclude that constitutive hyperdopaminergia is sufficient to produce specific and profound alterations in endocannabinoid signalling. Re-establishing endocannabinoid levels and neurotransmission alleviates hyperlocomotion in DAT KO mice and might constitute an alternative therapeutic strategy for disorders associated with hyperdopaminergia. In this process, VR1 receptors seem to play a key role and represent a novel promising pharmacological target.

CB_1 CANNABINOID RECEPTORS IN THE BRAIN AND GONADS OF AN AFRICAN CICHLID FISH, PELVICACHROMIS PULCHER

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Following the molecular cloning of two CB_1 genes in a bony fish (Fugu rubripes, Yamaguchi et al.,**1996**), the phylogeny of the cannabinergic system has become of great interest for neurobiologists. The comparative study of the cannabinoid receptors gives in fact the opportunity to use animal models other than mammals in the analysis of gene functions of endocannabinoids to better understand the physiological role of such signalling system and investigate their therapeutic potential in humans. Therefore in the present study the expression and distribution of the cannabinoid CB_1 receptor in the brain and gonads of a teleost fish, the African cichlid Pelvicachromis pulcher, has been analysed. By means of RT-PCR we cloned part of the CB_1 coding sequence and found an aminoacid sequence identity of 94.9% and 80.4% with Fugu rubripes CB_1A and CB_1B, respectively. Immunohistochemistry on brain sections showed immunostained neurons and abundant fibers and varicosities in the olfactory bulbs, rostral telencephalon and magnocellular preoptic nucleus of the preoptic area. Positive cell bodies and nerve terminals were observed in some diencephalic areas such as the lateral infundibular lobes of the caudal hypothalamus. A number of stained cells was also observed in the distal lobe of the pituitary gland. Intensely immunoreactive nerve cells and fibers were found in the mesencephalic tegmentum as well as the transitional area between diencephalon and mesencephalon containing pretectum and posterior tuberculum. By using different GnRH antisera on sections contiguous to those treated with anti CB_1, a large co-distribution between CB_1 and GnRH immunopositivities was shown.

By means of RT-PCR, in situ hybridisation and immunohistochemistry, we also found CB_1 positive cells in the gonads of Pelvicachromis pulcher, according to the results of Wenger et al. (**2001**) on mammals.

In conclusion, our results strongly suggest a participation of endocannabinoids in the control of fish reproduction at both central and peripheral levels.

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ENDOCANNABINOIDS CONTROL FEEDING IN CARASSIUS AURATUS

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The endocannabinoids anandamide and 2-AG have been identified in species from many animal phyla, but not in fish, where orthologs of mammalian cannabinoid CB_1 and CB_2 receptors have been described (Yamaguchi et al., Genomics, **1996**; Elphick, Biol. Bull., **2002**). Since endocannabinoids appear to regulate feeding behaviour even in very simple animals like the coelentarate Hydra (De Petrocellis et al., Neuroscience, **1999**), we investigated the presence and possible role of these compounds on food intake in the teleost fish Carassius auratus (goldfish).

Experiment I: Five groups of satiated goldfish [11.8±0.06 g average body weight (bw), n=5-15 goldfish/group] were intraperitoneally injected with 10 µl vehicle/g weight of anandamide at the following doses: 0.001, 0.01, 0.1, 1 or 10 µg/g. Fish were anesthetized before the IP injection in water containing tricaine methanesulfonate (MS-222, 1/10000) and the injections were performed when loss of equilibrium was observed. Individual goldfish were transferred to 5 l aquaria. Fish recovered equilibrium and normal swimming activity in anesthetic-free water within 1-2 min after the injections. Once recovered, the animals received preweighed food in excess (5% bw). Food intake (FI) was measured at 2 and 8 h postinjection, and calculated as follows: $FI= W_i - (W_f \times F)$, were W_i = initial dry food weight and W_f = remaining dry food weight. F was previously calculated in absence of fish to determine the reduction in the weight on food pellets due to water dissolution after remaining 2 and 6 h in the aquaria ($F= W_{i0} / W_{f0}$). Experiment II: Goldfish (34.68±1.63 g average body weight) fed with a daily ratio of 1% bw food, were divided into three experimental groups (n=12 goldfish/ group): (1) control group: receiving food in excess 2h before sacrifice; (2) fasting group: deprived of food for 24h, and (3) fasting and refed group: deprived for 24h and receiving food in excess 2h before sacrifice. Fish were sacrificed by decapitation, and the cerebellum, telencephalon and hypothalamus were dissected on ice and immediately stored at -80ºC until endocannabinoid extraction and analysis by isotope dilution-liquid chromatography-mass spectrometry. For each data point (N=4 per group) the corresponding brain regions from three fishes were pooled.

The dose of 0.01 µg/g of anandamide significantly (3-fold) increased food intake during the 0-2h interval ($P<0.001$), but not during the 2-8h interval. The lowest dose of anandamide (0.001 µg/g) also increased food intake, although the effect did not reach statistical significance ($P=0.06$), whereas the high doses were inactive. Both anandamide and 2-AG, as well as anandamide biosynthetic precursor N-arachidonoylethanolamine, were detected in all brain regions of C. auratus, in levels compatible with a possible effect on food intake at the doses described above. Food deprivation was accompanied by a significant increase of anandamide, but not 2-AG, levels only in the telencephalon, which in fish contains the circuits responsible for the incentive, rather than appetitive, aspects of food intake. Refeeding did not significantly re-establish anandamide levels in this region. Our data suggest that endocannabinoids play a role in the control of feeding after food deprivation in fish, as previously shown for mammals.

PARTIAL PURIFICATION AND CHARACTERIZATION OF A FATTY ACID AMIDOHYDROLASE (FAAH) FROM TETRAHYMENA PYRIFORMIS

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FAAH is a membrane-bound enzyme, found in a variety of mammalian cells, plants and some invertebrates. The enzyme is responsible for the catabolism and therefore the inactivation of endocannabinoids, including the neuromodulatory amide, anandamide (N-arachidonylethanolamine). In an earlier study we reported that Tetrahymena pyriformis, a unicellular eukaryote, was able to secrete a FAAH-like activity in starvation medium [V.Karava, L.Fasia, A.Siafaka-Kapadai, FEBS Letters (**2001**) 508: 327-331]. In this study we showed that [^{3}H]anandamide was metabolized by Tetrahymena pyriformis cell homogenate in a time and concentration dependent manner, apparently through the action of a FAAH. Amidohydrolase activity was maximal at pH 9-10, was inhibited by PMSF and arachidonyltrifluoromethylketone and was Ca^{2+} and Mg^{2+} independent. The enzyme had an apparent Km of 2.5 μM and V_{max} 20.6 nmol/min×mg protein. Subcellular fractionation in a discontinuous buffered sucrose gradient, showed the presence of the activity in every fraction with the highest specific activity in microsomal as well as in non microsomal membrane fractions. Attempts to solubilize the enzyme using various detergents were not successful since the activity was inhibited. Selected subcellular fractions though, after freezing and thawing cycles were subjected to immunoblot analysis, using polyclonal anti-FAAH antibody; two bands were detected, one of which had a molecular mass ~ 66kd which is the molecular mass of the mammalian FAAH. In conclusion, the presence of a FAAH in a lower unicellular organism was clearly shown, perhaps indicating the importance of the enzyme throughout the evolution and supporting the notion that Tetrahymena species are very good models for metabolic studies.

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HIPPOCAMPAL ENDOCANNABINOID SYSTEM MODULATES ENCODING OF SPATIAL INFORMATION

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Endogenous cannabinoids such as anandamide and 2-AG are present in the hippocampus, where they may play an important role in the modulation of memory, possibly through CB_1 receptor activation. Studies using the CB_1 selective antagonist SR141716A (SR) resulted in different outcomes: 1) SR reversed deficits in learning and memory induced by the CB_1 agonists Δ^9-THC and WIN-55,212-2; 2) SR alone in various doses had no effect; 3) SR acted like an inverse agonist and facilitated memory. However, it remains unclear from studies using antagonists and CB_1 mutant mice whether endocannabinoids are activated during the encoding or consolidation of memory. We thus studied the effects of SR and Tween 80 on different phases of memory formation in rats trained for spatial reference memory in the water maze.

SR was administered daily intraperitoneally (ip – Exp. 1+2) or intrahippocampally through micro-osmotic pumps (ihc – Exp. 3+4) either pre- or post-acquisition of the task. For all experiments male Lister hooded rats were used with a maximum swim time of 90s in a 1.5m-white perspex pool and 30s time on the platform. In Exp. 1 rats were ip-injected with SR (3mg/kg) or Tween 30 minutes prior to each of the 4 training sessions (4 trials in each session). Probe trials (no platform, 60s max) were performed drug free 1, 4 and 7 days following acquisition. In Exp. 2, SR or Tween were administered immediately after the last trial of each day of acquisition (6 trials, 4 days) with probe trials 1, 4 and 7 days later. For Exp. 3 rats were implanted with ihc cannulae connected to osmotic minipumps releasing SR (8.9ng or 0.089ng per day) or Tween and trained for 4 days (2 trial per day) followed by probe tests 1 and 4 days later. Finally in Exp. 4 animals were trained for 4 days prior to surgical implantation of ihc minipumps containing SR (0.089ng per day) or Tween. While under drug, they performed reversal learning and a series of probe trials post-surgery.

Systemic infusion of SR pre-acquisition impaired performance compared to controls. By contrast, post-training injection of SR had no effect relative to controls suggesting participation of CB_1 receptors during the encoding of spatial learning. Ihc infusion of SR (0.089ng) revealed a learning enhancement, which was particularly evident in the early stages of acquisition, whereas SR (8.9ng) had no effect on performance. Finally ihc infusion of SR (0.089ng) post-acquisition was found to have no effect on retention of previously learned spatial location of the platform. Also, it did not affect reversal learning but prolonged retention for the platform location acquired during reversal learning.

Collectively the results reveal a distinction between systemic and locally administered drug effects on spatial memory formation that require further study. They have in common that endocannabinoid function in memory formation pertains to the encoding, but not consolidation phase

THE EFFECT OF Δ^9-THC AND SR 141716 ON SENSORIMOTOR GATING IN THE RAT

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Consumption of cannabis is often associated with the development of psychosis, with some symptoms of cannabis intoxication resembling those of schizophrenia (Emrich et al., Pharmacol. Biochem. Behav. **1997**, 56, 803). Prepulse inhibition (PPI) of the acoustic startle response (ASR) is an operational measure of sensorimotor gating, deficiencies of which are present in schizophrenia and contribute to symptoms such as hallucinations and cognitive fragmentation. The acute effects of Δ^9-THC on sensorimotor gating in rats were investigated. The effects of haloperidol and clozapine, antipsychotics with clinical efficacy in the treatment of schizophrenia, as pretreatments to Δ^9-THC were also investigated for their effects on PPI, using saline as the vehicle control. Δ^9-THC (0.3 - 3 mg/kg i.v.) dose-dependently decreased the ASR, while 3 mg/kg THC significantly disrupted PPI. Haloperidol (0.1 mg/kg i.p.) was not able to significantly reverse Δ^9-THC-induced decreases in ASR or PPI. Clozapine (3 mg/kg i.p.) significantly reversed the Δ^9-THC-induced decrease in PPI; with a non-significant trend towards restoration of the decreased ASR. Clozapine possesses strong 5-HT_{2A} antagonist activity; a profile which may be responsible for its ability to reverse Δ^9-THC-induced disruption of PPI. The effect of the CB_1 receptor antagonist SR 141716 on PPI was studied using the lipophilic vehicle Intralipid® as the vehicle control. In contrast to experiments using saline as the vehicle control, when Intralipid® was administered 45 min prior to Δ^9-THC (0.1 – 3 mg/kg i.v.) there was no significant effect of Δ^9-THC on PPI, although the ASR was decreased, albeit not significantly. SR 141716 (0.3 - 3 mg/kg i.p.) had no effect on PPI or ASR when administered alone, suggesting that endogenous cannabinoid tone is not crucial in the regulation of sensorimotor gating in the rat. However, 3 mg/kg SR 141716 administered as a pre-treatment to 3 mg/kg Δ^9-THC elicited disruption of PPI. These results suggest that Δ^9-THC is capable of disrupting sensorimotor gating in the rat when administered with a saline vehicle pre-treatment, possibly via indirect activation of serotonergic neurotransmission. The effect on PPI of Δ^9-THC in conjunction with a lipohilic vehicle indicates that cannabinoid effects on sensorimotor gating may be altered by the vehicle used. The decreased PPI observed with Δ^9-THC and SR 141716 in combination suggests a disruptive effect on sensorimotor gating of this combination, possibly due to the respective partial agonist and inverse agonist activity of these drugs.

THE EFFECT OF SOCIAL ISOLATION ON SENSORIMOTOR GATING AND CB_1 RECEPTOR AND FATTY ACID AMIDE HYDROLASE MESSENGER RNA EXPRESSION IN THE RAT

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Prepulse inhibition (PPI) of acoustic startle provides an operational measure of sensorimotor gating in which a weak stimulus presented 30-500 ms prior to a startling stimulus reduces the startle response. Disruptions in PPI observed in patients with schizophrenia can be mimicked in rats by individual housing from weaning until adulthood (Geyer *et al.*, Biol. Psychiatry **1993**, 34, 361). The present study used male Sprague Dawley rats that were either reared in single cages from weaning for 8 weeks (isolated rats) or in group cages housed 6 per cage (grouped rats). Following this housing procedure, the effect of the CB_1 receptor agonist Δ^9-tetrahydrocannabinol (Δ^9-THC) on PPI was determined using startle response chambers. In addition, brains from vehicle treated isolated rats and grouped rats were removed and dissected for determination of CB_1 receptor and fatty acid amide hydrolase (FAAH) messenger RNA (mRNA) expression by real time PCR analysis. Vehicle treated isolated rats had significantly lower levels of PPI when compared to grouped rats. Δ^9-THC (0.3-3 mg/kg i.v.) had no significant effect on PPI of grouped rats. In contrast, Δ^9-THC (0.3-3 mg/kg i.v.) dose dependently potentiated the disruption of PPI in isolated rats. The disruption produced by THC 3 mg/kg i.v. was restored to vehicle control levels by pretreatment with the CB_1 receptor antagonist N-piperidino-5-(4-chlorphenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716) indicating a CB_1 receptor mediated effect. Real time PCR analysis revealed no significant difference in expression of CB_1 receptor mRNA in the globus pallidus, nucleus accumbens or substantia nigra in isolated rats compared with grouped rats. However, in the hippocampus of isolated rats there was a significant reduction in CB_1 receptor mRNA expression compared to that of grouped rats. There was no significant difference in the level of FAAH mRNA in socially isolated rats compared to grouped rats in any of the brain regions studied. These results suggest that isolated rats have increased sensitivity to the effects Δ^9-THC on PPI, as well as a decrease in CB_1 receptor mRNA expression in the hippocampus compared to grouped rats. However, this decrease in CB1 receptor mRNA expression in isolated rats is not accompanied by a change in FAAH mRNA expression in any of the brain regions studied.

ATTENUATION OF THE *IN VIVO* INDUCTION OF LONG-TERM POTENTIATION IN THE CA1 REGION OF THE HIPPOCAMPUS FOLLOWING PROLONGED CANNABINOID TREATMENT: ASSOCIATION WITH SPATIAL MEMORY DEFICITS

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Chronic, long-term, consumption of cannabinoids typically produces some degree of impairment in cognitive abilities, especially in tasks involving short term memory recall. To examine whether there are electrophysiological correlates of this impairment, we examined synaptic transmission and long-term potentiation (LTP) *in vivo* in the CA1 region of the hippocampus in animals following a fifteen day treatment with 100 μg/kg of the potent CB_1 receptor agonist HU-210. Our results indicate that this procedure produces deficits in a long delay matching-to-sample version of the Morris Water Maze, but not in a short delay task, indicating that behavioral performance is indeed impaired. Furthermore, while synaptic transmission was unaffected, LTP induction was significantly reduced in animals that had received chronic cannabinoid treatment. These results suggest that the memory deficits that are seen following chronic cannabinoid exposure are more sensitive to delay based memory tasks and may be related to deficits in synaptic plasticity that impair the consolidation of memory.

CELLULAR AND SYNAPTIC SPECIFICITY OF ENDOCANNABINOID SIGNALING IN THE NEOCORTEX

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Retrograde synaptic signaling has long been recognized as an important feature of neural systems. We have focused our efforts on identifying the specific neocortical cell types and circuits that utilize endocannabinoids (eCBs) as retrograde signaling molecules. In the cortex, as in several other brain regions, eCBs mediate a form of presynaptic depression termed depolarization-induced suppression of inhibition (DSI). In the present study, we investigated the cellular and synaptic specificity of eCB-mediated DSI in slices of mouse sensory cortex using single and dual whole-cell recordings. We report that GABAergic interneurons that depolarize in response to cholinergic agonists provided the majority of DSI-susceptible inputs to layer 2/3 pyramidal neurons (PNs). This subclass of interneurons generated large, fast synaptic currents in PNs that were transiently suppressed by postsynaptic depolarization. Neocortical DSI required the activation of type-1 cannabinoid receptors, but not metabotropic glutamate or GABA receptors. Using focal drug application, we found that the DSI-susceptible afferents preferentially form synapses on the perisomatic membrane of PNs, and not on the apical dendrites. Our current efforts are aimed at examining eCB signaling at the level of unitary synaptic connections between interneurons and PNs. Preliminary data confirm that GABAergic synapses that do not show DSI are unaffected by exogenous cannabinoids. Moreover, these cannabinoid- and DSI-insensitive interneurons do not respond to cholinergic stimulation. Together, these results suggest that eCB signaling in the neocortex provides a mechanism for PNs to transiently suppress perisomatic, inhibitory input from interneurons that are excited by the cholinergic system. Although the physiological implications remain to be explored, this selective suppression of inhibition may alter the excitability of principal neurons during behavioral states when ascending cholinergic neurons are active.

A BRIEF TRAIN OF ACTION POTENTIALS ENHANCES PYRAMIDAL NEURON EXCITABILITY VIA ENDOCANNABINOID-MEDIATED SUPPRESSION OF INHIBITION

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Endocannabinoids (eCBs) act as retrograde signaling molecules and mediate a form of short-term synaptic plasticity called depolarization-induced suppression of inhibition (DSI). Endocannabinoid-mediated DSI results from the calcium- and depolarization-dependent release of eCBs from postsynaptic neurons. In the neocortex, the release of eCBs from pyramidal neurons (PNs) appears to play an important role in regulating somatic inhibition from a select subpopulation of GABAergic inputs that express the type-1 cannabinoid receptor (CB_1R). Although postsynaptic control of afferent inhibition may directly influence the integrative properties of neocortical PNs, little is known regarding the patterns of activity that evoke eCB release and the impact such disinhibition may have on PN excitability. Whole-cell somatic current clamp recordings taken from individual layer 2/3 PNs demonstrated that a brief train of postsynaptic action potentials (APs) induced considerable eCB-mediated retrograde inhibition of spontaneous inhibitory synaptic inputs. We found that the magnitude and time course of this suppression was directly related to the frequency and number of postsynaptic APs in the train. Endocannabinoids mediated the observed DSI because suppression was blocked by the CB_1R antagonist AM251 and potentiated by the eCB uptake inhibitor AM404. Additionally, we explored the functional consequences of neocortical DSI by examining the responsiveness of PNs to synaptic stimulation. We found that eCB-mediated disinhibition induced by a single train containing as few as 5 APs, markedly and transiently enhanced the responsiveness of PNs to excitatory synaptic input and promoted an increase in AP discharge that paralleled the peak expression of DSI. The transient increase in AP firing that followed the induction of DSI was completely blocked by AM251 and occluded by the GABA-A antagonist SR 95531. Taken together, these results suggest that eCB release from PNs is likely to play an important role in information processing in the neocortex.

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EFFECT OF CANNABINOIDS ON INHIBITORY SYNAPTIC TRANSMISSION BETWEEN FAST SPIKING NEURONS (FSNS) AND MEDIUM SPINY NEURONS (MSNS) IN THE CAUDATE PUTAMEN

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There is a high density of CB_1 cannabinoid receptors in the caudate-putamen (Tsou et al., Neurosci, 83:393-411, **1998**). A fraction of these receptors is synthesized by parvalbumin expressing FSNs (Hohmann and Herkenham, Synapse, 37:71-80, **2000**). FSNs give the predominant GABAergic input to MSNs. We hypothesised that activation of CB_1 receptors on axon terminals of FSNs depresses synaptic transmission between FSNs and MSNs. We used the patch clamp technique for brain slices. The major problem was the identification of FSNs, because FSNs make up only 3-5 % of the neurons in the caudate-putamen. To solve the problem, we used transgenic mice in which parvalbumin synthesizing neurons express green fluorescent protein (Meyer et al., J Neurosci, 22:7055-7064, **2002**). Thus, FSNs could be identified due to their green fluorescence. FSNs were silent, but fired action potentials at rates more than 120 Hz during depolarizing current injections. In order to study neurotransmission, we recorded simulatenously from FSNs and MSNs. Single action potentials in FSNs were elicited by depolarizing current injections. Action potentials in FSNs were accompanied by inhibitory postsynaptic currents in MSNs (IPSCs; 64 ± 18 pA; *n*=15). The mixed CB_1/CB_2 cannabinoid receptor agonist WIN55212-2 (5 µM) decreased the amplitude of IPSCs by 72 ± 8 % (n=6; $P < 0.05$). The inhibition was due to an increase in failure rate; the amplitude of successful IPSCs was not changed. The inhibition by WIN55212-2 was abolished by the CB_1 cannabinoid receptor antagonist SR141716 (1 µM). WIN55212-2 (5 µM) decreased the frequency of miniature IPSCs recorded in MSNs in the presence of tetrodotoxin by 36± 8 % (n=10; $P < 0.05$) but did not change their amplitude. WIN55212-2 (5 µM) did not change the firing of FSNs elicted by depolarizing current injections.

The results show that activation of CB_1 receptors inhibits GABAergic neurotransmission between FSNs and MSNs. The lack of effect of WIN55212-2 on amplitude of successful IPSCs and miniature IPSCs excludes a postsynaptic effect as a mechanism of synaptic depression. Lowering of the success rate of neurotransmission points to a presynaptic mechanism. The decrease in frequency of miniature IPSCs indicates that presynaptic inhibition was partly due to inhibition of the vesicle release machinery. In contrast to the inhibition of transmitter release from terminals of FSNs, cannabinoids did not interfere with somadendritic mechanisms involved in regulation of the firing rate of FSNs. The current work shows cannabinoid action at a synapse which is difficult to study but plays an eminent role in movement control.

Abbreviations: SR141716, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-3-pyrazolecarboxamide; WIN55212-2, (+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)-methanone mesylate

STIMULATION OF LC NORADRENERGIC NEURONAL ACTIVITY BY CANNABINOIDS: A PRE- OR A POSTSYNAPTIC EFFECT?

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Previous work in this laboratory demonstrated that cannabinoid administration positively modulates the spontaneous firing rate of locus coeruleus (LC) noradrenergic (NAergic) neurons in rats. Additionally, we showed that intravenous injection of WIN55212-2 (WIN) is also able to revert LC NAergic cells evoked inhibition from the nucleus prepositus hypoglossi (PrH), which provides the main GABAergic afferents to the LC. In the present investigation we further tested the hypothesis of a GABAergic role in cannabinoid-mediated excitation of the LC. By using *in vivo* single-unit electrophysiology/iontophoresis techniques we observed the effect of WIN in the presence of bicuculline, a $GABA_A$ antagonist which is also known to excite NAergic neurons. As expected, iontophoretic application of bicuculline (5mM, 1-20 nA ejection current) induced a powerful current-dependent stimulation of LC NAergic neuronal activity. This effect was rapidly reversible and reproducible over time. In the presence of bicuculline (10 nA), systemic administration of WIN (0.5 mg/kg i.v.) further stimulated the firing rate of LC NAergic neurons. Given that bicuculline failed to occlude WIN-induced actions, at this stage we cannot exclude alternative mechanisms other than GABAergic. Nonetheless, experiments are still in progress to verify if a more complete blockade of LC $GABA_A$ receptors would attenuate the action of WIN. While not conclusive, these results suggest that cannabinoids might affect LC NAergic transmission via both trans-synaptic and post-synaptic mechanisms.

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SELECTIVE MODULATION OF TRANSMITTER RELEASE AT RAT NEUROMUSCULAR JUNCTIONS BY Δ^9-TETRAHYDROCANNABINOL

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Cannabinoid effects on transmission at neuromuscular junctions (NMJs) depend on the preparation, species and cannabinoids examined (see Aldridge & Pomeranz, **1977**; Turkanis & Karler, **1988**; Hoekman et al., **1976** and Van der Kloot, **1994**). Most of these studies were performed before cannabinoid receptor subtypes were identified and often used high cannabinoid concentrations (~10^{-4} M). We have therefore begun pharmacologically characterising the effect at rat NMJs, using quantal analysis (no. synaptic vesicles released/stimulus, i.e. quantal content - QC) and lower concentrations of cannabinoid receptor agonists/antagonists.

Male Sprague Dawley rats (150-250 gm) were killed by stunning and cervical dislocation (Schedule 1 killing, UK Animals (Scientific Procedures) Act, **1986**) and hemidiapragm/phrenic nerve preparations excised. Some preparation were paralysed by blocking muscle Na channels with 2 μM μ-conotoxin GIIIB, then sharp intracellular electrodes recorded the effects of cannabinoid receptor agonists and/or antagonists on transmitter release at NMJs. In other preparations, NMJs were visualised with fluorescent bungarotoxin, fixed in 1-4% paraformaldehyde and processed for immunofluorescence labelling of CB_1 receptors at NMJs with N-terminus or C-terminus primary antibodies, with or without permeabilisation.

Consistent with previous studies (Hoekman et al., **1976**), Δ^9-tetrahydrocannabinol (THC – CB receptor agonist) prolonged postsynaptic potentials (0.1-10μM). 100nM THC increased the time for postsynaptic potentials to return to 50% of peak value (T50%) from 2.4 ± 0.1 ms (mean ± SE, 30 NMJs (N) from 6 muscles (m)) to 2.8 ± 0.1 ms (N=16, m=3. $P<0.01$, Student's t-test). In the same muscles, QC increased from 25.7 ± 1.7 to 33.8 ± 3.3 ($P<0.02$). Interestingly, neither WIN55,212-2 (potent CB_1/CB_2 receptor agonist) nor its inactive stereoisomer WIN 55,212-3 produced a similar enhancement of release (e.g. 5 μM WIN55,212-2; 27.2 ± 2.7, N=17, m=3. $P>0.2$). Since all drugs were dispersed in Tween-80 (1:2, Tween:cannabinoid (v/v)) and only THC produced these effects, it is unlikely that the vehicle produced the observed enhancement. THC effects on T50% and QC were not blocked by the potent CB_1 receptor antagonist/inverse agonist SR 141716A (1μM, $P<0.02$ vs drug-free NMJs, N=17, m=3) and SR alone was without effect on these parameters (N=10, m=2). Immunofluorescence labelling for CB_1 receptors produced no consistent evidence of CB_1 receptor localisation at NMJs (N=50, m=5).

The data indicate THC enhances transmission at rat NMJs by increasing QC and prolonging postsynaptic potentials. The pharmacological profile and lack of CB_1 receptor labelling suggest neither CB_1 nor CB_2 receptors mediate these effects.

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SR141716 INCREASES BUT VCHSR DOES NOT AFFECT TRANSMITTER RELEASE IN GUINEA-PIG HIPPOCAMPUS

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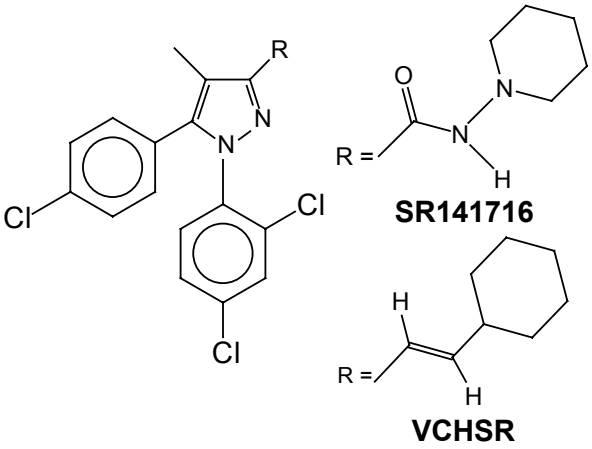

The cannabinoid CB_1 receptor inverse agonist and/or antagonist SR141716 (rimonabant) facilitates transmitter release in several experimental models (Schlicker and Kathmann, Trends Pharmacol. Sci. **2001**, 22, 565). This phenomenon might be attributed to (i) its inverse agonistic effect or (ii) the interruption of a feedback built up by endogenously formed cannabinoids. One would expect that a neutral antagonist will mimic the facilitatory effect of SR141716 only if the second but not if the first possibility holds true. Recently, VCHSR was identified as a neutral antagonist at human CB_1 receptors (Hurst et al., Mol. Pharmacol. **2002**, 62, 1274).

In guinea-pig cortex membranes, SR141716 and VCHSR showed pK_i values at CB_1 receptors (labelled by ^{3}H-SR141716) of 8.73 and 8.16, respectively. Transmitter release was studied in superfused guinea-pig hippocampal slices preincubated with ^{3}H-noradrenaline or ^{3}H-choline, in which the electrically evoked tritium overflow represents quasi-physiological noradrenaline and acetylcholine release, respectively. The pA_2 values for SR141716 and VCHSR at the CB_1 receptor involved in inhibition of noradrenaline release were 8.17 and 7.23, respectively. To compare the effect of both drugs on transmitter release, we used them at concentrations exceeding their antagonist dissociation constants by a factor of 20. SR141716 0.12 µM increased noradrenaline and acetylcholine release by 43 and 136 %, respectively, whereas VCHSR 1 µM did not affect the release of either transmitter.

Our results suggest that activation of the constitutively active CB_1 receptor is the more likely explanation for the facilitatory effect of SR141716 on hippocampal transmitter release.

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Keywords: Cannabinoid CB_1 receptor – SR141716 – VCHSR - noradrenaline release – acetylcholine release

CB_1 AGONISTS ADMINISTRATION MODIFY 5-HT RELEASE IN THE NUCLEUS ACCUMBENS AND STRIATUM OF FREELY MOVING RATS

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Evidence suggest that administration of Δ^9-tetrahydrocannabinol (THC) or synthetic CB_1 receptor agonists determinate behavioural effects such as modification of locomotor activity and food intake, induction of anxiety and hypothermia, as well as reinforcement effects. It is reported that injections of CB_1 receptor agonists modify extracellular 5-HT levels in specific brain areas and therefore serotoninergic could play an important role in modulation of these behavioural effects.

In the present study, using the "in vivo" microdialysis technique, we investigated the effect of systemic THC and WIN 55,212-2 administration on extracellular 5-HT levels in the shell of the nucleus accumbens and in the dorsal striatum brain areas involved in modulating several central effects of cannabinoids. An increase in accumbal 5-HT extracellular levels occurred after iv administration of WIN 55,212-2 or THC (0.15 mg/kg iv) with respect to basal levels. On the contrary, a higher dose (0.3 mg/kg iv) elicited a significant decrease of 5-HT levels after WIN 55,212-2 but not after THC. Pretreatment with the specific cannabinoid antagonist SR 141716A reversed the WIN 55,212-2- and THC-induced modification of 5-HT release in the shell of the nucleus accumbens. On the other hand, SR 141716A alone did not modify basal 5-HT extracellular levels during the entire experimental period. Finally we found very similar results in the dorsal striatum of our animals.

Our data demonstrated that cannabinoids modify 5-HT extracellular levels in the nucleus accumbens and the striatum suggesting an involvement of the serotoninergic neuronal system in behavioural effects induced by cannabinoid agonists administration.

EFFECTS OF ANANDAMIDE AND METHANANDAMIDE ADMINISTRATION ON MESOLIMBIC DOPAMINE TRANSMISSION A MICRODIALYSIS STUDY IN THE RAT

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Reinforcing effects of cannabis derivatives in animals have always been hard to assess, despite their well-known pleasurable effects in humans. We recently developed a reliable animal model of cannabis dependence, which has provided important preclinical insights into cannabis abuse and addiction. Indirect evidence of reinforcing effects of cannabinoids has come from conditioned-place-preference studies and neurochemical studies. In-vivo microdialysis studies have shown that the active ingredient of cannabis, THC, activates the mesolimbic dopaminergic pathway, selectively increasing extracellular dopamine (DA) levels in the shell of the nucleus accumbens in a similar manner to other drugs abused by humans. Activation of DA transmission in this area, indeed, is believed to play an important role in the motivational and addictive effects of drugs. While many studies on the reinforcing effects of cannabinoids have focused on THC and a few other synthetic CB_1 agonist-type drugs, only a few preclinical studies have dealt with the reinforcement-related effects of endogenous cannabinoids. The discovery of a brain cannabinoid receptor and of its endogenous ligands has suggested the existence of a brain cannabinoid system. Recently, a role for the cannabinoid system in some aspects of opioid and nicotine abuse has been suggested. However no data is available about the effects of endogenous cannabinoid ligands or drugs able to increase their availability in the brain on mesolimbic DA neurotransmission. For this reason, we studied the neurochemical effects of anandamide and of its metabolically stable analog, methanandamide, on DA transmission in the nucleus accumbens shell of freely moving rats. We found that cumulative doses of anandamide or methanandamide, 0.3 to 10 mg/kg i.v. administered 1hour apart, dose-dependently and significantly increased DA levels in the accumbens shell. Although the maximum increase in DA output was not significantly different between the two drugs, surprisingly, the effects of anandamide on DA levels lasted longer that those of methanandamide, an unexpected finding based on different metabolic rate. Since the mesolimbic DA system plays a role in motivational-rewarding effects of drugs, these data suggest that endogenous cannabinoids possess reinforcing effects, and support the hypothesis that activation of the cannabinoid system plays a role in the reinforcing effects of other drugs able to elevate its endogenous tone.

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FLUOROMETRIC DETECTION OF CANNABINOID MODULATION OF GLUTAMATE RELEASE FROM RAT HIPPOCAMPAL SYNAPTOSOMES

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Cannabinoids (both classical and non-classical) can modulate transmitter release in cultured rat hippocampal neurones via blockade of presynaptically located N- and P/Q type calcium channels (Twitchell, et al., (**1997**) *J. Neurophys*. 78(1), 43-50). The distribution pattern of cannabinoid receptors is consistent with such cannabinoid-induced effects (Pertwee et al., (**2002**) *Prostaglandins, Leukot Essent Fatty Acids.* 66 (2&3), 101-121), since cannabinoid administration alters cognitive/memory functions linked to the cerebral cortex, particularly the hippocampus (Hajos & Freund, (**2002**) *Chem and Physics of Lipids.* 121, 73-82). However, at least two different cannabinoid sensitive presynaptic receptors regulate network activity in the hippocampus (Freund, et al., (**2003**) *Physiol. Rev.* 83(3), 1017-66). CB_1 receptor expression within the hippocampus is heterogenous and has been localised to the presynaptic terminals of a subset of cholecystokinin containing GABA-ergic neurones (Tsou, et al., (**2000**) *Neurosci.* 93 969-975; Irving, et al., (**2000**) *Neurosci.* 98, 253-262). There is evidence suggesting that novel non-CB_1 cannabinoid-sensitive receptors are located on hippocampal excitatory axon terminals, mediating inhibition of glutamate release in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. These non-CB_1 receptors have distinct pharmacological characteristics, including a lower sensitivity to the synthetic CB_1/CB_2 receptor agonist, R-(+)-WIN55,212-2 (WIN2) and a sensitivity to vanilloid (TRPV1) receptor ligands (Hajos & Freund, (**2002**) *Neurosci.* 106(1), 1-4). This receptor may be a novel GPCR, since anandamide and WIN2 stimulate [35S]GTP-S binding in brain tissue from both CB_1 knockout and wild-type mice. The current study aims to further characterise the receptor modulating release from hippocampal glutamatergic terminals. At least two different cannabinoid sensitive presynaptic receptors regulate network activity in the hippocampus (Freund, et al., (**2003**) *Physiol. Rev.* 83(3), 1017-66). There is evidence that cannabinoids (both classical and non-classical) modulate transmitter release via blockade of presynaptically located N- and P/Q type calcium channels in cultured rat hippocampal neurones (Twitchell, et al., (**1997**) *J. Neurophys*. 78(1), 43-50). CB_1 receptor immunoreactivity has been found to be selectively localised on the presynaptic terminals of a subset of cholecystokinin containing GABA-ergic neurones (Tsou, et al, **2000**; Irving, et al., (**2000**) *Neurosci.* 98, 253-262). CB_1 receptor expression within the brain is heterogenous and the distribution pattern of these receptors is consistent with effects induced by cannabinoids (Pertwee et al., (**2002**) *Prostaglandins, Leukotrienes, and Essential Fatty Acids.* 66 (2&3), 101-121). This investigation was carried out specifically on hippocampal neurones since administration of cannabinoids alters cognitive/memory functions linked to the cerebral cortex, particularly the hippocampus (Hajos & Freund, (**2002**) *Chem. and Physics of Lipids.* 121, 73-82). To do this, cannabinoid modulation of glutamate release from adult rat hippocampal isolated nerve terminals (synaptosomes) was quantified using enzyme-linked fluorescence detection. Calcium dependent, 4-aminopyridine-evoked glutamate release was inhibited by 43 $\pm$ 9% (n = 6; $p < 0.01$) in the presence of WIN2 (1μM; as compared to vehicle control (0.01% DMSO)). The CB_1 receptor antagonist/inverse agonist SR 141716A (100nM; 0.01% DMSO), completely blocked the WIN2-induced inhibition of glutamate release, the glutamate release in the presence of WIN2 + SR 141617A being 101 $\pm$ 22%. The cannabinoid agonist CP55,940 (1μM; 0.01% DMSO) also reduced calcium dependent glutamate release by 21 $\pm$ 13% (n = 3).

These results demonstrate that 4-aminopyridine induced, calcium dependent glutamate release from adult rat hippocampal synaptosomes is inhibited by various cannabinoid receptor agonists. Further, at least some of these effects are SR 141716A-sensitive. Clearly, further studies are required to fully understand the cannabinoid-mediated modulation of glutamate release from hippocampal nerve terminals.

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PHARMACOLOGICAL MODULATION OF THE ENDOCANNABINOID SYSTEM IN A VIRAL MODEL OF MULTIPLE SCLEROSIS

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Theiler's virus infection of the central nervous system (CNS) induces an immune-mediated demyelinating disease in susceptible mouse strains and serves as a relevant infection model for human multiple sclerosis (MS). Cannabinoids have been shown to exert beneficial effects on animal models of MS and evidence suggests that the endocannabinoid system plays a role in the tonic control of spasticity. In this study we show that OMDM1 and OMDM2, two selective inhibitors of the putative endocannabinoid transporter, and hence of endocannabinoid inactivation, provide an effective therapy for TMEV-induced demyelinating disease (TMEV-IDD). Treatment of TMEV-infected mice with OMDM1 and OMDM2 ameliorated motor symptoms. This was associated with a downregulation of inflammatory responses in the spinal cord. In addition we show that OMDM1 and OMDM2 down-regulate macrophage function by: i)decreasing the surface expression of MHC class II molecules, ii) inhibiting NOS-2 expression, and iii) reducing the production of the pro-inflammatory cytokine IL-1β. Taken together, these results point to the manipulation of endocannabinoid system as a possible strategy to develop future MS therapeutic drugs.

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CANNABINOID-MEDIATED NEUROPROTECTION IN *IN-VIVO* AND *IN-VITRO* DEMYELINATION

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Multiple sclerosis (MS) is increasingly being recognised as a neurodegenerative disease triggered by inflammation in the central nervous system (CNS). Axonal or neuronal damage is a primary cause of disability and has thus far not been successfully targeted by available drug therapies. Anecdotal evidence, and now experimental data, has shown that cannabinoids are useful in the symptom management of MS, specifically for control of pain, spasticity bladder-related symptoms and tremor. Using the *in-vivo* chronic relapsing experimental allergic encephalomyelitis (CR-EAE) model of MS and *in-vitro* aggregate myelinating cell culture model there was evidence of evidence of cannabinoid-mediated neuroprotection.

3-D Neurosphere brain cultures containing neurons, astrocytes, oligodendrocytes and microglia were generated *in vitro* from embryonic telencephalon of mice. These neurons were allowed to myelinate and were then demyelinated using interferon-gamma. Whilst cultures from wildtype mice subsequently remyelinated, cultures from CB_1KO mice exhibited production of the death effector- active caspase 3 and loss of neurofilament indicating that endogenous activation of the CB_1 receptor may confer neuroprotection. In CR-EAE, neurofilament levels indicative of axonal loss were significantly reduced through the disease course, with levels being consistently lower in spinal cords from CB_1 receptor-knockout animals (CB_1-R KO). Furthermore activity deficits were markedly increased in CB_1R-KO mice following a single attack, such deficits occurred after about 3-4 attacks in wildtype mice. Whilst some cannabinoids can have immunosuppressive effects, treatment with WIN-55,212-2 failed to prevent the development of relapsing paralytic disease. However, the underlying residual deficit occurring as a consequence of inflammatory attack indicative of the ongoing neurodegeneration was significantly ameliorated and the loss of mobility was reduced compared to vehicle treated controls.

This study further indicates the neuroprotective potential of the cannabinoid system, which may be utilised in the control of neurodegenerative diseases such as multiple sclerosis.

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EXPRESSION OF CB_1 RECEPTOR PROTEIN IN THE STRIATUM OF ANIMAL MODELS OF PARKINSON'S DISEASE

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Parkinson's disease (PD) is a major problem in contemporary neurology and psychiatry, represents a significant financial burden on society and has a serious negative impact on the lifestyle and socio-economic status of those affected. The principal approach to treatment is dopamine replacement, most commonly with the dopamine precursor 3,4-dihydroxypheylalanine (L-DOPA). However, L-DOPA therapy leads to the development of unwanted, debilitating, involuntary movements "L-DOPA-induced dyskinesia" (LID) in 40-80% of patients within 5 years. Rats lesioned unilaterally with 6-hydroxydopamine (6-OHDA) represent a very good model of the nigrostriatal neurodegeneration seen in Parkinson' s disease (PD) and long-term administration of levodopa in these rats results in symptoms equivalent to levodopa-induced dyskinesia (LID). These animal models of PD and LID have previously been employed to detect changes in mRNA levels by *in situ* hybridization (Zeng BY *et al*., Neurosci Lett. **1999**; 276(2):71-4). Our aim was to assess whether changes in mRNA were translated to CB_1 protein content by Western blotting. Male Sprague-Dawley rats were unilaterally lesioned with 6-OHDA (12.5 μg) applied stereotactically into the medial forebrain bundle. Sham-operated animals were also prepared. Post-operation, the animals were treated with levodopa (15 mg/kg plus benserazide 3.5 mg/kg, i.p.) or vehicle twice daily for 21 days. The brains were removed, rapidly frozen and sectioned. The striata were carefully isolated and removed from the sections, homogenized and used for Western blot analysis (7.5% SDS-PAGE gel, rabbit polyclonal antibody raised against the N-terminus of the CB_1 receptor). In agreement with the results from the aforementioned *in situ* hybridization study, we did not detect any differences between 6-OHDA-lesioned and non-lesioned striatum. However, in contrast with findings of Zeng *et al* where an elevation of CB_1 mRNA was described, we found a decrease in CB_1 receptor protein content in the striatum of 6-OHDA-lesioned rats that have previously received long-term levodopa treatment when compared to non-lesioned striatum of the same group of animals. This study highlights three important issues: 1. the importance of measuring both CB_1 mRNA and protein levels; 2. the potential effect of dosing regime of levodopa on CB_1 function, i.e. application of 15 mg/kg of levodopa twice daily in our study vs. 50 mg/kg in one dose in study by Zeng *et al*.; 3. the time of assessment of CB_1 levels in relation to behaviour: in our study animals were killed at the time levodopa was exerting its maximal action, i.e. at the time equivalent to dyskinesia, whereas in the study of Zeng *et al.* animals were killed 3 hours after the last dose of levodopa when the effect would have subsided.

ALTERATIONS IN ENDOCANNABINOID LEVELS ACCOMPANYING PARKINSONISM AND LEVODOPA-INDUCED DYSKINESIA IN THE MPTP-LESIONED MACAQUE

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Endocannabinoids have a diversity of effects, mediated via CB_1, VR_1 and other mechanisms, that can modulate neuronal and glial signalling within the basal ganglia. It has previously been hypothesised that enhanced levels of endocannabinoids might contribute to: 1) mechanisms compensating for loss of dopamine in pre-symptomatic PD, 2) generation of parkinsonian symptoms in PD and 3) generation of levodopa-induced dyskinesia (LID) in PD. Here, we determined the levels of the endocannabinoids 2-arachidonyl glycerol (2-AG) and anandamide (AEA) throughout the basal ganglia in the MPTP-lesioned macaque model of Parkinson's disease (PD) and levodopa-induced dyskinesia (LID).

Twenty-seven female macaques (2.8-3.4 kg) were assigned to 4 groups: 1) normal (n=6), 2) parkinsonian, untreated (n=5), 3) parkinsonian, acute L-DOPA, non-dyskinetic (n=6) and 4) parkinsonian, chronic L-DOPA, dyskinetic (n=10). Animals in group 1 received only vehicle treatment. Animals in groups 2, 3 and 4 were rendered parkinsonian by repeated daily MPTP administration (0.2 mg/kg / day, for 14-18 days). Following stabilisation of parkinsonism (4 months), group 4 animals received twice daily L-DOPA treatment, tailored to provide maximal anti-parkinsonian benefit in each individual animal, for 6 months and killed 1 hour after their last L-DOPA administration. These animals demonstrated LID characterised by an idiosyncratic mix of chorea and dystonia. Group 3 animals received a single dose of L-DOPA sufficient to alleviate parkinsonian symptoms 1 hour prior to being killed. Brain regions were dissected and frozen immediately. The levels of 2-AG and AEA were assessed by isotope dilution atmospheric pressure chemical ionisation liquid chromatography-mass spectrometry.

AEA, but not 2-AG, was increased in GPe (149±9%; $p=0.01$) of parkinsonian, untreated compared to normal animals. Reversal of parkinsonism with acute L-DOPA administration had no significant effect on this rise. On the other hand, the generation of LID was accompanied by a reduction of AEA in SN (-49±15%; $p=0.06$), and of 2-AG (-37±8%; $p<0.01$) in Gpe, compared to parkinsonian animals.

These data support: 1) a role for reduced endocannabinoids in the generation of LID, and 2) the hypothesis that elevated endocannabinoids might contribute to a failed mechanism attempting to compensate for loss of dopamine in parkinsonism, as well as possibly generating motor symptoms in this disorder, as suggested by previous studies.

INCREASED ENDOCANNABINOID TRANSMISION IN THE BASAL GANGLIA IN A GENETIC MODEL OF PARKINSON'S DISEASE

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The endocannabinoid transmission becomes overactive in the basal ganglia in Parkinson's disease (PD), as reported in postmortem brains of PD patients (Lastres-Becker et al., Eur. J. Neurosci. 14, 1827-1832, **2001**) and in different animal models of this disease, such as MPTP-treated marmosets (Lastres-Becker et al., Eur. J. Neurosci. 14, 1827-1832, **2001**), 6-hydroxydopamine-lesioned rats (Romero et al., Life Sci. 66, 485-494, **2000**), or reserpine-treated rats (Di Marzo et al., FASEB J. 14, 1432-1438, **2000**), thus suggesting that the blockade of the cannabinoid CB_1 receptor might be useful to reduce hypokinesia characteristic of PD. In the present study, we have examined the binding capacity and mRNA levels for the cannabinoid CB_1 receptor in different basal ganglia structures in a genetic model of PD, such as Park-2 knockout mice. These mice exhibit a reduced release of dopamine but relative preservation of monoamine levels and cellular markers of the nigrostriatal dopaminergic neurons, so they could be considered representative of early and presymptomatic parkinsonian deficits (see Itier et al., Hum. Mol. Genet. 12, 2277-2291, **2003**, for a recent report on the behavioral and neurochemical abnormalities of Park-2 knockout mice). In these mice, we also found an increase in the population of CB_1 receptors in the substantia nigra compared to wild-type animals with no changes in other basal ganglia structures. However, despite this increase, the acute administration of the cannabinoid agonist, Δ^9-tetrahydrocannabinol (5 mg/kg), produced a motor depression that was of similar magnitude in Park-2 knockout and wild-type mice, and also equivalent changes in several neurotransmitters in the basal ganglia. In summary, in contrast with the data obtained in humans and models of neurotoxin-induced lesions in rodents and non-human primates, the changes in CB_1 receptors observed in parkin deficient mice seem to be produced by dopaminergic dysfunction and would not require massive destruction of dopamine neurons in the substantia nigra. In addition, these changes did not result in differences in behavioral responses to cannabinoid agonists between Park-2 knockout and wild-type mice.

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EFFECTS OF MODULATION OF VANILLOID AND ENDOCANNABINOID SIGNALLING ON LOCOMOTION IN NORMAL ANIMALS AND IN ANIMAL MODELS OF PARKINSON'S DISEASE

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The vanilloid TRPV1 receptor has been implicated in the control of voluntary movement by the basal ganglia. Capsaicin and the endocannabinoid anandamide (AEA) bind and activate the TRPV1 receptor at an intracellular site. AEA is transported into the cell by the putative anandamide membrane transporter (AMT) and inactivated by the enzyme fatty acid amide hydrolase (FAAH). While FAAH inhibitors enhance the actions of AEA at both cannabinoid and TRPV1 receptors, AMT inhibitors are expected to exert opposing, indirect actions on these two receptors. We hypothesised that direct (capsaicin), or indirect (by FAAH inhibition) stimulation of TRPV1 would modulate locomotor activity, and that modulation of TRPV1 signalling might have value in the treatment of movement disorders, such as Parkinson's disease. Locomotion was assessed in normal or parkinsonian rats. In the parkinsonian state, the effects on L-DOPA induced locomotion were assessed. In normal rats, drugs or vehicle were administered (i.p.) and behaviour assessed immediately for 30 minutes. Rats were rendered parkinsonian with reserpine (4mg/kg) and 18 hrs later, they were injected with L-DOPA (125mg/kg) in combination with either drugs or vehicle and locomotion was assessed for 4 hours. The drugs administered were the TRPV1 receptor agonist, capsaicin (0.1, 0.5 or 1mg/kg), the TRPV1 antagonist, capsazepine (10mg/kg), the FAAH inhibitor, URB597 (10mg/kg) and the AMT inhibitor, OMDM-2 (5mg/kg). Locomotion was measured using an open field arena in an automated movement detection system. In normal rats, capsaicin (1mg/kg) or URB597 caused a significant reduction in movement in both the horizontal (locomotion) and vertical (rearing) planes (-45% and -53% respectively with capsaicin; -33% and -37% for URB597; all $P<0.05$). Capsaicin-induced hypolocomotion was attenuated by capsazepine, suggesting that this effect was mediated by the TRPV1 receptor. L-DOPA treatment of parkinsonian rats elicited high levels of motor activity in both the horizontal and vertical planes. Horizontal activity was attenuated by capsaicin (1mg/kg, -60%, 0-1h post-injection), but not by URB597 or OMDM-2. Vertical activity was attenuated, in a dose-dependent manner by capsaicin (-61% to -73%, 2-3h post -injection) and by URB597 (-54%, 0-1h), but not by OMDM-2. URB597 also reduced a behaviour characterized by rapid running from the front to the back of the arena (-72%, 0-1h)**.** In conclusion, activation of the TRPV1 system by exogenous, and possibly endogenous, agonists can suppress spontaneous locomotion in normal animals. Different components of behaviour, at different times post-L-DOPA, involve different neurotransmitter systems and neural circuits. Thus, the differences in the effects of drugs interacting, in different ways, with the vanilloid and cannabinoid system highlight the multiple actions of these systems in the function of the basal ganglia and support the concept of targeting these systems in the treatment of movement disorders such as Parkinson's disease and L-DOPA-induced dyskinesia.

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CRH- AND DEPOLARIZATION-INDUCED INCREASE IN BDNF EXPRESSION IS INHIBITED BY CB_1 ACTIVATION

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Both the corticotropine-releasing hormone (CRH) system as well as the cannabinoid system have been demonstrated to affect synaptic plasticity. Activation of CRH receptor type 1 (CRHR1) leads to increases in cAMP production, and subsequent phosphorylation of the transcription factor cAMP response element-binding protein (CREB). The cannabinoid receptor type 1 (CB_1) however, is negatively coupled to the cAMP signaling cascade and inhibits voltage-dependent calcium channels. In this study, we analyzed a putative cross-talk between these two systems focussing on the regulation of the expression of brain-derived neurotrophic factor (BDNF), a CREB-regulated gene that also affects neuronal survival and synaptic plasticity. *In situ* hybridization revealed a high degree of coexpression of CRHR1 and CB_1 receptors in cerebellar granule cells, a necessary prerequisite for any cross-talk. We analyzed the effects of CRH and the CB_1 agonist WIN-55,212-2 on BDNF expression in primary cerebellar neurons from rats and mice. Semiquantitative RT-PCR and ELISA revealed increased levels of BDNF mRNA and protein 48 hours after application of CRH. This effect was inhibited by simultaneous administration of WIN-55,212-2. WIN-55,212-2 alone had no effect on BDNF expression. Moreover, at the level of intracellular signaling, short-term application of WIN-55,212-2 inhibited CRH-induced cAMP accumulation and CREB phosphorylation. Depolarization-induced BDNF synthesis was inhibited by long-term application of WIN-55,212-2 and was absent in cultures from CB_1-deficient animals. These data highlight a cross-talk between the CRH and the cannabinoid system in the regulation of BDNF expression by influencing different signaling pathways.

FUNCTIONAL PLASTICITY OF CANNABINOID RECEPTORS IN THE RAT PILOCARPINE MODEL OF ACQUIRED EPILEPSY

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Epilepsy affects 1-2% of the population, making it one of the most common neurological disorders. Cannabinoids have been shown to be anticonvulsant in a number of different animal seizure models (Karler and Turkanis **1981**). It has been shown recently in the mouse maximal electroshock model of seizure that the anticonvulsant nature of both exogenous and endogenous cannabinoids are mediated by the CB_1 receptor, the most abundant G-protein coupled receptor in brain (Wallace, Wiley et al. **2001**; Wallace, Martin et al. **2002**). Recently published work from our laboratory demonstrated an anticonvulsant effect of cannabinoids via a CB_1 receptor-dependent mechanism in the rat pilocarpine model of acquired epilepsy (Wallace, Blair et al. **2003**). In addition, epileptic animals displayed a significant increase in hippocampal CB_1 receptor protein expression as measured by Western blot analysis. Further evaluation of CB_1 receptor expression using immunohistochemical techniques revealed a selective increase in the synaptic regions of CA2-3 with a concomitant decrease in the molecular layer of the dentate gyrus. These findings demonstrate long-term CB_1 receptor plasticity in association with the pathophysiological state of epilepsy. However, it is not known whether this shift in receptor protein expression results in subsequent alterations of functional receptor G-protein coupling. To address this question, we utilized [^{35}S]GTPγS autoradiography, a well-accepted technique for measuring G-protein activation in brain sections (Sim, Selley et al. **1995**).

Approximately one year after the induction of epilepsy, WIN55,212-2 stimulated [^{35}S]GTPγS autoradiography was carried out on both epileptic and naïve control animals. Analysis revealed that the net stimulated [^{35}S]GTPγS binding in the whole hippocampus was significantly greater in epileptic animals ($n=7$-10 per group, $p<0.05$). In addition, it was shown that net stimulated [^{35}S]GTPγS binding of the dentate gyrus molecular layer significantly decreased with epilepsy, while binding in the stratum radiatum of CA2-CA3 increased. These results indicate that the shift in CB_1 receptor expression is functional, resulting in an altered pattern of G-protein activation throughout the hippocampus. The plasticity of the CB_1 receptor during epilepsy may play a crucial role in the anticonvulsant nature of cannabinoids.

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THERAPEUTIC POTENTIAL OF UCM707, AN INHIBITOR OF THE ENDOCANNABINOID TRANSPORT, IN ANIMAL MODELS OF VARIOUS NEUROLOGICAL DISEASES

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To date, UCM707, N-(3-furylmethyl)eicosa-5,8,11,14-tetraenamide, has the highest potency and selectivity in vitro (López-Rodríguez et al., J. Med. Chem. 46 : 1512-1522, **2003**) and in vivo (de Lago et al., Eur. J. Pharmacol. 449 : 99-103, **2002**) as inhibitor of the endocannabinoid transport, which might enable this compound to potentiate endocannabinoid transmission, with minimal side-effects, in the treatment of several neurological disorders. In the present study, we wanted to examine whether the treatment with UCM707 produced beneficial effects in several rat models of neurological diseases such as Huntington's disease (HD), Parkinson's disease (PD) and multiple sclerosis (MS). UCM707 exhibited a notable antihyperkinetic activity in the rat model of HD generated by intrastriatal application of 3-nitropropionic acid, while it also provided a certain neuroprotective activity in rats with striatal injury generated by unilateral application of malonate, another animal model of HD. By contrast, UCM707 did not provide neuroprotection in rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by 6-hydroxydopamine, a rat model of PD, possibly because UCM707 is devoid of antioxidant properties, and it was also unable to reduce the neurological decline of rats with experimental autoimmune encephalomielitis, a rat model of MS, possibly because it lacks of direct affinity for the vanilloid VR1 receptors. In summary, UCM707 might be a promising compound in HD to alleviate motor symptoms as well as to delay neurodegeneration, which represent important goals considering the lack of efficient pharmacological treatments in this basal ganglia disorder.

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MECHANISMS OF CANNABINOID RECEPTOR-DEPENDENT PROTECTION AGAINST EXCITOTOXICITY

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Recently, we have shown that the endocannabinoid system provides physiological protection against excitotoxic insults in mice. We demonstrated that the endocannabinoid anandamide and CB_1 receptors expressed in glutamatergic neurones mediate the protection against excitotoxicity. Here, we extend these studies.

Using mutant mice, we show that another target of anandamide, the non-specific cation channel TRPV1 vanilloid receptor, is not involved in the mechanisms of kainic acid-induced behavioural seizures. Furthermore, conditional mutant mice lacking expression of CB_1 receptor in GABAergic cells are not different from their wild-type littermates in kainic acid-induced seizures indicating a prominent role of CB_1 receptors on glutamatergic neurons regarding the protection from excitotoxicity.

In a series of in vitro studies, we have evidenced a key role of the brain-derived neurotrophic factor (BDNF) in CB_1 receptor-dependent protection against excitotoxic insults. In organotypic hippocampal slice cultures, prepared from CB_1 knockout mice or wild-type mice treated with the CB_1 antagonist SR141716A, a significantly increased level of neuronal cell death was observed after kainic acid treatment as compared to control explants. Treatment of wild-type explants with SR141716A also inhibited kainic acid-induced increase of BDNF protein levels. Importantly, exogenous application of BDNF to explants treated with SR141716A largely alleviated excessive neuronal cell death induced by kainic acid.

Taken together, these results underline the importance of the cell-type specific activation of CB_1 and the dynamic regulation of cellular pathways in the cannabinoid receptor-dependent protection against excitotoxicity.

NEUROPROTECTIVE EFFECTS OF SR 141716 IN A MODEL OF TRANSIENT GLOBAL CEREBRAL ISCHEMIA IN MONGOLIAN GERBILS

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First described as a selective CB_1 receptor antagonist, SR 141716 has been shown to block the effects of the cannabinoid agonists in a competitive manner (Rinaldi-Carmona et al., FEBS Lett., **1994**, 350,240). However, when given alone, SR 141716 can produce in laboratory animals effects that are opposite to those shown by cannabinoid agonists: locomotor stimulation (Compton et al., J. Pharmacol. Exp. Ther., **1996**, 227, 586), enhancement of memory, EEG arousal (Santucci et al., Life Sci., **1996**, 58, 103), hyperalgesia (Richardson et al., Eur. J. Pharmacol., **1997**, 319, R3) and food intake decrease (Arnone et al., Psycopharmacolgy, **1997**, 132, 104; Colombo et al., Life Sci., **1998**, 63, PL113; Simiand et al., Behav. Pharmacol., **1998**, 9, 179). Recently, SR141716A, by itself, attenuated evoked [^{3}H] glutamate release from hippocampal synaptosomes of rats and CB_1-null mutant mice, suggesting a potential neuroprotective effect independent of the CB_1 receptor (Kofalvi et al., Eur. J. Neurosci. **2003**, 18,1973).

Since during ischemia a massive release of glutamate has been reported to be responsible for neuronal death (Hampson et al., Proc. Natl. Acad. Sci., USA, **1998**, 95,8268) and endocannabinoid system has been shown to exert neuroprotective functions in a model of excitotoxicity *in vivo* (Marsicano et al., Science **2003**, 302,84), the aim of the present work was to investigate the effect of SR 141716 on transient global cerebral ischemia in gerbils using a wide range of doses (0.05 - 3 mg/kg). Starting from 30 min to 7 days after recirculation, gerbils, previously submitted to bilateral carotid occlusion, were monitored for different parameters, known to be hardly influenced by cerebral ischemia: electroencephalographic (EEG) mean total spectral power, spontaneous motor activity, cognitive function, rectal temperature and hippocampal neuronal count. SR 141716 was given i.p. 5 min before carotid occlusion.

SR 141716 (0.25-0.5 mg/kg) completely reversed the ischemia-induced EEG flattening, evaluated 7 days after ischemia. The same doses antagonized ischemia-induced hyperlocomotion, tested 24 hours and memory impairment, tested 3 days after ischemia. Histological examination of CA1 hippocampal field revealed a significant survival of neurons in comparison to vehicle treated gerbils, 7 days after ischemia. Rectal temperature did not change during the first 2 hours after recirculation, except for a significant decrease with the highest doses (1-3 mg/kg). Preliminary results indicate that SR 141716, even if partially, protected against the ischemic injury also when given 30 or 60 min after recirculation.

Even if the mechanism of SR 141716 is still to be elucidated, the present findings demonstrate a potential therapeutic effect.

Acknowledgements: We thank Sanofi-Synthelabo for generous gift of SR 141716

CANNABINOIDS PROTECTS THE STRIATUM AGAINST MALONATE TOXICITY THROUGH A CB_2 RECEPTOR-MEDIATED MECHANISM

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Compelling evidence supports the hypothesis that cannabinoid-related compounds could provide neuroprotective effects in neurodegenerative disorders involving impairment in energy metabolism and secondary excitotoxicity. Here, we examined this hypothesis in a rat model of Huntington's disease (HD) produced by intrastriatal injection of the mitochondrial complex II inhibitor malonate. To this purpose, we tested the potential neuroprotective effects of the following compounds: (i) cannabidiol (CBD), a plant-derived cannabinoid with low affinity for cannabinoid receptors but having a considerable antioxidant capability, (ii) the CB_1 receptor agonist, arachidonyl-2-chloroethylamide (ACEA), (iii) and the CB_2 receptor agonist, HU308. The results showed that neither CBD nor ACEA modified neurochemical deficits induced by malonate injection. In marked contrast, HU308 did protect striatal projection neurons from malonate-induced death. It reduced the depletion of GABA concentrations produced by the neurotoxin in the striatum, globus pallidus and, to a lesser extent, in the substantia nigra pars reticulata. In line with this, HU308 treatment significantly reduced the malonate-induced loss of mRNA for neuronal-specific enolase in the striatum. This neuroprotective effect of HU308 was likely related to its effect on CB_2 receptors since co-administration of HU308 and the selective CB_2 receptor antagonist, SR144528, led to no significant protection. In addition, preliminary immunohistochemical studies suggest that CB_2 receptors, which were apparently absent of the non-lesioned caudate-putamen, seem to be induced in pathological conditions. Co-localisation studies suggested that CB_2 receptors were in glial cells. Our results demonstrate that stimulation of CB_2 receptors protects the striatum against malonate toxicity. The present findings also indicate that, surprisingly this effect may require an up-regulation of the expression of CB_2 receptors in glial cells. Altogether our results support the hypothesis that CB_2 receptors could be a therapeutic target to slow down neurodegeneration in HD (or other neurodegenerative diseases).

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INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN NEURONAL APOPTOSIS INDUCED BY HIV-1 COAT GLYCOPROTEIN GP120

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Human immunodeficiency virus type-1 coat glycoprotein gp120 causes delayed apoptosis in rat brain neocortex. Here, we investigated the possible role of the endocannabinoid system in this process. It is shown that gp120 causes a time-dependent increase in the activity and immunoreactivity of the anandamide (AEA)-hydrolyzing enzyme fatty acid amide hydrolase (FAAH), paralleled by increased activity of the AEA membrane transporter and decreased endogenous levels of AEA. The AEA-synthesizing phospholipase D and the AEA-binding receptors were not affected by gp120. None of the changes induced by gp120 in the cortex were induced by bovine serum albumin, nor were they observed in the hippocampus of the same animals. Also the activity of 5-lipoxygenase, which generates AEA derivatives able to inhibit FAAH, decreased down to ~25% of the control activity upon gp120 treatment, due to reduced protein level (~45%). In addition, the FAAH inhibitor methyl-arachidonoyl fluorophosphonate significantly reduced gp120-induced apoptosis in rat brain neocortex, whereas selective blockers of AEA membrane transporter or of AEA-binding receptors were ineffective. Taken together, these results suggest that gp120, by activating FAAH, decreases endogenous levels of AEA, and the latter effect seems instrumental in the execution of delayed neuronal apoptosis in the brain neocortex of rats.

THE ENDOCANNABINOID ANTAGONIST, SR141617A, AFFECTS NEUROLOGICAL AND COGNITIVE FUNCTION IN THIOACETAMIDE INDUCED HEPATIC FAILURE AND ENCEPHALOPATHY IN MICE

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Introduction: Hepatic encephalopathy (HE) involves functional changes in endogenous neurotransmitters, including GABA-ergic, opiodergic and serotonergic. Endocannabinoids are endogenous compounds found in the central nervous system that act as neuromodulators via CB_1 receptors. Endocannabinoids and their receptors are also found in the immune system, gut and vascular endothelium. Recent studies have demonstrated that endocannabinoids are involved in the hemodynamic derangement in chronic liver disease, acting via CB_1 receptors on vascular endothelium.
Aim: To assess whether endocannabinoids are involved in the encephalopathy of fulminant hepatic failure.
Methods: Three groups of mice 2 with fulminant hepatic failure (FHF) induced by intraperitoneal injection of the hepatotoxin thioacetamide (TAA) and control group were studied. Neurological functions were determined using a 14 point scale, based on reflexes, balance and motor activity. Determination of neurological score was performed daily beginning 24 hours after the initial injection of TAA. Activity and performance were determined using open field and eight arm maze respectivly. The TAA groups were treated with 1mg/kg intraperitoneal injection of SR141617A, an antagonist to the CB_1 cannabinoid receptor or vehicle. Animals were treated with subcutaneous injection of 5% dextrose to avoid hypoglycemia. Body temperature was maintained by intermittent exposure to infrared light.
Results: Treatment with SR141617A, resulted in significant improvement of neurological and cognitive function and reversed the decrease in activity index in mice with FHF. SR141617A treatment significantly improved survival when administered following induction of FHF.
Conclusions: Treatment with endocannabinoid antagonist improves neurological and cognitive functions and reversed activity index and survival in an animal model of FHF. These results suggest that endocannabinoids play a role in this clinical syndrome. As a neuromodulatory system they may contribute to the development of HE, either directly or by way of interaction with the opiodergic system in the brain. SR141617A may also counteracts the deleterious hemodynamic effects attributed to endocannabinoids in liver disease. SR141617A might be a possible treatment for the clinical syndrome.

THE EFFECT OF CB_1 RECEPTOR ANTAGONISTS ON HYPEREMIC RESPONSE INDUCED BY WHISKER STIMULATION

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It is known that increases in local neuronal activity are accompanied by increases in blood flow (hyperemic response) within the active region. Since both neurons and astrocytes release endocannabinoids and activation of the CB_1 cannabinoid receptor dilates cerebral arteries and increases cerebral blood flow, we hypothesized that endocannabinoids contribute to neurovascular coupling. Here, we examined the role of CB_1 receptors in hyperemic responses in the whisker-barrel cortex of the rat.

Male Sprague-Dawley rats (250-350g) were anesthetized with ketamine/xylazine (100/7 mg/kg, intramuscular; supplemented as required). The left femoral artery was cannulated for blood pressure recording. The animals breathed room air with body temperature maintained at 37^{o}C using a controlled heating pad. Local cerebral blood flow was measured by Laser Doppler Flowmetry (LDF). Briefly, a LD probe was positioned over an open cranial window (with intact dura) in the right whisker-barrel cortex (2mm caudal and 5mm lateral to bregma) and adjusted until a LDF response was elicited following contralateral whisker stimulation (lateral movement of approximately 5mm at 10Hz, on for 13s and off for 27s).

Control LDF responses were measured at the beginning of experiments. Test responses were obtained from the same rats at 15, 30, 45 and 60 min after subdural injection of a CB_1 receptor antagonist (SR141716 or AM251) or its vehicle. Whiskers were stimulated for 8-15 times at each data point. The magnitude of hyperemic responses was expressed as the area under curve of mean LDF responses during whisker stimulation (i.e. % change from baseline X unit time). Data are reported as mean±s.e.m.; n≥5 rats. Statistical analysis was performed by one-way repeated measures analysis of variance, followed by Dunnett's post-hoc tests.

Subdural injection of SR141716, at 1μM, attenuated hyperemic responses to whisker stimulation (control, 354±78s; 15min, 217±48s, P<0.05; 30min, 133±34s, P<0.01; 45min, 123±45s, P<0.01; 60min: 182±46s, P<0.01). Neither its vehicle nor 100nM SR141716 inhibited the responses. A second CB_1 receptor antagonist AM251 (1μM) also significantly reduced the hyperemic response (control, 317±48s; 15min, 222±46s; 30min, 186±44s, P<0.05; 45min, 258±43s; 60min: 200±24s, P<0.05). In addition, SR141716, AM251 (both at 1μM) or the vehicle had no significant effect on basal cerebral blood flow or arterial blood pressure at all the time points.

In conclusion, this study shows that local application of CB_1 receptor antagonists decreases the hyperemic response elicited by whisker stimulation. This is consistent with the hypothesis that activation of the CB_1 receptor, presumably by endocannabinoids, modulates the recruitment of blood flow during neuronal stimulation in the somatosensory cortex of the rat.

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COMPARATIVE STUDIES ON THE EFFECTS OF ANANDAMIDE AND 2-ARACHYDONOYL-GLYCEROL ON PROLCATIN HORMONE SECRETION IN CB_1 KO MICE

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The function of endocannabinoids in the regulation of reproduction was investigated in CB_1 receptor inactivated mice. The aim of the study was to compare the different effects of AEA and 2AG, if any, on the regulation of prolactin hormone (PRL) secretion. Homozygote $CB_1^{+/+}$ (wild type) and $CB_1^{-/-}$ (mutant) adult, male mice were used.

AEA and SR141716A (SR1) or 2AG and SR1 were injected in a dose of 0.01mg/kg i.p. (SR1 30 min prior to agonists) to both $CB_1^{+/+}$ and $CB_1^{-/-}$ male mice. The putative vanilloid type 1 (VR1) receptor antagonist, capsazepine (Cap) was also administered prior to the endocannabinoids. Serum PRL content was measured by RIA. Basal PRL level was lower in $CB_1^{-/-}$ then that of wild mice. Receptor agonist AEA significantly decreased serum PRL content in wild mice and a tendency of decrease was seen in KO mice, too. 2AG decreased PRL content in wild mice but had no significant effect on KO animals. SR1 was able to prevent the decreasing effect of 2AG and AEA in wild mice, but that of this later with a less extent. Cap decreased PRL levels in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, and had no effect on 2AG treated animals.

The difference between the effects of AEA and 2AG on PRL secretion suggests that VR1 receptors are also involved in the regulation of PRL release and shows that both endocannabinoids affect the regulation of PRL secretion.

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EFFECTS OF N-ARACHIDONOYLGLYCINE ON UTERINE CONTRACTIONS IN THE RAT

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The relationship between chronic pelvic pain, uterine contractility, and the chemical messengers that regulate uterine contractions and pain are not well understood. Anecdotal evidence suggests that cannabinoids have been a useful therapeutic for pelvic pain for centuries though the mechanism of action is unknown. Dimitrieva and Berkley (**2002**) reported that activation of cannabinoid receptors increases uterine motility. Last year at this meeting we showed that anandamide and N-Arachidonoylglycine (NAGly) are both present in the rat uterus and that the levels of each increase significantly on the morning of estrus compared to all other stages of the hormonal cycle. Additionally, the levels of NAGly were significantly higher than anandamide throughout the cycle and showed a more dramatic increase the morning of estrus. The morning of estrus is the period following ovulation and represents a time of dynamic change in uterine physiology. The uterus is quiescent the day prior to estrus (proestrus) with contractions commencing during the morning of estrus at a frequency 1-2/min declining during the day to a frequency of 0.5-1/min by the following morning (metestrus).

The aim of this study was to examine any role NAGly may have on the regulation of uterine contractions. Urethane anesthetized rats were catheterized in the femoral artery and a water-filled balloon attached to a catheter was inserted into the left uterine horn. The pressure exerted on the balloon measured via a pressure transducer monitored uterine contractions. All rats in the current study were in the stage of estrus and weighed 250-300g. Following a 1 h baseline period during which spontaneous contractions were recorded, vehicle (2.5% ethanol, 2.5% emulphor in saline) or 400ug NAGly was delivered via the femoral artery. The contractions were monitored and digitized for and additional 4 h following drug delivery. There were no differences in uterine contractions observed during the first two hours after injection, however, during the third and fourth hour, when contractions naturally decrease as metestrus approaches, animals treated with NAGly exhibited significantly elevated rates of contractions compared to vehicle controls. These data suggest that NAGly plays at role in regulating the rates of uterine contractions in the rat.

CHARACTERISATION OF THE ENDOCANNABINOID SYSTEM IN EARLY HUMAN PREGNANCY

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In recent years it has been demonstrated that high circulating levels of anandamide, resulting from low expression of its metabolising enzyme fatty acid amide hydrolase (FAAH), may contribute to spontaneous miscarriage, and poor outcome in women undergoing in-vitro fertilisation. The site of action of anandamide has not however been determined. Our aim was to characterise the endocannabinoid system in first trimester human placenta. In this study we have examined the expression of CB_1 and CB_2 receptors and FAAH in human first trimester placental tissues by RT-PCR and immunohistochemistry. We have further quantified the expression of FAAH by real time PCR.

This study was approved by the regional Ethics Committee. First trimester placentae were collected from elective termination of normal pregnancies with informed consent. Tissue was collected from 14 placenta aged between 9 weeks and 3 days and 13 weeks and 2 days of gestation as determined by ultrasound. cDNA from placental samples were subjected to PCR amplification using oligonucleotide primer sets corresponding to either FAAH or cannabinoid receptors. FAAH primer sets yielded PCR products of the expected size in 13 of the 14 first trimester placental samples. In contrast to FAAH, no CB_1 receptor mRNA was detected in these samples. The CB_2 receptor primer set yielded PCR products of the expected size in 10 of the 14 placentae tested. Quantitative differences in FAAH mRNA levels were examined utilising real-time PCR with results normalised to 18S rRNA expression. A range of FAAH expression levels were observed. Expression levels increase up to 11 weeks of gestational age, prior to decreasing again beyond this time point.

Strong FAAH immunoreactivity was localized to the villous cytotrophoblasts. Intense labelling was also observed in extravillous trophoblast columns. Weaker FAAH immunoreactivity was localized to the syncytiotrophoblast layer. A subset of cells within the placental villous stroma were strongly immunoreactive; these were subsequently identified as macrophages, by double-label immunofluorescence for FAAH and CD14. CB_2 positive labelling was only observed in a subset of cells within the villous stroma. These cells were identified as villous macrophages by double immunofluorescence labelling with antibodies to CB_2 and CD14.

Here we show that FAAH is expressed throughout the human first trimester placenta, and appears to be regulated during gestation with levels peaking at 11 weeks. CB_2 receptors were localised only to placental macrophages. Interestingly, the cannabinoid receptor CB_1 was not identified in first trimester placenta, despite having previously been shown to be present in placental tissues at term. These findings suggest that the placenta may form a barrier to maternal-fetal transfer of anandamide.

THE EFFECTS OF TETRAHYDROCANNABINOL (THC), THE PRIMARY PSYCHOACTIVE CANNABINOID IN MARIJUANA, ON *IN VITRO* HUMAN SPERM MOTILITY

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Marijuana use may impair male fertility by inhibition of sperm function imperative for fertilization. In sea urchins, THC has been found to affect sperm motility and reduce the sperm's ability to bind to the egg. The aim of this study was to assess the effects of THC on human sperm motility parameters *in vitro*. Semen samples (n=51) were obtained from men attending the Regional Fertility Centre. Sperm were separated into 45% and 90% fractions using a two-step discontinuous Percoll gradient. The prepared sperm were divided into two aliquots. One was incubated, at 37°C for 3h, with THC at 1.5 μgml^{-1} (for comparison with work performed using sea urchin), and the other was incubated in the absence of THC. Sperm motility was assessed by computer-assisted analysis (Hamilton Thorne IVOS; USA). THC caused a marked decrease (-23%, $P<0.001$) in percentage progressive motility in the 90% fraction. The 45% fraction showed a greater decrease (-45%, $P=0.01$) in progressive motility in the presence of THC compared to controls. A decrease in the ALH, (- 10%, $P=0.007$) was also observed in the 90% fraction in the presence of THC. In the 45% fraction, a 10% decrease in ALH was observed but this did not reach significance. None of the remaining motility parameters (VAP, VSL, VCL, BCF and linearity) was significantly altered by THC in either fraction. THC (at the concentration tested) leads to a decrease in the percentage and the quality of human sperm motility *in vitro.* This may subsequently have consequences for reproduction. Further dose dependent studies will be performed to determine if these effects persist at a range of concentrations from the therapeutic to the supraphysiological.

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EFFECTS OF THE CANNABINOID CB1 RECEPTOR ANTAGONIST, SR141716, ON PAIN-RELATED BEHAVIOUR AND NERVE DEMYELINATION IN RATS WITH CHRONIC CONSTRICTION INJURY

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Neuropathic pain has become a focus of major attention due to the clear need for the development of new agents able to give satisfactory therapeutic treatments. Cannabinoid receptor agonists significantly inhibit nociceptive responses in a large number of behavioural studies in animal models of chronic pain, even if their psychotropic effects have complicated the full assessment of their therapeutic potential. The selective CB_1 receptor antagonist, SR141716, possesses some intrinsic activities; recent study has shown that this antagonist prevented the ulcers induced by indomethacin with a mechanism CB_1-independent (Croci et al., Br. J. Pharmacol., 141, 115-122, **2003**) so showing anti-inflammatory activity. Thus we tested the effect of SR141716 on hyperalgesia induced by the chronic constriction injury of the sciatic nerve (CCI) in the rat, a model of neuropathic pain. CCI animals were orally treated with SR141716 (1, 3 and 10 mg/kg) once a day for one week starting from day 7 after surgery. Before the surgery, on day 7 and on day 14, thermal and mechanical paw withdrawal thresholds were measured in both hind paws. SR141716 relieved hyperalgesia associated with the peripheral neuropathy in a dose-dependent fashion. Consistent with this finding, we showed that: 1) the increase in plasma prostaglandinE2 (PGE2) levels found in CCI rats was reversed by SR141716 2) the overproduction of lipoperoxides and nitric oxide (NO) showed in CCI animals was abolished by the repeated treatment with the antagonist 3) the over-expression of both the inducible and neuronal isoforms of nitric oxide synthase in the sciatic nerve, which is responsible for the NO increase, was completely reversed by SR141716. Many evidence points to a role of cytokines and especially tumor necrosis factor alpha (TNFα), in the generation of hyperalgesia in neuropathic pain; it is also strongly involved in primary demyelination and axonal degeneration of sciatic nerve. Here we shown that a significant enhancement in TNFα occurred in the spinal cord of CCI rats and that SR141716 repeated treatment brought this level down to that found in sham animals. To ascertain whether the cannabinoid antagonist can affect the peripheral demyelination, which is associated to painful neuropathy, we performed an histological analysis on semi-thin sections of sciatic nerve stained with toluidine blue for quantification of myelinated fibers. On day 7 after the injury, we demonstrated a significant reduction of the mean density of intact myelinated fibers. This reduction was still present on day 14 in CCI animals treated with vehicle for 7 days, whereas it was less evident or no more detectable after treatment with SR141716, depending on the dose used. Taken together these findings suggest that SR141716 is not only effective in alleviating neuropathic pain, but also seems to influence the nerve regenerating processes.

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THE CB_1 RECEPTOR ANTAGONIST SR141716A ATTENUATES FEAR-CONDITIONED ANALGESIA IN RATS

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Fear-conditioned analgesia is an important survival response mediated by supra-spinal sites controlling nociception and aversion. Cannabinoid$_1$ (CB_1) receptors and their endogenous ligands play an important role in nociception and aversion, however, the role of the endocannabinoid system in fear-conditioned analgesia has not been investigated. This study investigated the effects of systemic administration of the CB_1 receptor antagonist SR141716A (1 mg/kg, i.p.) or vehicle (ethanol:cremophor:saline; 1:1:18) on fear-conditioned analgesia and conditioned aversion in male Lister-hooded rats (250-300 g). The fear-conditioning paradigm used was footshock paired with context (10 x 1 s footshocks, 0.4 mA, administered at 1 min intervals; non-footshocked controls were also included) and the formalin test (intra-plantar injection of 50 µl, 2.5 % formalin or 0.9% saline into the right hindpaw) was used to assess nociceptive behaviour 30-45 min post-formalin. Twenty-four hours after receiving footshock, rats exhibited reduced formalin-evoked nociceptive behaviour (Fig. 1a) and increased freezing (Fig. 1b) when tested in the footshock apparatus, compared with non-footshocked formalin-injected rats. Systemic administration of SR141716A immediately after intra-plantar injection of formalin attenuated fear-conditioned analgesia (Fig. 1a) and freezing (Fig. 1b) from 1-7 min. Importantly, SR141716A had no effect on formalin-evoked nociceptive behaviour in rats not receiving footshock. SR141716A had no effect on contextually-induced freezing during the first half of the test trial in rats receiving intra-plantar injection of saline. Administration of SR1417176A did, however, attenuate short-term extinction of contextually-induced freezing (Fig. 1b) and ultrasound emission (8.0 ± 7.8 s vs 165.4 ± 72.9 s; $P < 0.01$) in rats receiving intra-plantar saline compared with vehicle-treated saline controls.

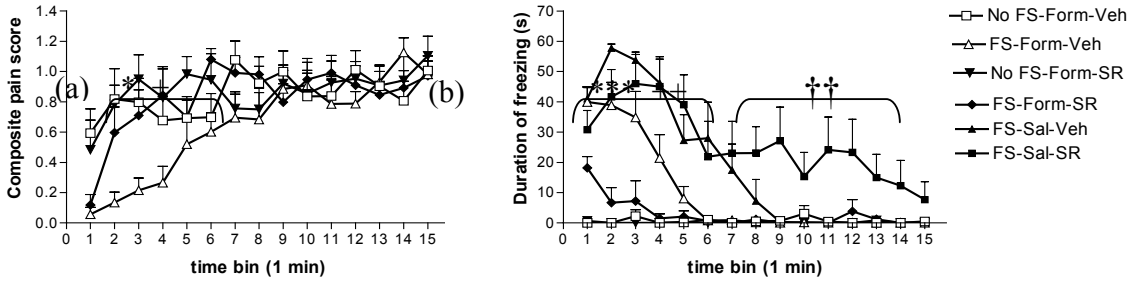

Figure 1 Effect of SR141716A on (a) fear-conditioned analgesia and (b) contextually-induced freezing in rats receiving intra-plantar formalin or saline. Data are means ± SEM (n = 6-7). $^{***}P<0.001$, $^{*}P < 0.05$ for FS-Form-Veh vs No FS-Form-Veh and $^{++}P < 0.01$, $^{+}P < 0.05$ for FS-Form-SR vs FS-Form-Veh from 1-7 min; $^{††}P < 0.01$ for FS-Sal-SR vs FS-Sal-Veh from 7-15 min (two-way ANOVA and Bonferroni post-hoc). FS (footshock); Form (formalin); Veh (vehicle); SR (SR141716A).

These data suggest an important role for the endocannabinoid system in mediating fear-conditioned analgesia. The data also provide evidence for differential modulation of conditioned aversive behaviour by the endocannabinoid system during tonic, persistent pain.

LACK OF CROSS-TOLERANCE TO THE ANTINOCICEPTIVE EFFECTS OF SYSTEMIC AND TOPICAL CANNABINOIDS IN MORPHINE TOLERANT MICE

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Opioid and cannabinoids produces antinociception through spinal, supraspinal and peripheral actions. Chronic administration of opioid and cannabinoids produces tolerance to their antinociceptive effects. Previous studies have demonstrated a functional interaction between cannabinoid and opioid systems in the development of opioid tolerance. To investigate the existence of cross antinociceptive tolerance to systemic and topical cannabinoids in systemic and topical morphine tolerant mice, we assessed systemic and topical antinociceptive effects of WIN 55, 212-2, a mixed CB_1 and CB_2 receptor agonist in mice made tolerant to morphine by s.c. implantation of morphine pellet and repeated topical administration of morphine, using radiant tail-flick test. Intraperitoneal and topical administered WIN 55, 212-2 produce dose dependent antinociception. However, mice rendered tolerant to the systemic morphine did not exhibit cross-tolerance to the antinociceptive effects of intraperitoneal and topical administered WIN 55, 212-2. Daily repeated topical administration of morphine produces tolerance within 3 days. However, mice rendered tolerant to the topical morphine did not exhibit cross-tolerance to the antinociceptive effects of topical WIN 55, 212-2. This observation implies that systemic and topical cannabinoid and opioid produces antinociception completely independent with each other.

INVOLVEMENT OF SPINAL 5-HT7 RECEPTORS IN THE SYSTEMIC CANNABINOID-ANALGESIA

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The antinociceptive effects of systemic administered cannabinoid are attenuated following spinal transection suggesting an important role for descending system from supraspinal to spinal sites in the systemic cannabinoid antinociception. It is well established that bulbospinal serotoninergic pathways performs a pivotal role in nociceptive information processing in the spinal cord. Currently, there are seven families of serotonin (5-HT) receptors. One of the most recently identified subtypes of 5-HT receptor is the 5-HT7 receptor. Suprisingly, there have been no attempts to directly examine the role of serotonergic system and spinal populations of 5-HT7 receptor in nociceptive processing. The present study was undertaken to examine the contribution of serotoninergic system and spinal 5-HT7 receptors in systemic cannabinoid antinociception in mice. The radiant heat tail-flick and hot plate tests were used to assess nociception. SB-269970, the selective 5-HT7 receptor antagonist was given intrathecally (i.th.). Subcutaneous administration of WIN 55, 212-2, CB_1 and CB_2 receptor agonist (1, 3 and 10 mg/kg), produced a dose dependent antinociceptive effects in tail-flick and hot plate test. The pretreatment of mice with 320 mg/kg of p-cholorophenalanine, inhibitor of serotonin biosynthesis, produced a significant decrease in the antinociceptice activity of WIN 55, 212-2. While i.th. administered SB-269970 (10 μg) alone did not produce any prolongation in tail flick and hot plate basal latencies, pretreatment with i.th. SB-269970 with WIN 55, 212-2 totally blocked the antinociceptive effects of systemic WIN 55, 212-2 in tail-flick and hot plate test. The results of present study demonstrate that the serotonergic system involved in systemic antinociceptive effects of cannabinoid and spinal 5-HT 7 receptors may play an important role in the descending pathways from supraspinal sites to spinal cord in the antinociceptive effects of systemic cannabinoids.

EFFECT OF CHRONIC TREATMENT WITH WIN 55,212 E AM404 ON SEROTONIN RELEASE AND NOCICEPTIVE BEHAVIOUR IN NEUROPATHIC RAT

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Introduction

A large body of evidence shows analgesic properties of exogenous and endogenous cannabinoids. In particular, cannabinoids result effective in several neuropathic pain models. Neuropathic pain is characterized by hyperalgesia and allodynia and represents a condition of pain still difficult to cure in clinical settings. Tricyclic antidepressants relieve neuropathic pain activating central monoaminergic system. In the present report we investigate the effect of chronic treatment with WIN 55,212-2 (a CB_1 and CB_2 receptors agonist) and AM404 (an inhibitor of anadamide transporter) on dorsal raphe (DR) serotonin release in neuropathic rats. Simultaneously, termoceptive behaviour has been monitored.

Method

Male Wistar rats, with chronic constriction injury (CCI) by sciatic nerve ligature (Bennet and Xie, **1988**) received AM404 (10 mg/Kg s.c.) or WIN 55,212-2 (0.1 mg/Kg s.c.) for 7 days. Rats were implanted with concentric microdialytic probes into the DR. The day after surgery, probes were perfused with ACSF and then, after 1 hour of equilibration period, samples of perfusate were collected every half an hour. Serotonin concentration was determined using HPLC fitted with electrochemical detector and expressed as fmol/20µl Simultaneously, microdialyzed rats were monitored for termoceptive behaviour through the plantar test. Thermal threshold was expressed as paw withdrawal latency (PWL, sec).

Results

In naïve rats DR serotonin concentration and thermal threshold was 27 ± 6 fmol and 9.3 ± 1.8 sec, respectively. Sham rats did not show significant changes in extracellular serotonin and thermal threshold (29 ± 8 fmol and 10.5 ± 1.1 sec, respectively) with respect to naives. CCI rats showed a significant increase of extracellular 5-HT (85 ± 11 fmol) and a significant reduction of thermal threshold (3.3 ± 0.8 sec). 7 days treatment with WIN 55,212-2 significantly increased DR serotonin in the shams (146 ± 12 fmol), but not in CCI rats (24 ± 12 fmol). The same treatment did not change thermal threshold in sham rats (8.9 ± 1.2 sec) but reverted thermal hyperalgesia in CCI rats (9.3 ± 1.8 sec). In the same way, AM404 significantly increase DR serotonin in the sham rats (106 ± 18 fmol), without changing DR serotonin (30 ±18 fmol) in CCI animals. This same treatment did not change thermal threshold in the shams (9.3 ± 1.3 sec) but reverted thermal hyperalgesia in CCI rats (8.4 ± 1.6 sec).

Conclusions

These data show that cannabinoids revert thermal hyperalgesia in neuropathic pain. In this condition DR serotonergic pathway results enhanced. Chronic treatment with cannabinoids returns the normal serotonergic outflow and increases, per se, serotonin release without changing the physiological pain sensitivity. Cannabinoids increasing DR serotonin and reverting hyperalgesia in neuropathic pain, may act like tricyclic antidepressant representing potential tools for therapeutic treatment of chronic pain.

References

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DIFFERENTIAL EFFECTS OF ENDOGENOUS AND EXOGENOUS CANNABINOID AGONISTS ON ISOLATED MURINE SENSORY NEURONS

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Cannabinoids modulate nociceptive impulses of primary afferent neurons through cannabinoid-1 receptors (CB_1R). One mechanism by which cannabinoids may decrease neurotransmission is through inhibition of voltage-dependent calcium channels. Therefore the K^+-evoked increase in the concentration of free intracellular calcium $[Ca^{2+}]_i$, measured by microfluorimetry, was used as a bioassay of effects of cannabinoids on isolated, adult murine primary afferent neurons 40-48 h after dissociation of dorsal root ganglia (DRG). Consistent with our previous observations in rat (Khasabova et al., 2002), the basal level of $[Ca^{2+}]_i$ did not differ between small (cell area < 475 μm^2) and medium-size (cell area 475-850 μm^2) murine DRG neurons. A brief pulse (10 sec) with KCl (50 mM) evoked more than a six-fold increase over the basal level of $[Ca^{2+}]_i$ in small neurons and a smaller increase in medium-size neurons (30%; $p<0.001$). The response to KCl was constant for 3 tests at intervals of 5 min in each population of neurons. Superfusion with the CB_1R agonist ACEA (1μM, 10 min) inhibited the response to KCl in medium-size neurons by approximately 40% after 10 min superfusion but had no effect in small neurons. Although the synthetic CB_1R agonist had no effect on small neurons, the endogenous cannabinoid agonist anandamide (AEA, 10 μM) inhibited the K^+-evoked increase in $[Ca^{2+}]_i$ by 60% within 5 min of superfusion in this population, and the effect was maintained through 10 min. Immunochemical studies of dissociated DRG neurons showed intense CB_1R-immunoreactivity (ir) in soma of medium-size DRG neurons and lighter staining in processes. CB_1R-ir co-existed extensively with RT97, a marker for myelinated A-fiber neurons. CB_1R-ir was rare in small, RT97-negative neurons. These results indicate that rat and mouse are similar in the differential response of two populations of DRG neurons defined by size to a CB_1R agonist, the magnitude of the response, and the occurrence of CB_1R-ir. Together the findings provide evidence for direct inhibitory effects of cannabinoids on primary afferent neurons. The cellular basis of the inhibitory effect of the endocannabinoid in small neurons remains to be determined.

DIFFERENTIAL MODULATION OF SENSORY NEURONE EXCITABILITY BY ANANDAMIDE

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Cannabinoids, by diverse mechanisms, can modulate the excitability of sensory neurones, and this may be of benefit in the treatment of pain disorders [Ross, et al., (**2004**) Current Neuropharmacology. 2, 59-73]. Using cultured dorsal root ganglion (DRG) neurones, we have previously shown dual effects of anandamide (AEA) on Ca^{2+} influx evoked by depolarisation with extracellular medium containing 30 mM KCl. AEA (1 μM) increased Ca^{2+} influx in one sub-population of DRG neurones and decreased Ca^{2+} influx in another population [Evans, et al., (**2004**) Br. J. Pharmacol., **In Press**]. The inhibitory responses to AEA were sensitive to pertussis toxin pre-treatment. In contrast the increases in Ca^{2+} influx evoked by AEA persisted after pertussis toxin pre-treatment. Studies on voltage-activated Ca^{2+} currents showed that AEA only produced inhibitory actions, suggesting that other conductances might be involved in the enhanced Ca^{2+} influx produced by AEA. In this study we have used cultured DRG neurones from 2-day old rats, the whole cell patch clamp recording method and fura-2 Ca^{2+} imaging to investigate the possible modulation of K^+ conductances by AEA. AEA (1 μM; with 0.01 % DMSO) inhibited voltage-activated K^+ currents evoked at +30 mV by 24 ± 6 % (n=7; $P<0.03$) and partial recovery was seen 10-30 minutes after removing the drug perfusion pipette from the bath. Similar results (24 ± 6 % inhibition; n=5) were seen when the neurones were bathed with Ca^{2+}-free solution, and when the neurones were pretreated for 16-24 hrs with pertussis toxin (500 ng/ml). AEA also reduced the multiple firing behavior of a sub-population (~20 %) of DRG neurones and caused a slowing of the repolarising phase of the action potential. Consistent with these results, pre-treating DRG neurones with 1μM AEA slowed the recovery of Ca^{2+} transients evoked by 60 mM KCl and this action was not altered by pertussis toxin. However, pre-treating neurones with the fatty acid amide hydrolase (FAAH) inhibitor phenylmethylsulfonyl fluoride (PMSF; 10 μM) did attenuate this effect of AEA indicating that AEA metabolism may play a critical role in the modulation of K^+ conductances and K^+-evoked Ca^{2+} transients in DRG neurones.

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MECHANISMS UNDERLYING HYPERALGESIA BY *N*-ARACHIDONOYL DOPAMINE (NADA)

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N-Arachidonoyl dopamine (NADA) is an endogenous molecule that is found in brain areas such as the stratum, hippocampus and brainstem, and is also present in the peripheral nervous system such as the dorsal root ganglion. It binds to CB_1 receptors with an affinity similar to those observed for other endocannabinoids, and is also one of the most potent endovanilloids in its ability to activate TRPV1 receptors. NADA administered peripherally induces behavioral hyperalgesia. Here we further characterize the effects of NADA via electrophysiological techniques. *In vivo* extracellular single-cell recording of nociceptive neurons in the spinal cord was conducted in urethane-anesthetized rats. Injection of NADA into the receptive field (in the hindpaw) increased the spontaneous and heat-evoked firing of spinal nociceptive neurons. The dose-response relationship was characterized, and the effects of pharmacological blockade by TRPV1 and CB_1 receptor antagonists were examined. The results show that the presence of NADA in the periphery activates nociceptive neurons in the classical spinal pain pathways which likely underlie the behavioral hyperalgesia and hypersensitivity.

PRS-211,375, A CB2 SELECTIVE CANNABINOID DEMONSTRATES ANALGESIC ACTIVITY IN A NEUROPATHIC PAIN MODEL

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Cannabinoid agonists reduce different types of pain sensation, by activating cannabinoid receptors CB_1 predominantly found in the central nervous system (CNS) and peripheral cannabinoid receptors CB_2 located mainly on cells of the immune system. Pharmos has developed a synthetic cannabinoid PRS-211,375 which is a selective CB_2 receptor agonist. The analgesic activity of PRS-211,375 was tested in the Bennet and Xie model for neuropathic pain and in taxol-induced peripheral neuropathy. The right sciatic nerve was loosely tied with 4 knots of 4-0 cat gut suture material. Two weeks later after verification of the establishment of neuropathic pain, PRS-211,375 was administered IP or SC at doses of 1, 5 or 10 mg/kg, vehicle or morphine 5 mg/kg serving as controls. One hour later the pain threshold was determined by testing thermal and mechanical allodynia. PRS-211,375 increased the pain threshold in a dose-related manner in all the tested parameters and was already significantly active at 5 mg/kg. The highest tested dose of 10 mg/kg had an analgesic effect similar to morphine, but in contrast to the non specific systemic effect of morphine PRS-211,375 selectively targeted the injured leg. Moreover, PRS-211,375 is expected to be safer than morphine avoiding side effects such as tolerance. Similar results were obtained in the taxol-induced neuropathy study.

There is a long unmet need for treatment of neuropathic pain, the existing medications being not fully satisfactory. The results of our studies indicate that PRS-211,375, a CB_2 selective agonist, may be a candidate for the treatment of neuropathic pain, with implications in conditions as various as diabetes, cancer, post-herpetic neuralgia and severe back pains.

SELECTIVE ACTIVATION OF CANNABINOID CB_2 RECEPTORS SUPPRESSES BEHAVIORAL SENSITIZATION EVOKED BY INTRADERMAL CAPSAICIN

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The present studies were conducted to test the hypothesis that activation of peripheral cannabinoid CB_2 receptors would suppress behavioral sensitization to thermal and mechanical stimulation evoked by intradermal administration of capsaicin, the pungent ingredient in hot chili peppers. The site of action was also identified using site-specific administration of the CB_2-selective cannabinoid agonist AM1241. Systemic administration of AM1241 (33, 330 μg/kg i.p.) suppressed the development of behavioral sensitization to thermal and mechanical stimulation in the capsaicin model of persistent nociception. AM1241 also produced a dose-dependent suppression of capsaicin-evoked nocifensive behavior, defined as licking and lifting of the capsaicin-injected paw. The actions of AM1241 were completely blocked by the CB_2 antagonist SR144528 but not by the CB_1 antagonist SR141716A. Local administration of AM1241 (33 μg/kg i.pl.) in the capsaicin-injected paw increased the latency for paw withdrawal to thermal stimulation and increased the frequency of paw withdrawal to mechanical stimulation. These effects are consistent with the ability of local injections of AM1241 to raise the threshold for paw withdrawal to punctuate mechanical stimulation. AM1241 suppressed capsaicin-evoked thermal and mechanical hyperalgesia and raised mechanical thresholds following local administration to the capsaicin-treated (ipsilateral) paw. The same dose was inactive following administration to the capsaicin-untreated (contralateral) paw. Our data indicate that AM1241 suppresses capsaicin-evoked behavioral sensitization to both thermal and mechanical stimulation through a local site of action. These data provide further evidence that actions at cannabinoid CB_2 receptors are sufficient to normalize nociceptive thresholds and produce antinociception in persistent pain states (Nackley et al., **2003**, Neurosci., 119, 747; Quartilho et al. **2003**, Anesthes.**,** 99**,** 955). Our data are also consistent with the results of electrophysiological studies demonstrating a CB_2-mediated suppression of C-fiber-evoked afterdischarge firing and windup induced by AM1241 in the absence and presence of inflammation.

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CANNABINOID CB_2 RECEPTOR ACTIVATION IN THE PERIPHERY INHIBITS MECHANICALLY EVOKED RESPONSES OF SPINAL NEURONES IN A RAT MODEL ON NEUROPATHIC PAIN

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Cannabinoid CB_2 receptor activation produces anti-nociception in the absence of psychoactive effects (Malan *et al.,* **2001**, Pain, 93, 239). Systemic administration of CB_2 receptor agonist AM1241 reduces tactile allodynia and thermal hyperalgesia in a model of neuropathic pain (Ibrahim *et al.*, **2003**, PNAS, 100, 10259). The aim of the present study was to investigate the effects of peripheral CB_2 receptor activation on cutaneous somatosensory processing in the spinal nerve ligation (SNL) model of neuropathic pain. Here we compare the effects of intraplantar injection of the selective CB_2 receptor agonist JWH-133 on mechanically-evoked responses of spinal neurones in sham-operated and neuropathic rats.

Tight ligation of L5-L6 spinal nerves was performed in male Sprague-Dawley rats. A control group of rats received sham surgery. 14-17 days post surgery, extracellular single-unit recordings of ipsilateral deep (laminae V–VI) dorsal horn neurones were made in isoflurane anaesthetised SNL and sham-operated rats. Innocuous (6, 8, 12 g) and noxious (21, 45, 80 g) mechanical stimuli were applied to the peripheral hindpaw and evoked responses of spinal neurones (spikes/s) were recorded. The effects of intraplantar injection of JWH-133 (5 μg or 15 μg in 50 μl) or vehicle (< 3% ethanol in distilled water with Tween 80) on mechanically-evoked responses of spinal neurones were recorded at 10 min intervals for 60 min. The ability of selective CB_2 antagonist SR144528 to block the effects of JWH-133 was also studied. SR144528 (10 μg in 50 μl, intraplantar) was injected 30 min before JWH-133 (15 μg in 50 μl, intraplantar). Data were analysed with Mann-Whitney test, $P < 0.05$.

Mechanical stimulation of the hindpaw receptive field produced graded stimulus-intensity dependent increase in neuronal firing in SNL and sham-operated rats. Intraplantar injection of the lower dose of JWH-133 (5 μg) in sham-operated rats significantly ($P < 0.05$) inhibited innocuous (6, 8, 12 g)- evoked responses of spinal neurones, but not noxious (21, 45, 80 g)-evoked responses of spinal neurones, compared to vehicle. Intraplantar injection of the lower dose of JWH-133 (5 μg) in SNL rats significantly ($P < 0.05$) inhibited noxious (21, 45, 80 g)-evoked responses of spinal neurones, but not innocuous (6, 8, 12 g)-evoked responses of spinal neurones, compared to vehicle. The higher dose of JWH-133 (15 μg) significantly inhibited both innocuous and noxious mechanically-evoked responses of spinal neurones in sham-operated and SNL rats, compared to vehicle. Intraplantar injection of SR144528 (10 μg) alone did not produce marked changes in mechanically-evoked responses of spinal neurones in SNL or sham-operated rats. Intraplantar pre-administration of SR144528 (10 μg) attenuated JWH-133 (15 μg)-mediated inhibition of mechanically-evoked responses of spinal neurones in both sham-operated and SNL rats.

These data demonstrate that local peripheral activation of CB_2 receptors produces marked inhibitions of mechanically-evoked responses of spinal neurones in a model of neuropathic pain. Importantly CB_2 receptor activation attenuated both innocuous and noxious mechanically-evoked responses, suggesting that the reported behavioural effects of CB_2 receptor activation on mechanical allodynia in neuropathic rats may be mediated by a local peripheral site of action. These inhibitory effects of CB_2 receptor activation in neuropathic and sham-operated rats may be mediated via an indirect inhibitory effect on immune responses (Facci *et al*., **1995**, PNAS, 92, 3376) and / or a putative direct site of action on peripheral nerves (Patel *et al*., **2003**, Brit J Pharmacol, 140, 261).

EFFECTS OF SPINAL ADMINISTRATION OF JWH-133 ON MECHANICALLY-EVOKED RESPONSES OF DORSAL HORN NEURONES IN NEUROPATHIC RATS

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Selective cannabinoid CB_2 receptor agonists have been shown to have antinociceptive effects in animal models of neuropathic and inflammatory pain, in the absence of CNS side-effects associated with CB_1 receptor agonists (Malan *et al.,* **2001**, Pain, 93, 293). CB_2 receptor mRNA is upregulated in spinal cord of neuropathic rats (Zhang *et al.,* **2003**, Eur J Neurosci. 17, 2750).

In the present study effects of spinal application of the selective CB_2 receptor agonist JWH-133 on mechanically evoked responses of dorsal horn neurones in neuropathic rats and sham operated rats were studied.

Tight ligation of spinal nerves L5 and L6 was performed in male Sprague-Dawley rats. A control group of rats received sham surgery. In isoflurane anaesthetised rats, extracellular single unit recordings of deep convergent dorsal horn neurones (Laminae V and VI) were made 14-17 days post surgery. Peripheral mechanically-evoked responses of neurones were measured (bending forces 8-100g) and effects of spinal administration (8, 78, 156 and 468ng/50µl) of JWH-133, or vehicle (0.005-0.3% ethanol in distilled water) on evoked responses were studied at 10 minute intervals for 60 minutes.

Mechanical stimulation of the peripheral receptive field of neurones produced a graded stimulus-intensity-dependant increase in responses of spinal neurones, in both neuropathic and sham-operated rats.

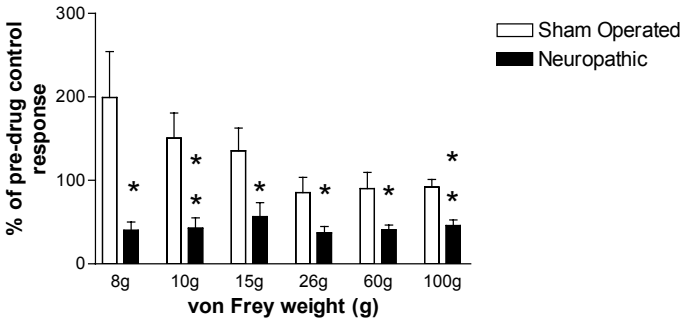

Fig 1. Effects of JWH-133 on mechanically-evoked responses of dorsal horn neurones in neuropathic (n=6) and sham-operated rats (n=6). Data are expressed as mean ± SEM. Statistical comparisons between neuropathic and sham rats performed using a Mann Whitney test. *p<0.05, **p<0.01

Spinal administration of lower doses of JWH-133 (8 – 78ng/50µl) attenuated mechanically-evoked responses of dorsal horn neurones compared to pre-drug controls, however these did not reach significance compared to effects in sham-operated rats.

Spinal administration of higher doses of JWH-133 (156 - 468ng/50µl) significantly attenuated mechanically-evoked responses of neurones in neuropathic rats, compared to pre-drug controls in SNL rats and JWH-133mediated effects in sham operated rats (Fig.1). The mean maximal inhibitory effects of JWH-133 were observed between 30 - 40 minutes post drug administration.

These data suggest that functional CB_2 receptors are present in the spinal cord of neuropathic rats, and that activation of these receptors attenuates innocuous and noxious mechanically-evoked responses of spinal neurones in neuropathic, but not sham-operated rats.

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ELEVATED LEVEL OF THE CHEMOKINE RANTES/CCL5 IN THE BRAIN DESENSITIZES THE ANALGESIS EFFECT OF THE CANNABINOID RECEPTOR AGONIST WIN 55, 212-2

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Cannabinoid and chemokine receptors are members of the G-protein-linked seven-transmembrane receptor family. These receptors, as well as the chemokine and cannabinoid ligands, are widely distributed in brain and periphery. Recently, we have demonstrated a heterologous desensitization of all 3 opioid receptor types by certain chemokines. The purpose of the present study is to investigate whether this interaction exists between cannabinoids and chemokines. In the present experiments, the chemokine Regulated on Activation Normal T cell Expressed and Secreted (RANTES/CCL5) was tested for its effect on nociception as well as for its possible interaction with WIN 55,212-2 induced analgesia. The cold-water tail-flick test was used to assess the analgesic effect of WIN 55,212-2 in male SD rats (250-300 g). A sterilized stainless steel C313G cannula guide (22 gauge, Plastics One Inc., Roanoke) was placed just above the periaqueductal gray (PAG). RANTES was microinjected into the PAG in a dose of 50, 100 or 200 ng, and tail-flick latency was then measured for 60 min after injection. RANTES (50-200 ng) evokes a dose-dependent hyperalgesia. RANTES (100 ng/µl), microinjected directly into the PAG 30 min before injection of WIN 55,212-2, reduced significantly the WIN 55,212-2 (1 mg/kg, i.m.)-induced antinociception (approximately 50 %). The present results show that RANTES/CCL5 microinjected directly into the PAG is able to lower the threshold to the perception of pain, and desensitizes the analgesic effect of the cannabinoid receptor agonist WIN 55,212-2, just as it desensitized the analgesic effect of opioid agonists.

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ENDOGENOUS CANNABINOIDS ACT AT CB_1 AND CB_2 RECEPTORS TO INHIBIT EMESIS IN THE FERRET

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Emesis is an upper gastrointestinal reflex regulated in the dorsal vagal complex of the brainstem. Previously, we have shown cannabinoid 1 receptor (CB_1r) and fatty acid amide hydrolase (FAAH) immunoreactivity in the neural sites involved in emesis and localised the anti-emetic effect of delta-9-tetrahydrocannabinol to the brainstem. The aim of the present study was to test whether the endogenous cannabinoid system functions to inhibit emesis induced by morphine 6 glucuronide (M6G, 0.05mg/kg) in the ferret. The endocannabinoid anandamide (2mg/kg) eliminated retching and vomiting induced by M6G alone (6.3 ± 0.6 episodes). This effect was reversed by the selective CB_1r antagonists SR141716 / AM251 (5mg/kg), but unaffected by the CB_2r selective antagonist AM630 (5mg/kg). 2-arachidonoyl glycerol (1mg/kg) also significantly reduced episodes of retching and vomiting by 86.3 ± 5.9% and this effect was reversed by both SR141716 and AM630. The endocannabinoid transport inhibitor, VDM11 (2mg/kg), reduced episodes of retching and vomiting by 65.1 ± 13.0%. The robust anti-emetic effect of VDM11 was reversed by both SR141716 and AM630. The FAAH inhibitor, URB597 (5mg/kg), reduced the number of episodes of retching induced by M6G by 48.4 ± 7.6%. The selective CB_2r agonists, JWH133 (1 and 5mg/kg) and AM1241 (1mg/kg), were also tested as anti-emetics. JWH133 did not inhibit emesis, but AM1241 reduced the number of episodes of retching by 31.7 ± 4.8%. These results show that endocannabinoids do not act solely at CB_1r to reduce emesis in the ferret. The CB_2r or another cannabinoid receptor may be mediating the anti-emetic effect of cannabinoids.

THE ENDOGENOUS CANNABINOID SYSTEM PROTECTS AGAINST COLONIC INFLAMMATION

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Excessive inflammatory responses can emerge as potential danger for organisms' health. Physiological balance between pro- and anti-inflammatory processes constitutes an important feature of responses against harmful events. Here, we show that CB_1 cannabinoid receptors mediate intrinsic protective signals counteracting pro-inflammatory responses. Both intrarectal infusion of dinitrobenzene sulfonic acid (DNBS) and oral administration of dextrane sulfate sodium (DSS) induced stronger inflammation in CB_1-deficient mice ($CB_1^{-/-}$) than in wild-type littermates ($CB_1^{+/+}$). Treatment of wild-type mice with the specific CB_1 antagonist SR141716A mimicked the phenotype of $CB_1^{-/-}$, showing an acute requirement of CB_1 receptors to protect from inflammation. Consistently, treatment with the cannabinoid receptor agonist HU210 or genetic ablation of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) resulted in protection against DNBS-induced colitis. Electrophysiological recordings from circular smooth muscle cells, performed 8 hours after DNBS-treatment, revealed spontaneous oscillatory action potentials in $CB_1^{-/-}$ but not in $CB_1^{+/+}$ colons, indicating an early CB_1-mediated control of inflammation-induced irritation of smooth muscle cells. DNBS-treatment increased the percentage of myenteric neurons expressing CB_1 receptors, suggesting an enhancement of cannabinoid signalling during colitis. Our results indicate that the endogenous cannabinoid system represents a promising therapeutic target for the treatment of intestinal disease conditions characterised by excessive inflammatory responses.

CANNABINOIDS INHIBIT CONTRACTILE RESPONSES TO ELECTRICAL FIELD STIMULATION IN RAT PROSTATE

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Cannabinoids produce alterations on reproductive organs (Gérard et al., Biochem J, **1991**, 279,129; Wenger et al., Life Sciences, **1999**, 65, 695). In the mouse and rat vas deferens cannabinoid CB_1 receptors, located on peripheral nerve endings, have been described. In the mouse vas deferens, cannabinoids inhibit contractile responses to to electrical field stimulation (Pertwee et al., Eur J Pharmacol, **1996**, 296,169; Christopoulus et al., Br J Pharmacol, **2001**, 132, 1281). In contrast, no effects in rat vas deferens have been reported (Lay et al., Eur J Pharmacol, **2000**, 391,151). Expression of functionally CB_1 receptors have been described in human prostate and in prostate human cells (Ruiz-Lorente et al., Prostate, **2003**, 54(2), 95). This study tested functional effects of two cannabinoid receptor agonists, methanandamide and WIN 55,212-2, on contractile responses to electric stimulation in rat prostate, compared with guanethidine, an adrenergic neuron blocking drug. Prostatic slices of male Wistar rats were cutted and mounted in organ baths (under a tension of 0.5 g) containing 5 ml of Krebs-Henseleit, maintained at 37ºC and bubbled with 95% O_2 and 5% CO_2 gas mixture Tissues were stimulated by single field pulses (70V, 0.2ms, 15Hz/1 min/5 min) delivered every 10 minutes. Isometric tension was recorded in a Power Macintosh (MacLab/4e program) through force-displacement transducers Grass FT07. Responses of to trains of electrical-field stimulation in the absence and presence of drugs were determined. Cumulative concentration-response curves of cannabinoids were constructed. We have evaluated the changes produced by methanandamide and WIN 55,212-2 (0.01 μM to 100 μM/10min), compared with guanethidine 10 μM. Only one agonist was used in each experiment. Results are expressed in % of contraction inhibition. The field stimulation induced contractions of the prostatic smooth muscle were markedly attenuated by guanethidine (75.5 ± 4.78%) and with a less potency by methanandamide and WIN 55,212-2, with the last concentration used (100 μM) (16 ± 1.64% and 23 ± 3.22% respectively). Very few data exist about the functional response of the prostate. Our data shows that cannabinoids has a dose-related inhibitory effect on the contractile response to electric-stimulation in the rat prostate, probably mediated through prejunctional cannabinoid CB_1 receptors, previously described in this tissue, then the effect of cannabinoids could be mediated through an inhibition of sympathetic noradrenergic pathways.

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VASORELAXANT EFFECTS OF 2-ARACHIDONYL GLYCEROL AND NOLADIN ETHER

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It is well established that the endocannabinoid anandamide is a hypotensive agent evoking vasodilatation in different vascular regions. Both 2-arachidonyl glycerol (2-AG), another endocannabinoid, and noladin ether, a metabolically stable analogue of 2-AG and itself a putative endocannabinoid, also produce hypotension. However, it is unclear if these compounds are also vasodilators. This study reports the relaxant effects of 2-AG and noladin ether in rat isolated mesenteric and coronary arteries.

Male Wistar rats (300-400g) were killed with sodium pentobarbital (120mg/kg, i.p.). The left anterior descending coronary, or small (third order) mesenteric, artery (2mm long) was mounted in a wire myograph in oxygenated Krebs-Henseleit solution with 10μM indomethacin. Arteries were precontracted with either 10μM methoxamine or 60mM KCl (mesenteric), or 3μM 5-HT (coronary). Endothelium (EC) was removed when required by rubbing the intima with a hair. $pEC_{50\%}$ is the negative logarithm of the concentration giving 50% relaxation. Data are given as mean±s.e.m; $n \geq 3$ rats. Statistical testing was by analysis of variance or Student's *t*-test.

In the rat mesenteric artery, EC removal inhibited 2-AG relaxation (+EC, $pEC_{50\%}$= 5.9±0.1; -EC, $pEC_{50\%}$=5.5±0.2, $P<0.05$). In EC-intact vessels, capsaicin (10μM) pretreatment caused a rightward shift of the 2-AG concentration-relaxation curve (control, $pEC_{50\%}$=5.9±0.1; capsaicin, $pEC_{50\%}$=5.4±0.1, $P<0.01$). Neither SR141716A (3μM; CB_1 receptor antagonist) nor SR144528 (1μM; CB_2 receptor antagonist) had any significant effect (relaxation to 10μM 2-AG: +EC; control, 89±3%; SR141716A, 90±3%; SR144528, 86±6%). Arachidonyl trifluromethyl ketone (ATFMK; 10μM), a fatty acid amide hydrolase (FAAH) inhibitor, reduced relaxation to 10μM 2-AG (+EC control, 88±2%; ATFMK, 59±5%, $P<0.01$: -EC control, 66±8%; ATFMK, 27±12%, $P<0.05$) but another FAAH inhibitor phenylmethylsulphonyl fluoride (PMSF; 200μM) had no effect (+EC control, 86±4%; PMSF, 91±3%). Noladin ether also caused EC-dependent relaxation of mesenteric arteries (relaxation to 10μM noladin ether: +EC, 65±11%; -EC, 26±7%, $P<0.05$). Precontraction of vessels with 60mM KCl significantly reduced responses to noladin ether (methoxamine, 52±14%; KCl, -2±2%, $P<0.05$) but not those to 2-AG (methoxamine, 82±5%; KCl, 29±26%). In the rat coronary artery, 2-AG and noladin ether only produced modest relaxant responses (responses at 30μM +EC: 2-AG, 40±13%; noladin ether, 41±14%).

Hence, 2-AG and noladin ether relax the rat small mesenteric artery by mechanisms which are unclear and, in the case of 2-AG, different from those of anandamide in their sensitivity to cannabinoid antagonists. Comparison of their effects indicates that mesenteric relaxation to 2-AG might be mediated in part by vasoactive metabolites. In contrast, in the rat coronary artery, the similarity of their responses suggests relaxation to 2-AG is independent of its degradation.

ANANDAMIDE VASORELAXATION IN RAT AORTA: METABOLISM, RECEPTORS AND L-ARGININE/NITRIC OXIDE PATHWAY

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Anandamide caused vasorelaxation mediated an unidentified endothelial mechanism. Wagner *et al* (Hypertension, **1999**, 33, 4529) proposed that anandamide acted on a receptor sensitive to SR141716A but not the CB_1 receptor: "anandamide receptor". Later, Offertaler and colleagues described that abnormal cannabidiol is a selective agonist of the endothelial "anandamide receptor" which is distinct from CB_1 and CB_2 receptors and is coupled through Gi/G_o to the PI_3 kinase/Akt signalling pathway (Mol Pharmacol, **2003**, 63, 699). Recently, Zoratti *et al* have shown that anandamide acts as a powerful stimulus for endothelial cells *vía* CB_2 receptor (Br J Pharmacol, **2003**, 140, 1351). The aim of this study was contribute to clarify and identify the precise mechanism by which anandamide causes vasorelaxation in rat aorta.

Aorta rings from male Wistar rats (250-300g) were mounted in a tissue bath containing Krebs-Henseleit buffer at 37ºC and bubbled with 95% O_2 and 5% CO_2. Isometric force-desplacement transducer for tension measurements were used. After an equilibration period of 90 min. (resting tension of 2 g), the arteries were precontracted with phenylephrine (Phe, 10^{-6} M) and anandamide concentration-response curves (10^{-9} M – 10^{-4} M) were carried out. The presence of endothelium, the contribution of CB_1 and CB_2 receptors (using SR141716A and SR144528), the implication of L-Arg/NO pathway (using L-NAME) and the metabolism of anandamide (using PMSF) were evaluated. Results are expressed as % of relaxation of Phe-induced tone. Data are given as the mean ± s.e.m (8-12 rings). A two-way ANOVA (Bonferroni/Dunn *post-hoc* test) was used (* $P \leq 0.05$).

Treatment	Anandamide (10^{-4}M)
Control	56.63 ± 9.67
Without endothelium	20.43 ± 3.1 *
Pertussis Toxin 300 mg/ml	25.63 ± 4.30 *
SR141716A 1µM,15min	43.07 ± 6.12
SR141716A 6µM, 15 min	41.28 ± 5.90
SR144528 1µM,15min	44.18 ± 5.89
SR144528 6µM,15min	33.58 ± 3.11 *
L-NAME 100 µM, 30min	13.29 ± 2.71 *
PMSF 200µM, 30 min	34.06 ± 4.28 *

Our results show that more than one receptor (CB_2 or other receptor distinct of CB_1 and CB_2?) are involved in the endothelium-dependent vasorelaxation produced by anandamide in rat aorta rings. Besides, it seems to be necessary its metabolism for exert the vasodilatory effect. Anandamide activates L-Arg/NO pathway to provoke vasodilation.

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COULD THE VEHICLE USED MODIFY THE VASORELAXATION CAUSED BY ANANDAMIDE?

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Studies in isolated blood vessels have described a vasodilator action of anandamide, however the precise mechanisms by which this cannabinoid produces vasodilation are not well-known and they seem complex and controversial. Previous studies about vascular actions of anandamide show different, non-reproducible and opposite results (Kunos et al., Chem. Phys. Lipids **2000**, 108, 159; Hogestatt & Zygmunt, Prostaglandins Leukot. Essent. Fatty Acids, **2002**, 66, 343). Among these studies, one of the most frequent differences is the use of distinct solvents that could modify vascular function that explain the controversial results described. The aim of this study was compare the vasorelaxation produced by anandamide dissolved in different vehicles commonly used in cannabinoid research.

Aorta rings from male Wistar rats (250-300g body weight) were used. The experiments were carried out in arteries with and without endothelium. After 90 min equilibration, arteries were precontracted with phenylephrine (Phe) 1μM. When a Phe stable tone was established, cumulative concentration-response curves of anandamide (10^{-9}M – 10^{-4}M) were constructed. The vehicles used were: Tween 80:ethanol at two different concentrations 1.3 ml/l and 0.65 ml/l, DMSO 0.5% and 1:1:18 (Tween 80:ethanol:saline). At the end of each concentration-response curve, 10μM of carbachol was added to verify the existence (>70%) or not (<10%) of functional endothelium. A two-way ANOVA (Bonferroni/Dunn *post-hoc* test) was used (*$P \leq 0.05$).

In intact arteries, anandamide produced a maximal vasorelaxation that was similar whatever it was the vehicle used (Tween 80 1.3ml/l: 43.91±6.43; Tween 80 0.65ml/l: 35.19±7.17; DMSO 0.5%: 56.63±9.67; 1:1:18: 40.11±14.06). By contrast, in denuded arteries significant differences existed in the maximal vasodilation produced by anandamide among the different vehicles used (Tween 80 1.3ml/l: 48.96±7.57 $P<0.05$ *vs* DMSO 0.5%; Tween 80 0.65ml/l: 61.23±5.21 $P<0.05$ *vs* DMSO 0.5%; DMSO 0.5%: 20.43±3.1; 1:1:18: 46.22±18.33 $P<0.05$ *vs* DMSO 0.5%). When the vasodilation produced by anandamide dissolved in the same vehicle were compared in arteries with and without endothelium, the results obtained were contradictory. While Tween 80 1.3ml/l and 0.65ml/l and 1:1:18 suggested an endothelium-independent component in the vasorelaxation, DMSO 0.5% only indicated an endothelium-dependent component in the vasorelaxation. Besides, it must be pointed out that all the vehicles used except DMSO 0.5% provoked a partial or total inhibition of carbachol-mediated relaxation in intact rat aorta rings (for 10μM of carbachol: Tween 80 1.3ml/l: 60.65±5.46; Tween 80 0.65ml/l: 69.93±6.40; DMSO 0.5%: 91.70±6.72; 1:1:18: -0.20±0.88). Our results conclude that the vasodilatory effect of the anandamide could be modified by the vehicle used to dissolve it. This could justify the different results described in the literature.

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VASCULAR EFFECTS OF Δ^9-TETRAHYDROCANNABINOL (THC) I: ANTAGONISM OF THE VASORELAXANT EFFECTS OF THE ENDOCANNABINOID ANANDAMIDE

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Individually, both THC and anandamide can cause vasorelaxation in rat mesenteric vessels. However, the interactions between endogenous and exogenous cannabinoid compounds are still largely unknown, and some evidence suggests that THC may act as an antagonist in certain situations (Bayewitch *et al*., J. Biol. Chem. **1996**, 271, 9902; Petitet *et al.*, Life Sci. **1998**, 63, PL1). In this study, the effects of THC on the vasorelaxant effects of anandamide have been examined.

Male Wistar rats (200-300 g) were killed by cervical dislocation. Small mesenteric resistance arteries (G3, 100-400 μm internal diameter) were isolated, cut into 2 mm lengths and mounted on a Mulvany-Halpern myograph. Vessels were bathed in oxygenated Krebs' solution at 37^{o}C. Vessels were set to a baseline tone of 0.5 g, and following equilibration, U46619 (10-300 nM) was added to increase tension by at least 0.5 g. The effects of the cannabinoids were assessed as concentration-response curves.

THC caused significant rightward shifts in the concentration response curves to anandamide in G3 vessels at 1 μM (control pEC_{50} 6.43 ± 0.22 cf 5.09 ± 0.57, P<0.05, ANOVA) and 10 μM (pEC_{50} 4.49 ± 0.20, P<0.01). THC (10 μM) did not affect responses to a non-cannabinoid vasorelaxant, verapamil (control pEC_{50} 7.01 ± 0.12; verapamil and THC 10 μM pEC_{50} 7.22 ± 0.09). The vasorelaxant responses to anandamide were not affected after exposing vessels to anandamide (1 μM) for 1 hour (anandamide pre-treatment pEC_{50} 6.64 ± 0.20). THC (10 μM) did not inhibit the actions of anandamide in the superior mesenteric artery (G0; control R_{max} 31.4 ± 5.2 % relaxation, pEC_{50} 5.39 ± 0.29; and THC 10 μM R_{max} 28.5 ± 5.5 % relaxation, pEC_{50} 5.44 ± 0.34). THC (10 μM) did not affect the concentration-response curve to the vanilloid receptor agonist capsaicin (capsaicin pEC_{50} 5.37 ± 0.11; and THC 10 μM pEC_{50} 5.45 ± 0.08), or the CB_1 receptor agonist, CP 55,940 (CP 55,940 pEC_{50} 5.86 ± 0.09; and THC 10 μM pEC_{50} 5.77 ± 0.18). When THC (10 μM) was applied in combination with endothelial-denudation in G3 vessels, THC inhibited the vasorelaxant effects of anandamide further than was seen with denudation alone (control pEC_{50} 6.43 ± 0.22; denuded vessels pEC_{50} 5.28 ± 0.29, P<0.05; denuded vessels and THC 10 μM pEC_{50} 4.25 ± 0.59, P<0.01). Anandamide did not have antagonist effects on vasorelaxation to THC (control R_{max} 70.8 ± 4.5 % relaxation, pEC_{50} 5.21 ± 0.13; and anandamide 10 μM R_{max} 76.2 ± 7.0 % relaxation, pEC_{50} 5.35 ± 0.20).

These data show that THC antagonises the vasorelaxant effects of anandamide. This does not appear to be through actions at the CB_1 receptor, the vanilloid receptor, or an endothelial target, and is confined to small resistance (G3) mesenteric vessels. This may implicate another unidentified site of action for cannabinoids in the vasculature.

VASCULAR EFFECTS OF Δ^9-TETRAHYDROCANNABINOL (THC) II: MECHANISMS OF VASORELAXATION

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Early research showed that THC produced prostanoid-mediated vasorelaxation of cerebral vessels (Ellis *et al*., Am. J. Physiol. **1995**, 269, H1859), but subsequent studies have shown a vasoconstrictor effect in the perfused mesenteric bed (Wagner *et al*., Hypertens. **1999**, 33, 429) and relaxant effect in the superior mesenteric artery (Fleming *et al*., Br. J. Pharmacol. **1999**, 126, 949). More recently Zygmunt *et al*. (J. Neurosci. **2002** 22, 4720) found that THC relaxed arteries entirely through sensory nerve activation. Since our work on the antagonist effects of THC on vasorelaxation to anandamide did not identify a site of action for THC, we have further investigated the mechanisms of vasorelaxation to THC.

Male Wistar rats (200-300 g) were killed by cervical dislocation. Small mesenteric resistance arteries (G3) or the superior mesenteric artery (G0) were isolated, cut into 2 mm lengths and mounted on a Mulvany-Halpern myograph. Vessels were bathed in oxygenated Krebs' solution at 37^{o}C. Vessels were set to a baseline tone of 0.5 g, and following equilibration, U46619 (10-300 nM) was added to increase tension by at least 0.5 g. The effects of THC were assessed as concentration-response curves.

THC caused vasorelaxation of G3 vessels which was less potent than that produced by anandamide (anandamide R_{max} 95.0 ± 10.8 % relaxation, pEC_{50} 6.43 ± 0.22; THC R_{max} 66.8 ± 7.6 % relaxation, pEC_{50} 5.31 ± 0.20, P<0.05). Anandamide also caused vasorelaxation in the superior mesenteric artery (R_{max} 31.4 ± 5.2 % relaxation, pEC_{50} 5.39 ± 0.29), but THC had no affect on the pre-constricted vessels up to 10 μM, and by 100 μM, produced an additional 11.9 ± 6.4 % contraction. Vasorelaxation to THC in G3 vessels was not inhibited by the CB_1 receptor antagonist AM251 (100 nM, R_{max} 56.7 ± 4.6 % relaxation, pEC_{50} 5.97 ± 0.19), pre-treatment with the vanilloid receptor agonist capsaicin (10 μM, R_{max} 76.1 ± 4.1 % relaxation, pEC_{50} 5.51 ± 0.12), removal of the endothelium (R_{max} 57.6 ± 3.6 % relaxation, pEC_{50} 5.16 ± 0.12) or by the anandamide transport inhibitor VDM11 (10 μM, R_{max} 68.8 ± 4.2 % relaxation, pEC_{50} 5.22 ± 0.12). Vasorelation to THC was inhibited by pertussis toxin (10 μM, R_{max} 34.9 ± 4.7 % relaxation) and ruthenium red (10 μM, R_{max} 49.5 ± 6.8 % relaxation). In the presence of indomethacin, G0 vessels showed enhanced vasorelaxant responses to THC in the lower concentrations ranges. The vasoconstrictor response to THC in G0 vessels was reversed by de-endothelialisation resulting in vasorelaxation (control –11.9 ± 6.4 % relaxation; de-endothelialisation 11.5 ± 2.2 % relaxation, P<0.05), and was antagonised by SR141716A (100 nM, control pEC_{50} 4.01 ± 0.90; SR141716A pEC_{50} 2.87 ± 0.90).

It is clear from these data that THC does not cause vasorelaxation through the same mechanisms as have been identified for anandamide, but act via an as yet unidentified site(s) of action. The heterogeneous vascular effects between arterial vessel types may help reconcile previous conflicting findings of both constrictor and relaxant effects of THC.

INITIAL CHARACTERIZATION OF THE CB2 PROMOTER REGION

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The cannabinoid receptor system has many physiological roles including altering appetite, locomotion, pain perception, inflammation, and vasodilation. The primary cannabinoid receptor of the immune system is CB_2, and it is found on T cells, macrophages, and B cells. CB_2 mRNA has a cycle of expression in macrophages going through various stages of an immune response, from low in resident cells, to higher levels in responsive cells, and back to low expression in activated macrophages. Therefore, understanding the regulation of CB_2 expression is important for determining when immune cells are receptive to cannabinoid ligand modulation.

To characterize the genetic regulation of CB_2, we have acquired a portion of mouse chromosome four containing exons 1-3, with exon 3 containing all protein coding information. We are currently scanning this piece of genomic DNA to define possible transcription factor binding sites via *in silico* searches. To elucidate the activity of the CB_2 promoter, we have made various deletion constructs of three putative promoter sites in a luciferase reporter system. In RAW264.7 macrophage-like cells, a 707-bp promoter construct upstream of exon 1 had activity of 3-fold over the negative control, while an 839-bp promoter construct upstream of exon 2 had activity 138-fold over the negative control. In addition, we are characterizing the 5'-end of the mouse CB_2 mRNA with a modified 5'-RACE (rapid amplification of cDNA ends) technique using RNA isolated from murine spleen and thymus, as well as RNA isolated from transformed cell lines. Future experiments will include creating constructs upstream of the third CB_2 exon and extending the luciferase experiments into other cell lines including another macrophage-like cell line (J774A.1) and a transformed T cell line (Jukat). Our goal is to define the promoter elements responsible for CB_2 expression in the immune cells.

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MACROPHAGES LACKING THE CB_2 RECEPTOR DISPLAY AN ALTERED MORPHOLOGY WHEN CHALLENGED WITH BACTERIA

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Macrophages are key effector cells involved in both innate and acquired immunity. One of the principle functions of macrophages in innate immunity is to engulf foreign particles, including microbes and cellular debris. Phagocytosis is a vital defense mechanism that helps protect the body from disease. Following macrophage contact with foreign bodies, a cascade of events is triggered in the immune system that is responsible for recruiting other cells involved in inflammation and humoral immunity. Cannabinoids have repeatedly been shown to alter overall macrophage function. Several investigators have reported a suppression of phagocytic activity by macrophages in the presence of Δ^9-tetrahydrocannabinol (Δ^9-THC) (Lopez-Cepero, et al., **1986** and Tang, et al., **1992**). Numerous findings also indicate a modification in the secretion of cytokines associated with both cell mediated immune responses and humoral immune responses. The lack of the CB_1 receptor, along with correspondingly high levels of the CB_2 receptor in macrophages, suggests a CB_2 receptor mediated immune response. Through the use of a unique mouse model that is CB_2 receptor deficient, we have investigated and reported alterations in cytokine secretion by macrophages lacking the CB_2 receptor, tumor necrosis factor alpha in particular. Furthermore, we are currently investigating whether phagocytic activity of macrophages is compromised in the presence of cannabinoids by using the CB_2 receptor knockout system. In order to examine the direct effect of macrophage phagocytosis of fluorescent bacteria, thioglycollate elicited peritoneal macrophages in wild type and CB_2 knockout mice were collected and treated with cannabinoids (anandamide, Δ^9-THC, and WIN55212-2), in vitro. The activated macrophages were allowed to adhere to glass coverslips, incubated with cannabinoids, and then challenged with the foreign particles. The coverslips were then mounted for visualization through fluorescent microscopy. Preliminary results suggest a variation in morphology in knockout macrophages as compared to wild type macrophages, especially in the presence of foreign particles. This finding may suggest an overall modification in phagocytic activity by macrophages lacking the CB_2 receptor. Such a study is important in gaining further insight into cannabinoid-mediated effects on the ability of macrophages to function normally in immuno-regulatory responses.

THE EFFECTS OF ECHINACEA PURPUREA ON MOUSE IMMUNE CELLS LACKING THE CB_2 RECEPTOR

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Echinacea purpurea is a large, daisy-like purple coneflower that has been used as an herbal remedy by Native Americans to treat colds and flu. According to many studies, E. purpurea is known for its ability to modulate the immune system; however, its mechanism remains unknown. It has been shown to enhance macrophage and natural killer cell function. In addition, some studies have revealed that E. purpurea affects lymphocyte function, but these studies remain controversial. Many studies have revealed that E. purpurea increases cytokines such as interleukin 1 (IL-1), IL-6, IL-10, tumor necrosis factor alpha (TNFα), and interferon gamma (INFγ). We have found that cannabinoids and the CB_2 receptor are also involved in the modulation of cytokine production.

To investigate if E. purpurea and cannabinoids modulate cytokine production through a common pathway, we studied cytokine secretion in splenocytes and macrophages derived from wildtype and CB_2 knockout mice. Mice splenocytes were treated with E. purpurea root extracts in the presence of Con A, a T cell activator, and INFγ and IL-4 were measured by enzyme-linked immunoassay (ELISA). Mice macrophages were treated with E. purpurea in the presence and absence of lipopolysaccharide (LPS), an agent that activates macrophage activity, and TNFα and IL-10 secretion levels were analyzed using ELISA.

We found that E. purpurea increased Con A stimulated INFγ secretion from splenocytes in wildtype. Con A-induced IL-4 secretion levels were variable in wildtype. E. purpurea stimulated TNFα secretion in wild-type and CB_2 knockout macrophages in the absence of LPS stimulation, but not in the presence of LPS. There was a significant difference in LPS-induced IL-10 production when treated with E. purpurea in both wildtype and CB2 knockout. Our findings show that E. purpurea increases secretion of the antiviral cytokine INFγ and the endogenous pyrogen TNFα, and that the CB_2 receptor is not involved in this pathway.

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THE CB_2 RECEPTOR SELECTIVE AGONIST JWH133 REDUCES COMPOUND 48/80-INDUCED PLASMA EXTRAVASATION *IN VIVO*

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In **1996**, Mazzari *et al*. (Eur J Pharmacol 300 [**1996**] 227-236) showed that orally administered palmitoylethanolamide, PEA, decreased substance P-induced plasma extravasation in mouse ear pinna and reduced carrageenan-induced hyperalgesia, carrageenan-formalin- and dextran-induced edema in both rat and mouse. In **1998**, Calignano *et al.* (Nature 394 [**1998**] 277-281) demonstrated that the effects of PEA against inflammatory pain could be reversed by the CB_2 antagonist / inverse agonist SR144528, but not by the CB_1 antagonist / inverse agonist SR141716A (rimonabant). Interestingly, however, PEA does not, at physiological concentrations, bind to CB_2 receptors (Lambert *et al.* Biochim. Biophys Acta 1440 [**1999**] 266-274). One explanation could be that PEA exerts its effects by interfering with the inactivation of other endocannabinoids, for example anandamide. Based on this, we wanted to examine the effects of palmitoylisopropylamide, PIA, a PEA-analogue known to interfere with AEA metabolism (Jonsson *et al.* Br. J. Pharmacol. 133 [**2001**] 1263-1275) and to have anti-inflammatory properties *in vivo* (Benvenuti *et al.*, Boll. Soc. Ital. Biol. Sper. 44 [**1968**] 809-13) and compare it with JWH133, a CB_2 receptor agonist, in an *in vivo* model of mast cell-dependent inflammation. The method by Mazzari *et al*. (ibid) was used with some modifications. Mice were injected i.p. with PIA, PEA, JWH133 or vehicle 30 min before a subcutaneous injection (2 µl) in each ear pinna with 100 µg compound 48/80, a mast cell degranulator, or water (control ear). SR144528 was injected i.p. 45 min before PEA, JWH133 or vehicle. Immediately after the ear injection, mice were given 2 mg Evans blue intravenously and killed after 2 h. After extraction of the ears in formamide, optical absorbance was measured at 620 nm. Compound 48/80 significantly induced plasma extravasation compared with ears injected with water, although there was a great variability between animals. In the first set of experiments JWH133 significantly reduced plasma extravasation at the concentration of 20 and 200 µg/animal, compared with control. In contrary, PIA did not affect plasma extravasation at any concentration tested (20, 200 or 600 µg/animal). In a following set of experiments SR144528 was tested against the effects of JWH133 and PEA. It was not possible, however, to interpret these data in terms of CB_2 mediated effects since the large variability in response between animals, together with the apparent tendency ($P<0.095$) of the SR144528 per se to reduce the response to compound 48/80, resulted in a lack of significant effect of JHW133 (200 µg/animal) and PEA (200 µg/animal). Preliminary experiments with a commercially available CB_2 receptor antibody gave immunoreactivity in the epidermis, rather than in the dermis. Although the immunoreactivity may not correctly identify CB_2 receptors, a possible explanation of our data is that the effect of JWH133 upon mast cell degranulation is either indirect, or non-CB_2 receptor mediated, or both. However, the compound did not prevent degranulation of RBL-2H3 rat basophilic leukaemia cells cultured on fibronectin in response to antigen stimulation. In conclusion, the present data suggest that the CB_2 agonist JWH133 can reduce the inflammatory response produced by mast cell degranulation *in vivo*, an effect that is not seen with PIA.

TEMPORAL VARIATION IN CB_2 RECEPTOR LEVELS IN RESTING VS ACTIVATED HUMAN PERIPHERAL BLOOD-DERIVED T LYMPHOCYTES

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Human lymphocytes express cannabinoid (CB) receptors and endogenous cannabinoids are thought to play a role as immune modulators. To examine the presence of CB_2 receptors in T cells, activation experiments were performed using different stimuli e.g., staphylococcal enterotoxin-B (SEB), phytohaemagglutinin (PHA) and anti-CD3/CD28. Peripheral blood mononuclear cells (PBMC) from normal, healthy human donors were variously activated and clonally expanded for up to 14 days with IL-2. Aliquots of T blasts were removed daily and whole cell lysates were made. Changes in the CB_2 receptor levels were detected by Western analysis. CB_2 protein levels increased after SEB activation and were maintained during the first week after IL-2 stimulation. However, CB_2 levels decreased after 7-10 days and remained weak for up to 14 days. Strong CB_2 expression was induced after anti-CD3/CD28 activation, which was maintained up to 14 days. T lymphocytes purified from human PBMCs, did not express CB_2 receptors at the protein level. We show that differences in responsiveness of activated T cells to endogenous vs synthetic cannabinoids, as measured by proliferation assays, appear to correlate with CB_2 receptor levels. Our results demonstrate that the CB_2 receptor expression is differentially increased upon cellular activation and that this modulation is dependent on the stimulus used.

EFFECT OF CANNABINOIDS ON IL-8-INDUCED NEUTROPHIL MIGRATION

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Post-ischemic inflammation is a contributing factor in the pathogenesis of stroke. As a result of an ischemic event, TNF-α and other pro-inflammatory cytokines are released from microglia. TNF-α acting on brain vascular endothelial cells stimulates IL-8 release which attracts circulating neutrophils from the blood stream across the blood-brain barrier into the brain. Accumulation of neutrophils in the penumbra contributes to the inflammatory process in the brain and causes swelling and release of cytotoxic contents from the neutrophils. Inhibition of neutrophil transmigration across the blood-brain barrier can thus be a therapeutic target in the treatment of stroke. We wanted to investigate possible effects of cannabinoids on IL-8-induced neutrophil migration since cannabinoids have been shown to have a positive effect in experimental stroke. Neutrophils were isolated from venous blood by MidiMacs separation using a CD15 antibody. Isolated neutrophils were labeled with Calcein AM and allowed to migrate against a IL-8 concentration in a 96-well ChemoTx chamber. Migration increased significantly with increasing IL-8 concentration, serum concentration and time. When suitable conditions had been established, the cells were allowed to migrate for one hour against 1 nM IL-8 in a cell culture incubator after which the amount of migrated cells were analysed by measuring the fluorescence intensity in the lower chamber using a Fluostar Galaxy spectrofluorometer. These conditions allow approximately 60% (± 10%) of the added neutrophils to migrate to the lower chamber. Fortyfive minutes prior to the start of the migration, the neutrophils were incubated with several different cannabinoids and related compounds. The endocannabinoid AEA and the homologues PEA and OEA as well as the synthetic cannabinoids CP 55,940, HU 210 and WIN 55,212-2 were tested in concentrations from 1 nM to 1 μM. Both CP 55,940 and WIN 55,212-2 produced a significant increase in IL-8 induced neutrophil migration, however, with no apparent dose-response relationship. None of the endocannabinoids tested produced any significant difference compared to vehicle treated cells. Thapsigargin, used as a positive control for inhibition of migration, produced a significant, albeit modest, decrease at 100 nM and 1 μM as compared to vehicle alone. Previously it has been shown that NSAIDs can inhibit TNF-α stimulated neutrophil transmigration across a monolayer of endothelial cells. For this reason we tested, in addition to cannabinoids, three different NSAIDs in this assay. None of the substances tested, diclofenac, ibuprofen and indomethacin, produced any significant difference in neutrophil migration compared to control in concentrations up to 10 μM. These results might imply that the effect seen on transmigrating neutrophils may be on the level of chemokine release from the endothelial cells and not a direct effect on the neutrophils.

CANNABINOIDS MODULATE THE IMMUNOLOGICAL ACTIVATION OF GUINEA-PIG MAST CELLS. THE ROLE OF NITRIC OXIDE AND EICOSANOIDS

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Introduction

The endogenous cannabinoid 2-arachidonyl glycerol (2AG) may exert receptor-mediated actions on the immune system through the CB_2 receptor. In guinea pig mast cells (MCs) we have shown that the release of histamine by antigen (ATG) was dose-dependently decreased by 2AG and CP 55,940, a synthetic CB receptor ligand, and antagonized by SR144528, a CB_2 receptor antagonist. However, L-NAME partially abrogated the inhibitory effect of 2AG and CP 55,940. Here we report on the interaction between cannabinoids, nitric oxide (NO) and prostaglandins on the modulation of the ATG-induced activation of guinea pig MCs.

Methods

Isolated purified MCs from actively sensitized guinea pigs were challenged in vitro with ATG in the absence and in the presence of the drugs under study. The experiments were carried out in the presence of the inhibitor of fatty acid amide hydrolase, PMSF (10uM). Histamine release and intracellular calcium were measured fluorimetrically; changes in cGMP levels and PgE_2 production were evaluated with EIA; the generation of NO was evaluated by the Griess reaction; the expression of iNOS and COX2 protein was evaluated by Western blot analysis.

Results

2AG and CP 55,940 concentration-dependently (1nM-1uM) decreased the ATG-induced histamine release. The inhibition induced by 2AG and by CP55,940 was partially reverted by L-NAME (10uM). Consistently, both 2AG and CP 55,940 generated NO from MCs and induced the expression of iNOS protein. The COX1/COX2 inhibitor indomethacin (10uM) and the COX2 inhibitor rofecoxib (10uM) also reverted the inhibitory action of both 2AG and CP 55,940, which, in turn, generated PgE_2 from MCs and induced the expression of COX2 protein. Both 2AG and CP 55,940 increased intracellular cGMP levels.

Conclusion

The data are consistent with the participation of NO and prostaglandins in the inhibitory effect of cannabinoids on the immunologic response of guinea pig MCs.

NO PROCOAGULATORY EFFECTS OF IN VIVO EXPOSURE TO Δ^9-TETRAHYDROCANNABINOL (THC) ON HUMAN PLATELETS

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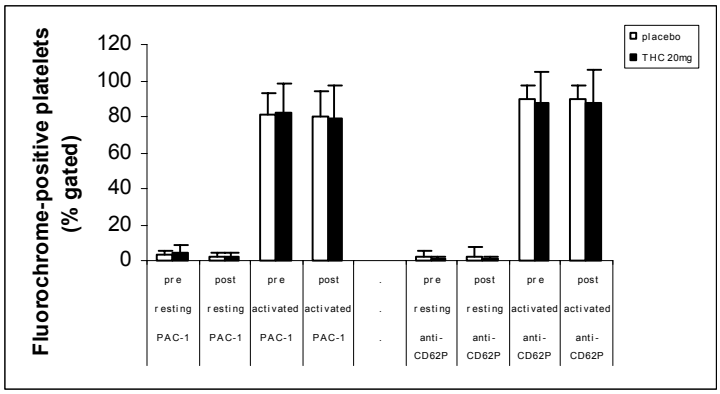
Objective: In vitro incubation of blood with Δ^9-tetrahydrocannabinol (THC) at 0.4 to 4 ng/ml has been shown to activate platelets[1]. The aim of the present study was to evaluate the effects of THC on platelets in vivo.

Design: Prospective, randomized, cross-over, double-blinded, placebo-controlled volunteer study.

Methods: Blood from 17 healthy female volunteers taking no drugs within 14 days before and during the study other than the blinded study medication was investigated. Blood was withdrawn before and 2 hours after oral intake of the standardized study medication (Cannabis extract, containing the standardized amount of 20mg of THC or active placebo, containing 5 mg of diazepam) administered in a randomized sequence. The expression of activated platelet fibrinogen receptor (glycoprotein IIb-IIIa) and P-selectin were determined on non-stimulated and on agonist-activated platelets using thrombin receptor activator peptide 6 (2 μM) by whole blood flow cytometry using the monoclonal antibodies PAC-1 and anti-CD62P (Becton Dickinson)[2].

Main Results: THC exposure during the study period had no statistically significant effect on the expression of glycoprotein IIb-IIIa and P-selectin on both resting and agonist-activated human platelets. There was no correlation between platelet reactivity and THC plasma levels (ranging from 1.05 to 7.92 ng/ml).

Conclusion: A single oral dose of cannabis extract containing 20 mg THC had no procoagulatory effects on platelets from healthy volunteers.



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ENDOCANNABINOIDS INTERFERE WITH DENDRITIC CELLS'LIFE CYCLE BY PREVENTING THE GENERATION OF MATURE DC

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Monocytes are circulating precursors of Dendritic Cells (DC), professional antigen presenting cells with a unique ability in priming innate and specific immune response. Recent findings report that endogenous cannabinoid system is present in human DC and can be regulated by cellular activation. We observed that: (i) anandamide reduces the number of immature DC generated from human CD14+ monocytes; (ii) anandamide interferes with activation/differentiation signals induced by LPS in immature DCs by reducing the release of pro-inflammatory cytokines and their terminal differentiation; (iii) these effects are not prevented by CB and VR antagonists. These observations provide novel evidence on the ability of endocannabinoids to modulate the immune response by interfering with DC lifecycle.

AJULEMIC ACID, A NON-PSYCHOACTIVE CANNABINOID ACID, DOWNREGULATES ACTIVATION of HUMAN SYNOVIAL CELLS

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Ajulemic acid (AJA), a synthetic non-psychoactive cannabinoid, prevents joint tissue damage in rats with adjuvant arthritis. Our observation that AJA suppresses IL-1β production in human peripheral blood and synovial fluid monocytes may help explain the protective effects seen in the animal model. In an effort to better understand mechanisms whereby AJA exerts its anti inflammatory action, we investigated its influence on prostaglandin levels (PG), production of other inflammatory cytokines, and regulation of cell activation and growth in human fibroblast-like synovial cells (FLS).

Methods: FLS were isolated from tissue obtained at surgery for joint replacement in patients with rheumatoid arthritis (RA). Cells were treated for 60 minutes with AJA (1-30μM), and then stimulated with TNFα. IL-8 and COX-2 mRNA were measured by ELISA in whole cell lysates 4 hr after stimulation. PGE_2 and 15d-PGJ_2 secretions were measured by ELISA in supernatants after overnight stimulation. Phosphorylation of IκBα, the cytoplasmic inhibitor of NFκB, was assessed by Western blotting at intervals 1-30 minutes after FLS stimulation. Nuclear binding of NFκB was determined by EMSA. Cell proliferation and cell counts were determined by measuring BrdU incorporation and MTT reduction, respectively. Apoptosis was assessed by determination of Annexin-V labeling (flow cytometry) and by DNA fragmentation (ELISA).

Results: Treatment with AJA increased the ratio of 15d-PGJ_2 to PGE_2, increased COX-2 mRNA, and modified IL-8 mRNA expression in stimulated FLS in a biphasic manner. AJA also suppressed TNFα-induced phosphorylation of IκBα and nuclear binding of NFκB. AJA reduced BrdU uptake and cell counts. These responses were accompanied by morphologic changes in FLS consistent with apoptosis. Increases in Annexin-V labeling and DNA fragmentation were also seen in AJA treated cells compared to untreated controls.

Conclusions: Treatment with AJA shifted prostaglandin production to an anti-inflammatory profile favoring 15d-PGJ_2. The altered PG profile may be due to increased COX-2 expression under these conditions. AJA also modified expression of the inflammatory cytokine IL-8, which is NFκB dependent. By inhibiting phosphorylation of IκBα, AJA may prevent nuclear translocation of NFκB, thus reducing production of inflammatory cytokines.

Others have found that 15d-PGJ_2 increases as inflammation resolves in an animal model (Gilroy et al. Nat Med, **1999**). The increase in 15d-PGJ_2, a known PPARγ ligand, suggests that AJA may exert anti-inflammatory effects through PPARγ-dependent mechanisms. In addition to its beneficial effects on the PG profile and altered cytokine expression, AJA induced apoptosis and inhibited proliferation of FLS. Thus, the joint-protective effects of AJA may be due in part to decreased formation of destructive pannus. These results suggest that AJA may be useful for the treatment of RA.

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ANANDAMIDE METABOLITES FROM BOTH CYCLOOXYGENASE ENZYMES CAUSE INHIBITION OF IL-2 SECRETION IN MURINE SPLENOCYTES

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Previous studies from this laboratory have shown that anandamide (AEA) causes inhibition of interleukin-2 (IL-2) secretion in activated splenocytes. Additionally, it has also been shown that arachidonic acid (AA) causes a concentration-dependent inhibition of IL-2, which was strikingly similar to that of anandamide. The IC_{50} values are also quite similar: 11.4 μM and 10.3 μM for AEA and AA, respectively. Pretreatment with flurbiprofen, a nonselective cyclooxygenase (COX) inhibitor, attenuated the inhibition of IL-2 secretion by both AEA and AA. The aforementioned data suggest that the COX enzymes may play a role in the inhibition of IL-2 secretion by AEA and AA. The overall goal of the present studies was to elucidate which of the COX enzymes are involved in the inhibitory effects of AEA and AA upon IL-2 secretion. Pretreatment with the COX-1 selective inhibitor, piroxicam, caused a partial attenuation of the inhibition of IL-2 by both AEA and AA, similar to that which was observed with flurbiprofen. Similarly, pretreatment with the COX-2 specific inhibitor, NS398, also attenuated the inhibitory effects of both AEA and AA upon IL-2 secretion. Additionally, the attenuation of the inhibitory effects of AEA by both piroxicam and NS398 is concentration-dependent, suggesting that the effects of both inhibitors is due to their specific activity at the COX enzymes and not at other nonspecific sites. Collectively, the data from the current studies suggests that both COX-1 and COX-2 are involved in the inhibition of IL-2 secretion by AEA and AA.

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THE CANNABINOID CB_1 RECEPTOR (CB_1R) ANTAGONIST SR141716A DOES NOT AFFECT FOOD INTAKE, PREFERENCE OR BODY WEIGHT IN CB_1R KO MICE

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The CB_1r antagonist SR141716A is known to cause hypophagia in mice, an effect abolished by genetic deletion of CB_1r (*DiMarzo et al., Nature 410: 822,* ***2001***). SR141716A has also been reported to preferentially suppress the intake of palatable, calorie-dense foodstuff, possibly due to a primary action on food 'reward' mechanisms in the CNS. In the present study, we have compared control (C57Bl/6) mice and CB_1r KO mice of the same background strain with regard to the relative intake of two different food types and body weight change after acute SR141716A treatment.

Age-matched control and CB_1r KO mice were adapted to *ad lib.* access to a choice of moist R3 lab chow ('wet mash') and a mixture of palatable calorie-dense food (cocoa fat, peanut butter, soft chocolate pastry; 'sweets') for a week prior to the experiment. Following overnight (12hrs) fast, the animals were then treated with vehicle or SR141716A (20µmol/kg ip) and again provided access to wet mash and sweets. Intake of both food types was monitored for 6hrs thereafter. Body weight was measured before start of the fasting period, immediately before start of the experiment, and 24hrs after start of the re-feeding session.

Total caloric intake was highest in the first 2hr-period, and did not differ between vehicle-treated control and CB_1r KO mice: 12.4 ± 1.48 and 11.1 ± 0.92 kJ, respectively. Wet mash intake constituted only ~15% of the total intake during this period, and there was no significant difference in food 'preference' (wet mash *vs*. sweets) between control and CB_1r KO mice. Compared to corresponding vehicle-treated groups, SR141716A suppressed the intake of sweets by 80-85%, but had no significant effect in CB_1r KO mice (<1.0% reduction). The intake of wet mash was not significantly altered after SR141716A treatment either in control or CB_1r KO mice. Total caloric intake in the 2-4 and 4-6hrs periods was lower than in the initial 2hr-period, but the overall pattern remained the same, both with respect to the relative intake of wet mash *vs*. sweets and to treatment effect of SR141716A in control but not in CB_1r KO mice. During the 12h fasting period preceding the food intake experiment, the mice lost an average of 1.4-1.6g in body weight. The weight loss was partly maintained in controls given SR141716A, whereas vehicle-treated controls as well as vehicle- and SR141716A-treated CB_1r KO mice regained or exceeded their pre-fasting weight.

Food deprivation-induced hyperphagia was similar in vehicle-treated controls and CB_1r KO mice, and the relative intake of wet mash *vs*. sweets did not differ between the two groups, suggesting that CB_1r are not essential to mechanisms mediating the orosensory characteristics of food under these conditions. Moreover, the fasting-induced effects on body weight was comparable in controls and CB_1r KO mice, tentatively suggesting that there may be no major alterations in basal metabolism in the latter despite the somewhat lower body weights in the CB_1r KO mice (~18g) *vs*. controls (~22g) prior to fasting. Putative non-specific effects of the currently used dose of SR141716A do not seem to play a major role in its food intake-suppressing action in control animals, as there was no effect of the compound in the CB_1r KO.

HYPOTHYROIDISM DOES NOT IMPACT THE WEIGHT-REDUCING EFFECTS OF SR141716A

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Chronic treatment with the selective CB-$_1$ antagonist, SR141716A (SR), produces weight loss that is greater than accounted for with the reduction in food intake, suggesting an effect to increase energy expenditure. Since cannabinoid agonists have been shown to inhibit the thyroid axis and cause hypothermia, we hypothesized that part of the effect of SR to decrease body weight could be due to activation of the thyroid axis. We therefore evaluated the effects of SR on body weight and fat mass after chemical ablation of the thyroid.

Diet-induced obese rats were rendered hypothyroid with the addition of 0.025% methimazole (MMI) in the drinking water (Tx, N=15) while another group was intact (Sham, N=15). Blood was collectecd on day 14 after MMI to verify thyroid status. After 2 weeks of MMI, both Tx and Sham groups were separated into 3 drug groups: vehicle, 2.5mg/kg SR and 25.0mg/kg SR (po). Food and body weights were recorded daily. Body composition was analyzed via QMR before treatment, 14 days after MMI and 10 days after SR treatment.

As expected, MMI treatment resulted in significantly greater levels of TSH (Sham, 0.98±0.04 vs Tx, 4.94±0.21 ng/mL, $p<0.05$) and lower T4 levels (Sham, 2.66±0.13 vs Tx, 0.26±0.08 ng/L, $p<0.05$) relative to the sham group. Minimal changes in food intake and body weight were seen in the Tx rats relative to Sham rats. While no differences in fat mass gain were evident (+18.2 ±3.2g for sham vs +13.9±1.1g for Tx, NS), an increase in body water content was evident in the Tx rats (11.0±1.6 to 17.3±1.8g, $p<0.05$).

Treatment with SR produced cumulative weight loss that, by day 10, was significantly greater in the Tx group (-30.4g for Sham+25mg/kg SR vs -42.2g for the Tx+25mg/kg SR, $p<0.05$). Likewise, a greater reduction in cumulative food intake was seen in Tx + SR treated groups. Body composition analyses reveal a dose-dependent decrease in fat mass that was similar in both Sham and Tx groups (-28.3±3.8g for Sham+25mg/kgSR vs -25.1±2.8g for Tx+25mg/kg, NS) and a non-significant decrease in lean mass. The major difference in body weight loss between these two groups appeared to be a reduction in body water content as determined by QMR (0.7±5.7 for Sham+25mg/kg vs -17.9±2.3 for Tx+25mg/kg, $p<0.05$).

These results suggest that the thyroid is most likely not involved in the metabolic effects of SR to reduce fat mass and body weight. The slightly larger loss in body weight seen in the hypothyroid rats appears to be simply due to changes in water balance.

OLEOYLETHANOLAMIDE IS AN ORALLY ACTIVE SATIETY-INDUCING ANOREXIANT

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Oleoylethanolamide (OEA) is a natural analog of anandamide that does not bind to cannabinoid receptors, but is a high-affinity ligand of the nuclear receptor PPAR-αFu et al., Nature **2003**, 425:90-3). When injected as a drug OEA inhibits feeding, reduces body weight and lower serum lipid levels (Rodriguez de Fonseca et al., Nature **2001**, 414:209-12). To explore the behavioral basis of OEA-induced hypophagia, we monitored feeding behavior in rats and mice using an automated system. Recorded data were analyzed as cumulative food ingested and as meal pattern profile (Gaetani et al., Neuropsychopharmacology **2003**, 28:1311-6). The results show that intraperitoneal administration of OEA (1-20 mg/kg) to rats causes a long-lasting inhibition of food intake, which is due to a delay of the eating onset. This delay is accompanied by an increase in food-probing episodes and is observed at doses that have no effects on locomotor activity in the open field test, ruling out the involvement of motoric inhibition. To test if the anorexiant effects of OEA are mediated by activation of PPAR-α receptors, we analyzed the feeding behavior of wild type and PPAR-α $^{-/-}$ mice after intraperitoneal administration of OEA (10 mg/kg). The drug effects were completely absent in PPAR-α$^{-/-}$μιχε. On the other hand, other PPAR-αagonists, such as Wy-14643 (40 mg/kg i.p.) and GW7674 (20 mg/kg i.p.), produced similar effects as OEA on total food intake and meal pattern in wild-type mice. Finally, to test if OEA is orally active, we analyzed feeding behavior in rats following oral administration of OEA, delivered either by gavage (50-200 mg/kg) or in capsules coated with a pH-sensitive resin (25-50 mg/kg) (Oveisi et al., Pharmacol Res **2004**, 49:461-6). When administered by gavage, the lowest effective dose of OEA produced a 20% inhibition of food intake over a 24-hour period. A similar inhibition was obtained with a 4-fold lower dose, when OEA was administered in pH-sensitive capsules. The effects of OEA capsules were significantly different from controls 5 hours after administration, a delay likely due to the transit of the capsules from the stomach to the small intestine. Parallel to food-intake inhibition, the analysis of meal pattern revealed that OEA capsules produced an increase in post-meal interval, but not in meal size, from 5 to 8 hours after administration. The analysis of OEA tissue distribution at the time of its maximal activity showed a 50-fold elevation in OEA levels within the initial segment of the gastrointestinal tract, while only a 2-fold increase was observed in plasma and liver. No changes of OEA levels were observed in the brain. In conclusion, the selective alterations induced by OEA on feeding onset after intraperitoneal administration and on post meal interval after oral administration strongly suggest that the primary contribution of this drug to normal feeding may be the regulation of satiety (the tonic state of inhibition over eating) rather than satiation (the phasic termination of feeding resulting from the act of food ingestion). Such contribution is mediated by the activation of PPAR-α receptors, most likely those present in the gastrointestinal tract.

OLEOYLETHANOLAMIDE REGULATES FEEDING AND BODY WEIGHT THROUGH ACTIVATION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-ALPHA

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The fatty acid oleoylethanolamide (OEA) is an anorexic factor and body-weight regulator (Rodríguez et al. Nature, **2001**, 414, 209-212. Gaetani et al, Neuropsychopharmacology, **2003**, 28, 1311-1316) that acts through peripheral vagal sensory nerves, but its molecular targets have long remained elusion. Though it is a natural analogue of endocannabinoid anandamide, OEA does not activate cannabinoid receptors (Schuel et al. CPL., **2002**, 121, 211-227). Here we show that OEA binds with high affinity (EC50=120±1nM) to peroxisome proliferator-activating receptor-α (PPAR-α), a ligand-activated transcription factor that regulates several aspects of lipid metabolism (Desvergne et al. Endocr. Rev., **1999**, 20, 649-688). Administration of OEA reduces food intake and body weight in wild-type mice, but not in mice deficient of PPAR-α. Two structurally distinct PPAR-α agonists, Wy14643 and GW7647 (Willson et al. J. Med. Chem., **2000**, 43, 527-550. Brown et al. PCT Int. Appl., **2000**, 32), exhibit similar effects. In contrast, the potent and selective agonists for PPAR-γ and PPAR-β/δciglitazone and GW501596 (Oliver et al. Proc. Natl Acad. Sci., **2001**, 98, 5306-5311. Chang et al. Diabetes, **1983**, 32, 830-838), are ineffective. OEA regulates the expression of several PPAR-α target genes in the small intestine and liver of wild-type mice, but not PPAR-α-null mice. OEA initiates the transcription of genes involved in lipid metabolism, such as fatty-acid translocase (FAT/CD36), fatty-acid transport protein 1 (FATP 1), fatty-acid binding protein (FABP) and uncoupling protein 2(UCP2), and represses transcription of inducible nitric-oxide (iNO) synthase, an enzyme producing NO that may contribute to the appetite stimulation. The functional similarity between OEA and PPAR-α agonists suggests that OEA is an endogenous ligand of PPAR-α. In the small intestine and the liver of wild-type mice, OEA levels are significantly lower during feeding than during satiety. The expression of PPAR-α parallels OEA concentrations, whereas the expression of iNOS shows the opposite pattern. Our results indicate that OEA regulates feeding and body weight by acting as a high-affinity ligand for PPAR-α, subsequently stimulating the lipid metabolism. The effects of OEA reveal an unexpected role for PPAR-α in the regulation of feeding behavior and may suggest that OEA may provide a scaffold for the creation of novel anti-obesity drug.

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SR141716 INHIBITS CULTURED MOUSE 3T3 F442A ADIPOCYTE CELL PROLIFERATION WITHOUT LIPID DROPLET ACCUMULATION

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Adipocyte cell proliferation (hyperplasia) is an important process in body fat mass development in obesity. The selective CB_1 receptor antagonist (SR141716) reduces food intake and body weight of obese rodents. Acrp30 (adiponectin) is an adipocytokine exclusively expressed and secreted by adipose tissue, which has been shown to regulate lipids and glucose metabolism and to play a key role in the body weight regulation and homeostasis. The plasma level of the protein Acrp30 and its mRNA expression in adipose tissue are decreased in obese and in type 2 diabetic subjects. We have previously reported that SR141716 stimulated Acrp30 mRNA expression in adipose tissue of obese fa/fa rats, by a direct effect on adipocytes (Bensaid et al., Mol. Pharmacol. 63, **2003**). Here we report that SR141716 (10 to 400 nM) inhibits cell proliferation of cultured mouse adipocyte 3T3 F442A in a concentration-dependent manner. In parallel to this inhibitor effect on adipocyte cell proliferation, SR141716 (10 to 100 nM) stimulates protein levels and mRNA expression of two late markers of adipocyte cell differentiation (Acrp30 and GAPDH) with a maximal effect at 100 nM, without inducing the accumulation of lipid droplets as monitored by Oil Red O staining. Furthermore, treatment of mouse adipocyte 3T3 F442A cells with SR141716 (100 nM) inhibits basal and serum-induced p42/44 MAP kinase activity. These results suggest that the inhibition of MAP kinase activity induced by SR141716 may be one of the mechanisms mediating the inhibition of cultured 3T3 F442A adipocyte cell proliferation and the stimulation of Acrp30 and GAPDH expression. The inhibition of adipocyte cell proliferation and the induction of adipocyte late "maturation" may participate in SR141716 anti-obesity effects and in particular the reduction of body fat mass.

ATTENUATION OF FOOD INTAKE IN MICE BY DIFFERENT CLASSES OF CB_1 RECEPTOR ANTAGONISTS

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Previous studies have implicated a role for endogenous cannabinoids in regulation of appetite. The purpose of this study was to investigate the effects of brain cannabinoid (CB_1) receptor agonists and antagonists on food consumption in mice. Mice were food-restricted for 24 hr and then allowed access to their regular rodent chow. Amount of food consumed in 1 hr was recorded. Locomotor activity was measured in separate groups of mice. Whereas the CB_1 antagonist SR141716A dose-dependently decreased food consumption at doses that did not significantly affect motor activity, Δ^9-tetrahydrocannabinol (THC) increased food consumption at lower doses that had no effect on motor activity and decreased food consumption concomitant with decreased motor activity at higher doses. Similarly, anandamide increased food intake at a dose that did not affect locomotor activity. In contrast, amphetamine (a known anorectic) and diazepam (a benzodiazepine and CNS depressant) only decreased food consumption at doses that also significantly increased or decreased motor activity, respectively. These results are consistent with those of previous research and anecdotal reports that suggest marijuana increases appetite and that SR141716A may produce the opposite effect. In addition, these results suggest that SR141716A may not be a "silent antagonist" -- i.e., it may have pharmacological effects of its own aside from antagonism of cannabinoid effects, including decreased feeding behavior and locomotor stimulation. O-3259, an analog of SR141716A, produced effects similar to those of the parent compound, suggesting that this effect may be related to the action of these compounds at the CB_1 receptor. We also evaluated the effects of O-2050, a sulfonamide analog of Δ^8-THC, in this model. O-2050 has good binding affinity for the CB_1 receptor and produces antagonist effects *in vitro*. In the feeding model, O-2050 significantly and dose-dependently reduced food intake at doses that produced only mild to moderate increases in locomotor activity. In summary, we have shown that two different classes of CB_1 receptor antagonists suppressed food intake in this model. In contrast, Δ^9-THC and anandamide increased food consumption. Although these results strongly suggest that CB_1 receptors may play a role in regulation of feeding behavior, further research is needed and is ongoing.

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REGULATION BY R-(+)- METHANANDAMIDE AND VANILLOID AGONIST OF PROSTATE CELLS PROLIFERATION

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Endocannabinods are now presented as new neuromodulators acting through specific cannabinoid receptors like CB_1 and CB_2. It has been shown that the endogenous cannaboid anandamide and other cannabionid agonists regulate cellular proliferation in many cell types including prostate cells (Sánchez. *et al.,* (**2003**) FEBS Letter 555: 561-566). Aditionally, anandamide can also activate the endogenous receptor for capsaicin, the purgent principle in hot pepper, named vanilloid VR1 receptor.

In this study, we demonstrate the presence of vanilloid receptor (VR1) in prostate tumoral cells and we examine here the involvement of cannabionids and vanilloid agonists in the proliferation of LNCaP prostate cells.

Cells were treated with different concentration of R-(+)-Methanandamide (MET), an anadamide analogue with higher metabolic stability than anandamide, which has been shown to bind also to VR1 receptors, and with capsaicin. The effect of cannabinoids and vanilloids on cell proliferation was studied by [^{3}H]-Thymidine incorporation into DNA for the last 16 h of the culture period. Radioactivity incorporated was monitored by liquid scintillation.

At doses greater than micromolar, MET inhibited the growth of prostate cells whereas at submicromolar concentrations, induced mitogenic effect. The maximal effect produced by MET was observed at 0.1μM and at 2 days of treatment. The stimulation of MET was blocked by the two cannabionid receptor antagonists, SR 141716 (antagonist of CB_1) and SR 144528 (antagonist of CB_2) at concentration of 0.5 μM as well as by the VR1 receptor antagonist, capsazepin at concentration of 1μM.

By other hand, capsaicin alone also had a mitogenic effect on LNCaP cells that was prevented by capsazepin.

These results suggest that cannabinodis and vanilloids regulate prostate cellular growth acting through vanilloid receptors expressed in prostate cells.

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BIOCHEMICAL MECHANISMS RELATED TO APOPTOSIS INDUCED BY CANNABIDIOL IN HUMAN GLIOMA CELLS

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Recent studies from our laboratory have demonstrated that the non-psychoactive cannabinoid compound cannabidiol (CBD) can induce, both in vitro and in vivo, inhibition of tumoral cell growth and trigger apoptosis in human glioma cells (Massi et al, J. Pharmacol. Exp. Ther., **2004**, 308) with evidence of an oxidative stress-based mechanism.

On this basis, the present study has been aimed at analysing the biochemical pathways activated by CBD to induce cell death, with the hypothesis of an activation of a "mithocondrial"-dependent apoptosis. Activation of the mithocondrial pathway of cell death is coupled to cytochrome c release, formation of the apoptosome, stimulation of the "initiator" caspase-9 and activation of effector caspases, such as caspase-3, the most important "effector" caspase. Because cytochrome c and caspases activation are early events in the apoptotic programme, to evaluate in detail the time-dependence of apoptosis induction, human glioma U373 and U87 cells were exposed for graded (1-24 h) periods of time to ineffective and effective concentrations of CBD, as estimated in antiproliferative and apoptotic studies. The initial assessment of cytochrome c release revealed a significant accumulation of cytochrome c of about 50% over the control in the cytoplasm of cells exposed to CBD, after 6 h from the exposure to the drug.

To verify whether caspase-9 is the downstream "initiator" caspase, we evaluated its activation in cells treated with CBD. A significant stimulation of caspase-9 of about 100% over the control was detected starting from 10-h exposure to CBD, as evaluated by spectrophotometric analysis of yellow pNA release caused by the cleavage of the synthetic tetrapeptide substrate LEHD-pNA.

CBD was also able to induce a significant activation of the "effector" caspase-3, representing the most important converging caspase in apoptosis induced by different stimuli. The maximal activation was seen starting from a 14-h exposure (280%), with a time course that followed the activation of caspase-9 and that preceded the appearance of nuclear sign of apoptosis, suggesting a temporal correlation among these events. The caspase-3 activity was selectively prevented by the pan-caspase inhibitor zVAD-fmk.

In conclusion, our data show that CBD leads to the activation of mithocondrial pathway and triggers downstream signals culminated to the caspase-3 activation, suggesting a possible route through CBD can induce apoptosis on human glioma cells.

Acknowledgements: CBD was supplied by GW Pharm

SYNTHESIS AND ANTITUMOR ACTIVITY OF QUINONOID DERIVATIVES OF CANNABINOIDS

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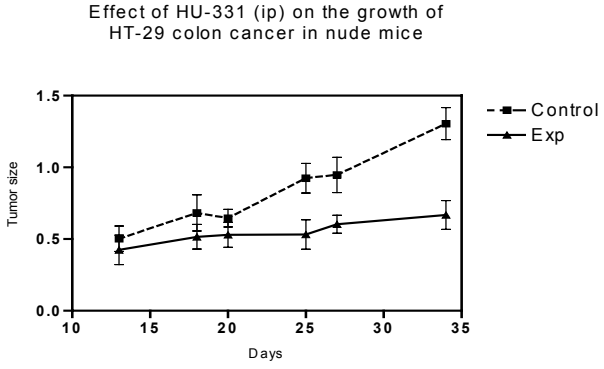
Three Cannabis constituents, cannabidiol, Δ^8-tetrahydrocannabinol and cannabinol were oxidized to their respective para-quinones cannabidiol-hydroxyquinone, Δ^8-tetrahydrocannabinol-quinone and cannabinol-quinone. In the 1960's Δ^8-tetrahydrocannabinol-quinone had been assigned a para-quinone structure (Mechoulam et al, Tetrahedron **1968**, 24, 5615), which was later modified to an ortho-quinone (Hodjat-Kashani et al, Heterocycles **1986**, 24, 1973) In order to distinguish between the two possible quinone structures a detailed NMR investigation was undertaken. The original para-quinone structure was confirmed. X-ray crystallography elucidated the structures of the crystalline cannabidiol-hydroxyquinone and cannabinol-quinone. All three compounds inhibited the *in vitro* growth of human cancer cell lines, albeit they differed in their potency. Δ^8-Tetrahydrocannabinol-quinone exerted the weakest anti-cancer activity since concentrations of 12.5 μg/ml or higher were required to inhibit by 50% or more the growth of cells tested. Cannabinol-quinone was a more potent anti-cancer reagent than Δ^8-tetrahydrocannabinol-quinone. The growth of Raji lymphoma cells was inhibited by over 50% at a concentration of 6.25 μg /ml. By far the most potent anti-cancer activity was displayed by cannabidiol-hydroxyquinone. An inhibition of 50% of the growth of the Raji and Jurkat lymphomas was obtained at a concentration of HU-331 as low as 0.2 μg /ml, while 50% inhibition of the growth of HT-29 colon cancer and of MCF-7 mammary cancer cells required a concentration of only 3.125 μg /ml. HU-331 displayed a marked anti-cancer activity not only *in vitro* but also *in vivo* in experiments with *nude* mice that received a subcutaneous inoculation of HT-29 colon carcinoma cells. The administration of HU-331 at a concentration that did not have observable adverse effects on the hosts (5mg/kg) resulted in significant inhibition of the growth of the tumors.



CB_2 RECEPTOR AGONISTS REDUCES THE ACCUMULATION OF HUMAN HEPATIC MYOFIBROBLASTS: A NOVEL ANTIFIBROGENIC PATHWAY IN THE LIVER

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Hepatic myofibroblasts are central for the development of liver fibrosis associated with chronic liver diseases, and blocking their accumulation may prevent fibrogenesis. Cannabinoids are the active components of marijuana and act via two G protein-coupled receptors, CB_1 and CB_2. Here, we evaluated expression of CB_2 receptors in the cirrhotic liver, and investigated their role in cultured human hepatic myofibroblasts.

In liver biopsies from patients with cirrhosis of various etiologies, immunohistochemistry showed the presence of CB_2 receptors in non-parenchymal cells located within and at the edge of fibrous septa. In contrast, CB_2 receptors were not detected in normal human liver. CB_2 receptors were expressed in hepatic myofibroblasts, as shown by immunohistochemistry in liver biopsies and in cultured cells, and functional, as evidenced in GTPγS binding assays. In cultured human hepatic myofibroblasts, activation of CB_2 receptors triggered growth inhibition and higher doses were apoptotic. Growth inhibition involved cyclooxygenase-2 (COX-2) and apoptosis resulted from oxidative stress. Indeed, on the one hand, the antiproliferative effects of the plant-derived cannabinoid Δ^9-tetrahydrocannabinol (THC) were blocked by a COX-2 inhibitor NS-398, and THC increased COX-2 expression. On the other hand, apoptosis elicited by THC was blocked by two antioxidants, N-acetyl-cysteine and a superoxide dismutase/catalase mimetic, EUK8; accordingly THC increased oxidative stress.

In conclusion, CB_2 receptors are up-regulated in hepatic myofibroblasts during chronic liver diseases. Activation of CB_2 receptors reduces accumulation of these cells by triggering potent growth inhibitory and apoptotic effects. These results suggest that activation of upregulated CB_2 receptors may limit fibrogenesis during chronic liver injury.

CANNABINOID CB_2 RECEPTORS ARE EXPRESSED BY NEURONAL AND GLIAL ELEMENTS OF THE HUMAN CEREBELLUM

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Two types of cannabinoid receptors have been characterized so far, named CB_1 and CB_2. While CB_1 receptors are present both in the CNS and in the periphery, CB_2 receptors have shown an almost exclusive distribution within the immune system. We report that CB_2 receptors may be present in different cellular types of the human cerebellum. Thus, immunohistochemical analysis of human cerebellar sections revealed an intense staining for CB_2 protein in different cell types. The staining produced by two anti-CB_2 antibodies (one generated against the N-terminal and another generated against the C-terminal of the human protein) showed a similar pattern, that was completely prevented by the pre- and co-incubation with their respective immunizing peptides. Purkinje neurones showed a high level of labelling in somas, dendrites and axonal projections while granule neurones exhibited a weaker staining. A strikingly selective pattern of staining could be noticed in almost all perivascular areas, including high and small diameter blood vessels. High magnification observations revealed that the external surface of the vessels appeared covered with a "dot-like" staining pattern. Specifically, CB_2-positive perivascular cells exhibited morphological features of microglia embracing the outer portion of the vessel walls. The phenotypic properties of these CB_2-positive cells were further characterized by double immunofluorescence labeling with an anti-HLA DP, DQ, DR antibody. In addition, digoxigenin-tagged riboprobes revealed the presence of CB_2-mRNA in granule and Purkinje neurones as well as in cells at a perivascular location. Tissue sections treated with the sense probe were completely devoid of any staining. Finally and for control purposes, rat brain tissue sections were used, lacking of any staining for CB_2 protein or mRNA. Our results open new perspectives on the role of the endocannabinoid system in the regulation of the immune response in the human CNS as well as on the motor effects of cannabinoids and related compounds.

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ANTI-ANGIOGENIC EFFECT OF CANNABINOIDS IN GRANULOMA FORMATION IN RATS

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Angiogenesis is the multistep process leading to new capillary formation from the pre-existing blood vessels, which is sustained by the "in loco" release of several mediators including cytokines, such as VEGF and TNF-α Angiogenesis plays a pivotal role in many both physiological and pathological conditions, in addition to solid tumour growth. The progress of chronic and proliferative inflammation, such as granuloma formation, also depend on angiogenesis which is required for the maintenance of tissue perfusion and to allow cellular traffic increase necessary for chronicity. Therefore, the discovery of new anti-angiogenic molecules may represent today a promising field in the therapy of cancer and chronic inflammatory diseases. Plant-derived cannabinoids from *Cannabis Sativa* and their synthetic analogues mediate a wide range of biological effects both in the central nervous system and in periphery, through the interaction with two G protein-coupled receptors, which have been identified, namely CB_1 and CB_2. The former is mainly distributed in central and peripheral neurons, while the latter found in cells of immune system is responsible of immune function modulation. Aim of this study was to evaluate the effect of synthetic cannabinoids in granuloma-associated angiogenesis in the rat. Granuloma was induced by subcutaneous implantation of two λ-carrageenin (1%)-soaked sponges on the back of male Wistar rats. Granulomatous tissue formation was evaluated as wet weight 96 hours after the implants; angiogenesis was evaluated both as haemoglobin (Hb) content in the tissue, and as the expression of VEGF and TNF-α in the exudated. Results of our study demonstrated that granuloma formation induced by λ-carragenin-soaked sponges was significatively reduced by local administration of the synthetic cannabinoid WIN 55-212-2 (10μg/100μl/site daily) (0.69±0.66 gr vs 1.2±0.076gr carrageenin control). WIN 55212-2 was more efficacy when given in loco respect to systemic administration (0.89±0.018 gr vs control). Time-course experiments of local WIN 55212-2 administration, i.e. (i) daily administration; (ii) single administration at time of implantation (time 0); single administration three days after implantation (time 3), showed that WIN 55212-2 (10μg/100μl/site) was more efficacy when given daily (42.5% inhibition) or at time 0 (41.7 % inhibition), whereas it was ineffective at time 3 (15.6% inhibition), when angiogenetic process have been already started. Local time 0 and daily administration of WIN 55212-2 was also effective in reducing Hb content in granulomatous tissue by 54.25% and 32.55% respectively. For all the further experiments we used local administration at time 0. *Western Blot* analysis showed that WIN 55212-2 (1-10 10μg/site) reduced the expression of both VEGF and TNF-α. In the attempt to discriminate the selective involvement of cannabinoid receptor subtype in mediate the observed anti-angiogenic effects, we used the CB_2 selective agonist, JWH-015 and the CB_1 selective agonist ACEA. JWH-015 (10μg/100μl/site), but not ACEA, inhibited granuloma formation when given locally at time 0 by 38%. In conclusion, our data demonstrate that cannabinoids are able to reduce granuloma-associated angiogenesis when given at the starting of the process. Moreover, selective activation of CB_2 receptors by synthetic cannabinoids are involved in the inhibition of granuloma formation, targeting their potential use in the therapy of angiogenic-related diseases.

CANNABINOID RECEPTORS IN THE EYE: THERAPEUTIC TARGETS IN GLAUCOMA

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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. IOP is determined by the rate of aqueous humor formation by ciliary epithelium and by the rate of aqueous humor outflow via trabecular meshwork (TM) and Canal of Schlemm. While all of these ocular structures express CB_1 cannabinoid receptors, the cellular mechanisms underlying the pharmacology of cannabinoids at these tissue sites is not known, nor is it clear which of these tissues contribute to the net hypotensive effects of cannabinoids in the eye.

Our studies are directed at understanding cannabinoid receptor activation in the TM, the major aqueous outflow pathway in the eye. Research has suggested that TM tissue is contractile, in a manner comparable to smooth-muscle, with both Ca^{2+}-dependent and Ca^{2+}-independent contraction and associated increases in phosphorylation of myosin light chain (MLC) protein and formation of actin-myosin filaments. Recent findings have shown that stimulation of isolated TM strips with muscarinic agonists increases intacellular Ca^{2+} and contracts TM tissue, while stimulation of TM with cannabinoid agonists relaxes pre-contracted TM tissue (for reviews see Thieme *et al.,* **1999**, and Chien *et al.,* **2003**)

We investigated the effects of muscarinic and cannabinoid agonists and their coupled signalling pathways on the contractile properties of isolated TM cells. In our experiments we use acutely isolated bovine TM cells as well as a cultured human TM cell line together with the following techniques: (1) *Immunohistochemistry and Confocal Microscopy:* To determine the expression of CB_1 and M3 receptors, as well as the expression and organization of α-smooth-muscle actin (α-SM actin). (2) *Western Blot Analysis*: To determine the effects of stimulation of cells with muscarinic (carbachol 1-10μM) and cannabinoid (WIN55,212-2;1-5μM) agonists on MLC phosphorylation (3) *Cell Contraction Assays:* To determine the agonist-induced alteration in cells size/contractility.

Our results show the expression of both CB_1 and M3 receptors in TM cells. Furthermore, stimulation of TM cells with both muscarinic and cannabinoid agonists increased MLC phosphorylation and immunocytochemical staining revealed the presence of α-SM actin. Our functional assays confirmed that stimulation of TM cells with muscarinic agonist, carbachol (1-10μM) contracted TM cells. Current experiments are investigating interactions between muscarinic and CB_1R signalling pathways in the regulation of TM cell contractility. In conclusion, our work demonstrated that individual TM cells are contractile and express α-SM actin. Agonists that activate both M3R and CB_1R can increase phosphorylation of contractile proteins in these cells and interactions between M3R and CB_1R may result in alterations in TM cell contractility. Changes in resistance of TM tissue could affect aqueous humor outflow.

ENDOCANNABINOID LEVELS IN NORMAL AND GLAUCOMATOUS HUMAN EYE TISSUES

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Glaucoma is a disease characterized by an increase in intra-ocular pressure (IOP) leading to retinal degeneration and blindness. Both cannabinoids and endocannabinoids are known to lower IOP. The endocannabinoid system is present in various structures of the eye, where it may be involved in the regulation of IOP. For example, anandamide were found in the human retina, ciliary process and trabecular meshwork tissue. 2-arachidonoylglycerol (2-AG) levels were higher than those of anandamide in the retinas of several species, but have never been investigated in the human eye. The IOP-lowering effects of cannabinoids and endocannabinoids are mostly due to the presence of the cannabinoid CB_1 receptors in eyes tissues. The cannabinoid CB_1 receptor is abundantly expressed in the two synaptic layers of the retina in several vertebrate species, such as monkey, mouse, rat, chick, goldfish and tiger salamander. By in situ hybridisation and RT-PCR analysis, it was found that cannabinoid CB_1 receptor is not restricted to the retina, but that it is also present in the ciliary body, iris and the choroid. Instead, the expression of the cannabinoid CB_2 receptor seems to be limited to the neurosensory retina. Fatty acid amide hydrolase (FAAH) activity was also found in porcine and bovine retina, choroid, optic nerve, iris and lacrimal gland. In addition, endocannabinoid degradation was detected in bovine iris-ciliary body and cornea homogenates. Immunohistochemical studies confirmed the presence of the FAAH in the retina, where it is abundantly expressed in the large ganglion cells with dendritic projection to a narrow band in the proximal inner plexiform layer, but also in the soma of the horizontal cells such as the dopamine amacrine cells and the large cells.

Here, we have quantified by isotope-dilution liquid-chromatography mass spectrometry the levels of anandamide and 2-arachidonoylglycerol in human cornea, iris, ciliary body, choroid and retina in both glaucomatous and normal post-mortem eyes. Among the various tissues, anandamide (20-250 pmol/g wet weight tissue) was significantly most abundant in the iris, whereas 2-arachidonoylglycerol (40-1400 pmol/g) was most abundant in the retina. 2-AG levels were higher than anandamide levels in the retina, ciliary body and choroid, but not in the iris and cornea. Although still preliminary because of the limited number of replicates analysed (n=4), the amounts of 2-AG in glaucomatous eyes appeared to undergo a significant ~30% decrease in the ciliary body, which is the eye tissue actively involved in the regulation of IOP. This observation, if confirmed by further experiments, might indicate that pathological alterations in the levels of 2-AG contribute to the enhancement of IOP typical of glaucoma.

CANNABINOID INDUCES VASODILATATION IN ISOLATED PRE-CONTRACTED BOVINE OPHTHALMIC ARTERIES

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The present experiments were designed to investigate the effects of some cannabinoid agonists like anandamide and WIN 55,212-2 on vascular smooth muscle in the bovine ophthalmic artery. The bovine ophthalmic artery rings were mounted in organ bath for isometric force measurement. The presence of functional endothelium in the arteries was tested by applying carbachol. Concentration-response curves to norepinephrine and serotonin were constructed and both ligands were potent constrictors giving pEC_{50} values of 6.54 and 7.22, respectively. The maximal effect was reached by 1 μM serotonin or 5 μM norepinephrine. The effect of cannabinoid endogenous anandamide (Devane et al., Life Sci. **1992**, 258, 1946) and cannabinoid agonist WIN 55,212-2 on vasoconstriction was investigated. Both anandamide and WIN 55,212-2 if added to the bath when vasoconstriction had reached a maximum steady level caused a highly significant relaxation of arteries. The vasodilation induced by anandamide and WIN 55,212-2 was concentration-dependent and reversible. This action of cannabinoid agonists occurred irrespective of which agent was used to induce vasoconstriction and was endothelium-independent. Moreover, a pre-incubation with the selective cannabinoid$_1$ (CB_1) receptor antagonist SR 141716A (Rinaldi-Carmona et al., Science **1994**, 56, 1941) blocked the vasodilation produced by anandamide e WIN 55,212-2 whereas a pre-incubation with the selective cannabinoid$_2$ (CB_2) receptor antagonist SR 144528 did not modify the relaxation response of arteries. In conclusion, these results provide strong evidence of the involvement of cannabinoid CB_1 receptors promoting the relaxation of the bovine ophthalmic artery.

GENETIC AND PHARMACOLOGICAL BLOCKADE OF CB_1 RECEPTORS MODULATES ANXIETY IN THE SHOCK-PROBE BURYING TEST

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Cannabinoids affect a variety of behavioral processes, including emotion and cognition. More specifically, the CB_1 receptor may be involved in anxiety and learning and memory. The exact role CB_1 receptors play in anxiety remains unclear and both genetic and pharmacological blockade of CB_1 receptors have produced inconsistent effects on anxiety. However, these studies examined passive avoidance as an index of anxiety. In the present study, we measured anxiety responses by examining both active and passive avoidance using the shock-probe burying test while CB_1 receptors were blocked either genetically or pharmacologically. In the shock-probe burying test, increased anxiety is reflected by increased burying (increased active avoidance) and increased freezing (increased passive avoidance). In addition, probe-contacts may reflect cognitive performance and/or passive avoidance. Since few studies have tested mice in the shock-probe burying test, we first validated the test pharmacologically. Systemic (i.p.) injections of chlordiazepoxide (4 mg/kg) or FG7412 (5 mg/kg) in mice decreased and increased burying behavior respectively, while not affecting freezing or the number of contacts made with the probe (Experiment 1). More importantly, both CB_1 knockout mice and mice injected (i.p.) with 3 or 10 mg/kg, but not 1 mg/kg, of the CB_1 receptor antagonist SR141716A had lower burying scores, fewer contacts with the probe, and similar freezing times compared to wild-type mice and mice injected with vehicle (Experiments 2 and 3). Collectively, these results suggest that CB_1 receptor blockade reduces some, but not all, aspects of anxiety. The increase in probe contacts induced by CB_1 receptor blockade may be due to enhanced cognitive performance.

THE ADOLESCENT SHR STRAIN AS A MODEL OF ADHD: IMPULSIVE BEHAVIOUR AND ITS MODULATION BY A CANNABINOID AGONIST

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Attention-deficit/Hyperactivity Disorder (ADHD) is a neuropsychiatric syndrome affecting human infants and adolescents. Two main behavioural features are reported: 1) impaired attention and 2) an impulsive-hyperactive behavioural trait. The latter was studied in a series of experiments, using as subject the spontaneously-hypertensive-rat (SHR) strain (a validated animal model for ADHD) by means of an operant paradigm. Food-restricted SHRs and their Wistar-Kyoto (WKY) controls were tested during adolescence (i.e. post-natal days 30 to 45), in operant chambers provided with two nose-poking holes. Nose-poking in one hole (H1) resulted in the immediate delivery of a small amount of food, whereas nose-poking in the other hole (H5) delivered a larger amount of food after a delay, which was increased progressively each day (0 to 100 sec). As expected, all animals showed a shift in preference from the large (H5) to the immediate (H1) reinforcer as far as delay length increased. Impulsivity can be measured by the steepness of this preference-delay curve. The two strains differed in spontaneous circadian activity in the home cage, SHRs being more active than WKYs at several time-points. During the test for impulsivity, inter-individual differences were completely absent in the WKY strain, whereas a huge inter-individual variability was evident for SHRs. On the basis of the median value of average hole-preference, we found a steep-slope ("impulsive") SHR subgroup, with a very quick shift towards the H1 hole, and a flat-slope ("non-impulsive") SHR subgroup, with little or no shift. The impulsive subpopulation also presented reduced noradrenaline levels in both cingulated and medial-frontal cortex, as well as reduced serotonin turnover in the latter. Also, cannabinoid CB_1 receptor density resulted significantly lower in the cortex of impulsive SHRs, when compared to both the non-impulsive subgroup and control WKYs. Interestingly, acute administration of a cannabinoid agonist (WIN 55,212, 2 mg/kg s.c.) normalized the impulsive behavioural profile, without any effect on WKY rats. In conclusion, two distinct subpopulations, differing for impulsive behaviour and specific neurochemical parameters, were evidenced within adolescent SHRs. These results support the notion that a reduced cortical density of cannabinoid CB_1 receptors is associated with enhanced impulsivity. This behavioural trait can be positively modulated by administration of a cannabinoid agonist. Present results confirm and extend previous literature, indicating that adolescent SHRs represent a suitable animal model for the pre-clinical investigation of the early-onset ADHD syndrome.

IS THE EMOTIONAL-LIKE RESPONSE INVOLVED IN Δ^9-TETRA-HYDROCANNABINOL-INDUCED CONDITIONED PLACE PREFERENCE (CPP)?

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Although cannabinoids have clear addictive potential at the human level, knowledge of the abuse liability of Δ^9–tetrahydrocannabinol (Δ^9–THC), the psychoactive constituent of cannabis, is still controversial. In the CPP paradigm, Δ^9–THC produced place aversion, no effect or place preference in both mice and rats, depending on different methodological details (Tanda and Goldberg, Psychopharmacology **2003**, 169, 115). Exogenous and endogenous cannabinoid agonists also induced both anxiolytic- and anxiogenic-like behavioural reactions in rodents depending on the dose and the context (Onaivi et al., J, Pharmacol. Exp. Ther **1990**, 253, 1002; Rodriguez de Fonseca, J, Pharmacol. Exp. Ther. **1996**, 276, 56; Akinshola et al., Neurochem. Res. **1999**, 24, 1233; Valjent et al., Br. J. Pharmacol. **2002**, 135, 564).

The aim of the present work was to investigate if the reinforcing/aversive properties of Δ^9–THC, evaluated in a CPP task, might correlate with its anxiolytic/anxiogenic effects studied in a plus-maze apparatus.

Pre-conditioned male Wistar rats were injected i.p. with increasing doses of Δ^9–THC (0.015-12 mg/kg) and after 10 min confined, for 30 min, in the drug-paired compartment. On the following day, rats received vehicle and confined to the opposite side. After eight of these conditioning sessions, CPP was assessed by allowing uninjected rats free access to both compartments and by measuring the time spent in each one. A further group of animals was submitted to the same CPP schedule but on the test day they were assessed in a plus-maze apparatus. The total number of entries and the time spent in the open and closed arms were recorded in a 5-min test. The role of CB_1 cannabinoid and opioid receptor was investigated pre-treating rats with SR 141716 (0.25-1 mg/kg) and naloxone (0.5-2 mg/kg), respectively.

Only in a range of doses from 0.075 to 0.75 mg/kg, Δ^9–THC produced a significant CPP which was completely antagonised by pre-treatment with SR 141716 or naloxone. An anxiolytic effect was shown only with a dose as 0.75 mg/kg.

These initial results are consistent with a lack of correlation between reinforcing and anxiolytic effects of Δ^9–THC.

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INTRAVENOUS SELF-ADMINISTRATION OF THE ENDOGENOUS ANANDAMIDE AND ITS SYNTHETIC ANALOGUE METHANANDAMIDE BY SQUIRREL MONKEYS

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In spite of its well-established abuse properties, documented in humans, our knowledge of the abuse liability of cannabis and of the reinforcing properties of its psychoactive ingredient, THC, is still limited. We have recently shown that the psychoactive ingredient in cannabis, THC, maintains high rates of i.v. self-administration behavior in squirrel monkeys without any history of pre-exposure to other drugs. Reinforcing effects of THC are mediated by cannabinoid CB_1 receptors and are highly dependent on associated environmental stimuli (cues). We have recently investigated whether anandamide, the endogenous ligand for cannabinoid CB_1 receptors, and its synthetic analogue R(+)-methanandamide serve as effective reinforcers of intravenous drug-taking behavior in non-human primates. Squirrel monkeys readily learned to press a lever for intravenous injections of anandamide under a ten-response, fixed-ratio schedule of reinforcement during daily sessions conducted Monday to Friday. Each session lasted one hour and there was a one-minute timeout period after each injection. Each dose of anandamide or methanandamide was studied for five consecutive sessions and there were five sessions with vehicle substitution between testing of different doses doses. Peak rates of responding for anandamide or methanandamide were as high as those we previously found with intravenous injections of THC, nicotine or cocaine under the same conditions. When the dose of anandamide was varied, we obtained a typical inverted U-shaped dose-response curve, with maximal rates of responding at a dose of 40μg/kg. Surprisingly, the long lasting, metabolically stable, synthetic analog of anandamide, R(+)-methanandamide, maintained self-administration behavior that was virtually identical to that maintained by anandamide over the same range of injection doses. When vehicle placebo was substituted for anandamide or methanandamide, drug-taking behavior immediately decreased to very low levels but was rapidly reinstated when anandamide or methanandamide injections were again made available. Anandamide self-administration behavior was significantly reduced but, unlike THC, was not completely blocked by the selective cannabinoid CB_1 receptor antagonist SR141716. The partial reduction of anandamide self-administration behavior by the CB_1 receptor antagonist indicates that both CB_1 and non-CB_1 mechanisms are involved in the reinforcing effects of anandamide. The high response rates maintained by self-administration of an endogenous cannabinoid directly demonstrate the involvement of endogenous cannabinoid systems in brain reward processes and provides further evidence of the reinforcing potential of cannabinoids in non-human primates.

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ENKEPHALIN-CONTAINING NEURONS IN THE CENTRAL NUCLEUS OF THE AMYGDALA ARE ACTIVATED BY ACUTE AND CHRONIC EXPOSURE TO Δ^9-THC

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Cannabis sativa is the most widely consumed illegal drug of abuse in North America and Europe. Delta 9-tetrahydrocannabinol (Δ^9-THC) is the active chemical in cannabis. To better understand the central effects of Δ^9-THC, we sought to identify brain areas and neuronal populations responsive to acute and chronic administration of different doses of Δ^9-THC. We used c-Fos positive immunoreactivity as a marker for Δ^9-THC-responsive neurons. *Acute Δ^9-THC treatment.* Rats were habituated to vehicle injections for 2 weeks, and killed two hours after receiving an IP injection on the 15th day of either vehicle or Δ^9-THC (5 or 10 mg/Kg). Brains were removed and processed for immuno-detection of c-Fos. In agreement with previous studies, Δ^9-THC was found to stimulate c-Fos expression in several brain areas. The central nucleus of the amygdala (CNA) showed the highest concentration of c-Fos expressing neurons in the brain. While all animals treated with 10 mg/kg of Δ^9-THC displayed high levels of c-Fos expression, a lower dose (5 mg/kg) of Δ^9-THC induced variability of c-Fos expression in the CNA across animals. These results indicate variability in the rat population in response to low doses of acute exposure to Δ^9-THC. *Chronic Δ^9-THC treatment.* Rats were injected for 2 weeks with daily single injections of 5 or 10 mg/Kg of Δ^9-THC. On the 15th day the animals received a final injection of either vehicle or Δ^9-THC, and two hours later were killed. All rats that were chronically injected with Δ^9-THC and a final injection of vehicle showed very low levels of c-Fos expression. In contrast, rats injected with Δ^9-THC had c-Fos immunoreactive neurons concentrated in the CNA. However, individual variable levels of c-Fos immunoreactivity were observed in CNA of rats exposed to 10 mg/kg of Δ^9-THC. Contrary to this finding, animals treated with 5 mg/kg showed less individual variability. These results indicate variability in the rat population in response to different doses of chronic Δ^9-THC administration and suggest individual dose dependent variability to the development of tolerance, measured as c-Fos induction by Δ^9-THC. *Phenotypic characterization of c-Fos immunoreactive neurons.* We used a combination of *in situ* hybridization and immuno-histochemistry to determine the phenotype of neurons activated by Δ^9-THC. We found that acute and chronic administtration of Δ^9-THC induced expression of c-Fos principally in neurons expressing pro-enkephalin (ENK). These results provide anatomical evidence indicating that within the CNA, GABAergic neurons that contain ENK are responsive to acute and chronic Δ^9-THC exposure. As cannabinoid receptors (CB_1) appear to be absent from the CNA, we are testing the hypothesis that Δ^9-THC may activate CB_1 receptors within the basolateral amygdala (BLA), lowering tonic inhibition on glutamatergic neurons located in the BLA that synapse on ENK neurons distributed in the CNA.

LONG-TERM EFFECTS OF ADOLESCENCE EXPOSURE TO CANNABINOIDS ON RAT MESOACCUMBENS DOPAMINE NEURONS: TOLERANCE AND CROSS TOLERANCE TO MORPHINE AND PSYCHOSTIMULANTS

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Cannabis derivatives are commonly abused from periadolescence and generally considered as harmless. On the other hand, previous studies demonstrated the ability of these compounds to induce long-term effects on behavioural and cognitive functions, when administered chronically especially during adolescence.

The mesoaccumbens dopamine (DA) system is involved in neural mechanisms of reward. In the rat, acute administration of cannabinoids causes excitation of ventral tegmental area (VTA) DA neurons, and an increased DA output in their terminal regions. We examined if early exposure to cannabinoids alters the basal electrical activity of DA neurons and their responsiveness to major drugs of abuse. Thus, we pretreated periadolescent male rats (5-6 weeks of age) with the CB_1 receptor agonist WIN55212-2 (WIN), for three days, twice daily, at increasingly doses (2-8 mg/kg, i.p.) or with corresponding volume of vehicle. In these animals, two weeks after last cannabinoid injection, we recorded the activity of antidromically identified mesoaccumbens DA neurons. We did not find any significant difference between two groups of animals in baseline electrophysiological features of DA neurons (antidromic latency, firing rate or pattern). However, the ability of WIN (0.0625-0.5 mg/kg, i.v.) to stimulate DA neurons was reduced in cannabinoid-treated animals as compared with controls. Thus, we studied the response of DA neurons to morphine and psychostimulants administration. Morphine (0.5-8 mg/kg, i.v.) elicited an increase in the electrical activity of DA cells recorded from controls, while was without effect on the firing rate of DA neurons recorded from treated animals. In these rats, DA neurons were also tolerant to the inhibitory effects of cocaine (0.125-4.0 mg/kg, i.v.) and amphetamine (0.0625-2.0 mg/kg, i.v.), as compared to controls.

Our study suggests that a long-lasting form of neuronal adaptation, occurs in DA neurons following sub-chronic cannabinoid intake at young age, which affects subsequent responses to drugs of abuse. It can be speculated that this cannabinoid-induced altered sensitivity might lead to enhanced vulnerability in selected individuals to more harmful drugs of abuse.

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REGULATION OF NOP RECEPTOR DENSITY BY DELTA-9-TETRAHYDROCANNABINOL TREATMENT IN SH-SY5Y CELLS

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The neuropeptide nociceptin (also named as orphanin FQ) is the endogenous ligand for the ORL-1 receptor, recently referred to as NOP. This receptor presents marked structural analogies with the three different opioid receptors, nevertheless it is not able to interact with the ligands for such receptors. The pharmacological characterization of this neuronal system allowed tosuggest that nociceptin acts as a functional antagonist towards the endogenous opioid system.

Previous studies showed a functional modulation between the opioid and cannabinoid systems and evidence has been provided that the endogenous opioid system is involved in the regulation of several effects elicited by cannabinoids, such as analgesia, reward, immunological responses or anxiety-like behaviour.

With the aim to better characterize the interplay between nociceptin/NOP and cannabinoid systems and in order to investigate the possibility of a cross-talk between CB_1 and NOP receptors, we used the human neuroblastoma SH-SY5Y cell line as a model to investigate the effects of delta9-tetrahydrocannabinol (Delta9-THC) on NOP receptor density.

The cells were exposed to 50 nM, 100 nM and 150 nM delta9-THC for 24 hours and NOP receptor density was measured by receptor binding assay by means of [leucyl-^{3}H]-nociceptin. A dose-dependent NOP receptor down-regulation was observed after exposure to 50-150 nM delta9-THC (73% and 67 % versus controls 100%, $p<0.05$ for the doses of 100 and 150 nM , respectively).

These results show that delta9-THC is able to affect NOP receptor levels in SH-SY5Y cells and that the mechanisms activated by occupation of CB_1 receptor can contribute to the regulation of NOP receptor. Furthermore, they indicate the existence of interactions between cannabinoid and nociceptin/NOP systems, thus suggesting a cross-talk between CB_1 and NOP receptors.

CROSS-TALK BETWEEN CANNABINOID AND OPIOID SYSTEMS IN RELAPSE TO DRUG-SEEKING

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Detoxification from drug addiction represents the major medical problem due to high relapse rates occurring even after a prolonged drug-free period. Extinction-reinstatement animal models of drug-seeking behaviour offer some promise toward understanding relapse to compulsive drug abuse long after detoxification. Since opioid and cannabinoid endogenous systems have been reported to interact in the modulation of addictive behaviour, we investigated the ability of cannabinoid and opioid agents to reinstate or prevent drug-seeking behaviour after a long period of extinction.

We originally found that cannabinoid primings reinstate heroin-seeking behaviour in rats after a long period of drug abstinence, while the cannabinoid (CB_1) receptor antagonist SR 141716A inhibits heroin-induced relapse to drug-seeking. In a more recent follow-up study it was found that (*i*) cannabinoid-induced reinstatement of heroin-seeking is attenuated by pretreatment with SR 141716A or naloxone, but completely prevented by co-administration of the two antagonists, (*ii*) the residual long-lasting effect induced by cannabinoid primings on heroin-seeking reinstatement is not affected by SR 141716A or naloxone pretreatment, and that (*iii*) after different periods of drug abstinence, cannabinoid may substitute for heroin in a time-dependent manner.

Finally, in a parallel set of experiments, we demonstrate that in rats previously trained to intravenously self-administer the synthetic CB_1 receptor agonist WIN 55,212-2, extinguished drug-seeking behaviour is reinstated by intraperitoneal priming injections of the same CB_1 receptor agonist as well as heroin, but not cocaine, primings, thus demonstrating the bi-directionality of cannabinoid-opioid interactions in modulating central mechanisms underlying relapse phenomenon. Moreover, drug-induced reinstatement of cannabinoid-seeking is fully prevented by pretreatment with both SR 141716A and naloxone.

Taken together, these findings strengthen our previous hypothesis of a strict functional interaction between the cannabinoid and opioid receptors in the modulation of central mechanisms triggering relapse to drug-seeking.

MODULATION OF THE REINFORCING AND DISCRIMINATIVE STIMULUS EFFECTS OF THC BY ALTERATIONS IN OPIOID SYSTEM ACTIVITY

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Many of the behavioral effects of THC and other cannabinoids in experimental animals can be blocked by opioid antagonists or are diminished or absent in opioid-receptor deficient mice (Maldonado and Rodriguez de Fonseca **2002**; Tanda and Goldberg **2003** for review). For example, the opiate antagonist naloxone blocks 1) self-administration behavior maintained by synthetic cannabinoid CB_1 receptor agonists (Navarro et al. **2001**; Braida et al. **2001**a; Braida et al. **2001**), 2) the enhancement of electrical brain-stimulation reward produced by THC (Gardner et al. **1989**), and 3) increases in dopamine levels in the nucleus accumbens produced by THC (Chen et al. **1990**; Tanda et al. **1997**). We have investigated in squirrel monkeys whether treatment with naltrexone alters either drug-taking behavior under a fixed-ratio schedule of THC injection or drug-seeking behavior under a second-order schedule of THC injection. Presession treatment with naltrexone markedly reduced peak levels of drug-taking and drug-seeking behavior, but did not completely block drug-taking behavior. These findings suggest a facilitatory modulation of cannabinoid reinforcement by endogenous opioid activity that is unmasked by treatment with an opioid antagonist. Also, THC-seeking behavior that had been extinguished by simple substitution of vehicle for THC under the second-order schedule could be rapidly and repeatedly reinstated by pre-session injection of either THC or morphine, but not by pre-session injection of cocaine. Reinstatement of extinguished THC-seeking behavior by morphine provides further evidence of opioid system modulation of THC's reinforcing effects.

In other experiments with rats that had learned to discriminate injections of THC from injections of vehicle, systemic administration of the opioid agonist morphine did not produce THC-like discriminative effects but markedly potentiated discrimination of THC. Conversely, the opioid antagonist naloxone reduced the discriminated effects of THC, confirming a facilitatory role for endogenous opioid systems in the discriminative-stimulus effects of THC. One possible unexplored mechanism for such opioid-cannabinoid interactions is that direct actions of THC or other cannabinoids at central CB_1 receptors have the secondary effect of releasing endogenous opioids such as β-endorphin, which results in a facilitation of cannabinoid effects. Using *in-vivo* microdialysis techniques, we found that THC produces large increases in extracellular levels of β-endorphin in the ventral tegmental area (VTA) and lesser increases in the shell of the nucleus accumbens. Bilateral microinjections of β-endorphin directly into the VTA, but not into the shell of the nucleus accumbens, markedly potentiated the discriminative effects of ineffective threshold doses of THC, but had no effect when given alone. This potentiation was blocked by naloxone. Altogether these results indicate that psychotropic effects of THC related to drug abuse liability are regulated by THC-induced elevations in extracellular β-endorphin levels in brain areas involved in reward and reinforcement processes. These findings suggest a novel mechanism that may underlie many previous observations of opioid system modulation of cannabinoid effects.

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EFFECT OF CANNABINOIDS ON REINSTATEMENT OF MORPHINE CONDITIONED PLACE PREFERENCE (CPP) IN MICE

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It has been reported that cannabinoid and opioid systems interact in modulating the behaviour of drug-abuse. In our laboratory we have recently shown that a single injection (priming) of WIN 55,212-2, the central cannabinoid receptors (CB_1) agonist, reinstated heroin-seeking behaviour in rats after a prolonged drug-free period, suggesting a strong correlation between cannabinoids and opioids in the mechanisms of relapse. In this study we have verified whether cannabinoids were also able to reinstate an extinguished CPP of morphine in mice.

The CPP apparatus consisted of eight rectangular opaque boxes, separated by a guillotine door into two distinct compartments of equal size. Each compartment had different visual and tactile cues. The place preference protocol was performed in four different phases: preconditioning (I), conditioning (II), extinction (III) and reinstatement (IV) phase, the total time required was 18 days. (I) On the first day, each mouse was allowed to explore both compartments for 15 min period. On the second day, the time spent in each compartment was recorded during 15 min. (II) From the third day onwards, mice were treated for 8 days with alternated sub-cutaneous (s.c.) injections of morphine or saline and immediately confined to the assigned (drug-paired) treatment compartment for 30 min. (III) As CPP developed, all mice received a saline injection for six consecutive days and were alternatively confined in the drug- and saline-paired compartment. (IV) The day following the extinction of CPP, one of the five morphine groups was treated with morphine, the second and the third were challenged with an intraperitoneal priming of Δ^9-THC and WIN 55,212-2, the fourth received SR 141716A 20 min before the test and the fifth morphine group was treated with SR 141716A+morphine given immediately before starting test session. At the end of each phase, time spent by the animals in drug-paired sides was recorded.

Our results showed that morphine induced a clear CPP after a period of conditioning. We also found that morphine, THC or WIN 55,212-2 primings reinstated an extinguished morphine-induced CPP, while pretreatment with the CB_1 antagonist SR 141716A did not restore CPP by itself, but antagonized the effect induced by morphine and cannabinoid primings. These results are in line with our previous studies and strengthen the hypothesis of a functional interaction between the cannabinoid and opioid endogenous systems in modulating reinstatement of drug-seeking behaviour.

REINFORCING PROPERTIES OF COCAINE IN MICE LACKING CB_1 CANNABINOID RECEPTOR

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Acute rewarding properties of cocaine have been shown to be preserved in CB_1 knockout mice by using both place conditioning (Martin et al. **2000**, Eur J Neurosci. 12:4038-46) and acute self-administration paradigms (Cossu et al. **2001** Behav Brain Res. 118:61-5). Accordingly, acute cocaine self-administration were not modified in mice lacking CB_1 cannabinoid receptor. However, most recent studies suggest that CB_1 cannabinoid receptors may mediate some processes related to drug addiction such as relapse to cocaine seeking behaviour (de Vries et al. **2001**, Nat Med. 7:1151-4). Therefore, we evaluated whether or not CB_1 KO mice would self-administer cocaine in operant conditions. CB_1 KO mice and their wild type littermates were trained to nose poke either for food, water or cocaine (1 mg/kg/infusion; i.v.) reward under FR1 (acquisition) and FR3 (maintenance) schedules of reinforcement. Results showed that CB_1 KO mice did not significantly differ from WT mice in the acquisition and maintenance of food and water self-administration under FR schedule of reinforcement. In contrast, while CB_1 KO mice acquired self-administration of cocaine as their wild type littermates, the maintenance of this behaviour reflected in the FR3 schedule, was attenuated in the CB_1 KO mice. Microdialysis studies were performed to elucidate if these behavioural differences may be correlated to changes in extracellular dopamine levels into the nucleus accumbens. Preliminary results show that acute injection of cocaine (20 mg/kg, i.p) produced same dopamine increase in both genotypes. These results showed that acquisition an acute effects of cocaine are maintained in mice lacking CB_1 cannabinoid receptor while the maintenance of drug seeking behaviour was modified in this mice.

INTERACTIONS BETWEEN DOPAMINE D2 RECEPTORS AND ENDOCANNABINOIDS MEDIATE THE EFFECTS OF COCAINE IN THE STRIATUM

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Compelling evidence indicates that endocannabinoids are implicated in drug addiction. In the present study, we have addressed the interaction between cocaine and endocannabinoid system by means of biochemical and electrophysiological experiments in rat brain slices. Our results show that cocaine, through the stimulation of dopamine D2-like receptors, increases the levels of the endocannabinoid anandamide in the striatum, a brain area primarily involved in the compulsive drug-seeking and drug-taking behaviours typical of addiction. The cocaine-induced increase in anandamide concentrations was attributable to both stimulation of its synthesis and inhibition of its degradation, and facilitated, through the activation of cannabinoid CB_1 receptors, the dopamine D2-receptor-dependent effects of cocaine. Enhanced function of CB_1 receptors likely contributed to this action, as suggested by the finding that also the binding properties of these receptors increased following cocaine administration.

Understanding the molecular and physiological effects of drugs of abuse in the brain is essential for the development of effective strategies against addiction.

THE DA D3 RECEPTOR ANTAGONIST SB-277011A ANTAGONIZES THC-ENHANCED BRAIN-STIMULATION REWARD IN RATS

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Marijuana is a widely used botanical with abuse liability, and there is no widely effective medication available to assist marijuana users in breaking the habit. Delta-9-tetrahydro-cannabinol (THC), the active constituent in marijuana, stimulates the brain mesolimbic dopamine (DA) system and enhances brain stimulation reward, like other drugs with abuse potential. The mesolimbic DA system is highly enriched with DA D3 receptors, which have extraordinarily high affinity for endogenous DA. We have previously shown that blockade of brain DA D3 receptors with the novel D3-selective receptor antagonist SB-277011A robustly attenuates cocaine-enhanced brain reward and cocaine-induced reinstatement of drug-seeking behavior. SB-277011A has also been previously shown to attenuate nicotine-triggered relapse to nicotine-seeking behavior in the reinstatement model. The purpose of the present study was to determine whether SB-277011A inhibits THC-induced reward, as assessed by attenuation of THC-enhanced electrical brain stimulation reward. Rats were trained to lever press for rewarding electrical brain stimulation of the medial forebrain bundle at the level of the lateral hypothalamus and tested on a rate-frequency curve-shift electrical brain-stimulation reward paradigm. THC (0.25 mg/kg i.p.) reliably shifted brain-reward stimulation curves to the left, lowering stimulation thresholds by around 15%. SB-277011A (12 mg/kg i.p., administered 1 hour prior to brain stimulation) attenuated THC-enhanced brain stimulation reward. These data suggest that DA D3 receptors play an important role in mediating marijuana-enhanced brain stimulation reward, and that the highly selective D3 receptor antagonist SB-277011A may be promising in the treatment of marijuana dependence.

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IMPAIRED PERFORMANCE ON A DECISION-MAKING TASK BY HEAVY MARIJUANA USERS: AN FMRI STUDY

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The majority of studies examining the consequences of long-term marijuana use on brain function have focused on aspects of memory and related cognitive processes. In contrast few studies have examined performance on other types of tasks, particularly those involving judgment and decision-making ability. A number of recent studies have shown that chronic exposure to abused substances such as psychostimulants, opiates and alcohol produces impaired decision-making task performance. The purpose of this study was to determine if chronic marijuana users would also display dysfunctional decision-making and if deficits would be reflected in patterns of neural activation.

We conducted an fMRI study using the Iowa Gambling Task (Bechara et al., Cognition, **1994**; 50:7) as adapted for use in the scanner. The Gambling Task measures the ability to balance immediate rewards against future negative consequences. Subjects select cards from one of four decks, two of which are advantageous resulting in a net gain and two disadvantageous resulting in a net loss. Eighteen heavy users (individuals who had smoked marijuana daily for at least 5 years) were compared to sixteen control subjects (individuals who had smoked marijuana no more than 50 times in their lives). Groups did not differ significantly in gender distribution, age, or nicotine, alcohol and caffeine use. The user group performed more poorly than controls (mean ± SEM; net score -25.1 ± 7, vs.18.6 ± 7) over the course of 100 card choices. Net score correlated significantly with age of first marijuana use (r_{xy} = .662, P < .01), but not duration of use (r_{xy} = -.318).

Users and controls displayed similar patterns of activation during task performance and included the occipital cortex (BA18), middle and superior frontal gyri (BA6, 8, 9), and the precuneus (BA40,7). Users, however, also displayed significant task-related deactivation within the anterior cingulate cortex (BA24, 32). Activity in this region was significantly correlated to gambling scores of users and controls (r_{xy} = .599, P < .001). These results show that chronic marijuana use is associated with impaired decision-making and that this is reflected in a failure to activate the anterior cingulate cortex. This implies that heavy marijuana use may be associated with a more general deficit in the processing of information related to conflicting outcomes.

Acknowledgments: This work was supported by DA10230 (LJP), DA12137 (AL) and DA07246 (CML)

A NOVEL COMPONENT OF CANNABIS EXTRACT POTENTIATES EXCITATORY SYNAPTIC TRANSMISSION IN RAT OLFACTORY CORTEX *IN VITRO*

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Cannabis has been proposed as a potential treatment for epilepsy, with some patients taking the herb illicitly to alleviate their symptoms (Grotenhermen and Schnelle, J. Cann. Ther. **2003** 3:17), although the few human studies available to support this use have given equivocal results. Cannabinoid CB_1 receptor agonists are currently considered primary targets for investigation (Croxford, CNS Drugs, **2003**, 17:179; Baker et al, Lancet Neurology, **2003** 2:291; Wallace et al **2002**, Eur. J. Pharmacol. 452:295). We have previously shown that a standardized cannabis extract (SCE), isolated Δ^9-tetrahydrocannabinol (Δ^9-THC), and even cannabis extract devoid of Δ^9-THC (Δ^9-THC-free SCE) can inhibit epileptiform bursting in a rat olfactory cortical brain slice model of muscarinic agonist-induced seizures (Postlethwaite et al J. Neurophysiol. **1998**, 79:2003), acting via a cannabinoid CB_1-receptor mechanism (Wilkinson et al, J. Pharm. Pharmacol. **2003**, 55:1687). Further work presented here demonstrates that although Δ^9-THC (1 μM) can produce a significant depression of evoked depolarizing postsynaptic potentials (PSP) in olfactory cortex neurones, which is fully reversed by the CB_1 antagonist SR141716A (1 μM), SCE's *both with and without* of Δ^9-THC (in an equivalent concentration) caused a significant *potentiation* of evoked PSP amplitude, again reversed by the CB_1 antagonist. The synaptic potentiating effect of Δ^9-THC-free SCE was *greater* than that produced by SCE. We also compared the effect of the Δ^9-THC-free SCE on evoked PSP's and artificial PSP's (aPSP) evoked electrotonically by a brief intracellular current injection, to determine whether the observed increase in background cell input resistance could account for this effect. In such experiments, the synaptic potentials were enhanced, whereas the aPSPs remained unaffected, suggesting that the effect was not due to changes in electrical membrane properties. Similar recordings were made using CB_1 receptor-deficient knockout mice ($CB_1^{-/-}$) and wild-type littermate controls. Control recordings confirmed that the cannabinoid or extract-induced changes in membrane resistance, cell excitability and synaptic transmission, were similar to those seen in rat olfactory cortex neurones. However, in $CB_1^{-/-}$ cells, Δ^9-THC, SCE and Δ^9-THC-free SCE produced no observable changes in cell membrane properties or synaptic amplitude. Interestingly, the potentiating effects and enhancement of cell excitability of the unknown extract constituent(s) on neurotransmission were capable of over-riding the predominantly suppressive effects of Δ^9-THC on excitatory neurotransmitter release. This phenomenon may possibly explain the preference by some patients for herbal cannabis rather than isolated Δ^9-THC (due to attenuation of some of the central Δ^9-THC side effects) and even account for the rare incidence of seizure episodes in some individuals taking cannabis recreationally.

Acknowledgements: BJW was supported by a School of Pharmacy Studentship. Thanks to David Baker for providing receptor-deficient knockout mice and wild-type littermate controls.

CANNABIDIOL AFFECTS mGluR- BUT NOT NMDA-MEDIATED CALCIUM RESPONSES IN HIPPOCAMPAL NEURONES

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Cannabinoids show both neuroprotective and neuromodulatory effects [1,2]; however the underlying mechanisms remain to be fully elucidated. Here, we investigated whether cannabidiol (CBD), the major non-psychotropic plant cannabinoid, can modulate Ca^{2+} signalling in primary hippocampal cultures.

The hippocampi of Sprague-Dawley neonates (1-3 days) were dissected and the tissue enzymatically dissociated. Cultures were matured for 5-12 days in medium containing 90% minimal essential medium (MEM) and 10% foetal bovine serum (FBS). One hour prior to imaging, cultures were loaded with Fura-2AM (6–10 μM). Cultures were perfused with HEPES buffered solution (HBS) (containing 0.5 μM TTX) and $[Ca^{2+}]_i$ measurements were calculated as ratio units (from images captured at wavelengths 350 nm and 380 nm).

Since glutamate-mediated Ca^{2+} rise can lead to excitotoxicity, we investigated whether CBD influenced this process. Application of CBD alone (1 μM) induced a Ca^{2+} response (range: 0.15-1 ratio units) in both neurones (46%; n=23/50) and glia (83%; n=55/66). Ca^{2+} signals evoked with glutamate (5-10 μM; n=58) and with the subtype-specific glutamate receptor agonist kainate (10-50 μM, n=64) were decreased by 35.4% and 49.5% ($P<0.0001$), respectively, in the presence of CBD (1 μM). However, Ca^{2+} responses induced with N-methyl-D-aspartate (NMDA, 10 μM; n=116), were not significantly affected. Interestingly, responses evoked with the metabotropic glutamate receptor group I agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 10-50 μM, n=30) were most potently affected by CBD and reduced to 17.8% of control ($P<0.0001$).

Further experiments with thapsigargin (2 μM; n=178), which depletes $[Ca^{2+}]_i$ stores, demonstrated the dependence of the CBD-induced Ca^{2+} rise on such stores. In addition, CBD (1 μM) was shown to lower $[Ca^{2+}]_i$ raised by thapsigargin (by 19%). In the presence of nifedipine (L-type Ca^{2+} channel blocker) and cadmium (a general Ca^{2+} channel blocker), the CBD-induced Ca^{2+} rise was significantly reduced (61% and 66% respectively), suggesting a possible link between $[Ca^{2+}]_i$ stores and L-type Ca^{2+} channels.

In conclusion, we present data on the mechanisms by which CBD may exert its neuroprotective effect. CBD can reduce glutamate-triggered Ca^{2+} responses and modulate $[Ca^{2+}]_i$ via intracellular stores. This further highlights the potential of CBD as a promising neuroprotective agent.

[1] Platt, B., Drysdale, A.J. (**2004**). Current Neuropharmacology, 2(1): 103-114.
[2] Drysdale, A.J., Platt, B. (**2003**). Current Medicinal Chemistry, 10(24): 2719-2732.

EVALUATION OF THE INTRANASAL DELIVERY OF CANNABIDIOL IN RATS

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Cannabidiol (CBD) is a cannabinoid that has shown promise as a treatment for multiple conditions, including multiple sclerosis, pain, psychosis, dystonic movement disorders, and epilepsy. Intranasal dosing has advantages over oral dosing, including a rapid onset of drug effect and bypassing the first-pass effect. Intranasal delivery may be the therapeutic answer for severely nauseated patients and those with other gastrointestinal complications. When rapid attainment of a drug blood level is necessary, intranasal delivery is superior to a drug injection because it is a noninvasive pain-free treatment that can improve patient compliance. Evaluation of cannabidiol intranasal bioavailability was completed in an anesthetized rat nasal absorption model. Plasma samples were analyzed by LC-MS after intravenous (I.V.) and intranasal (I.N.) dosing, and the data was analyzed with WinNonlin pharmacokinetic software. The intranasal formulation consisted of drug in propylene glycol, and the intravenous dose was solubilized in 5% propylene glycol:95% saline containing 3% Tween 80.

Pharmacokinetic Parameters Obtained After I.V. and I.N. CBD Dosing in Rats

Parameter	I.V. (n=4) Mean ± s.d.	I.N. (n=3) Mean ± s.d.
AUC (ng/mL*h)	112 ± 21	84 ± 43
α (h^{-1})	82 ± 31	1.6 ± 1.0
β (h^{-1})	0.38 ± 0.14	0.75 ± .21
$t_{1/2}$ (α) (h)	0.01 ± 0.001	0.55 ± .34
$t_{1/2}$ (β) (h)	2.19 ± 1.23	0.96 ± .26
C_{max}(ng/mL)	2438 ± 904	44 ± 8.4
CL_{tot} (L/h)	9.76 ± 2.36	13.6 ± 7.0
T_{max} (h)	--	0.5
MRT (h)	1.3 ± 0.2	1.5 ± 0.2
V_{ss} (L/kg)	11.6 ± 2.0	33.2 ± 27.2*
F bioavailability	1.0	0.75 ± .38

*V_{ss}/F

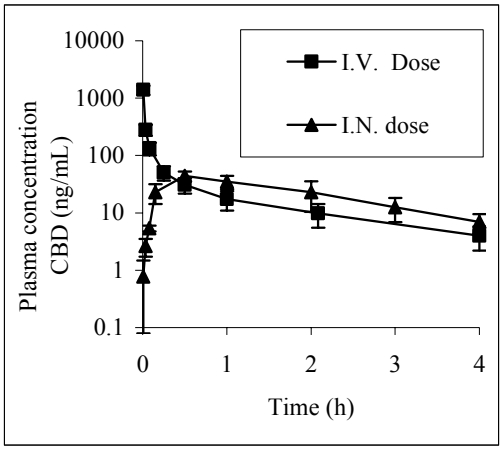

Figure 1. Plasma concentration-time profiles of cannabidiol after 1 mg/kg I.V. or I.N. drug dosing in rats

Cannabidiol was well absorbed from the nasal cavity, as observed from the 75% absolute bioavailability and the maximum drug concentration (44 ng/mL) occurrence at 30 minutes (T_{max}). The intranasal delivery route appears to be a viable option for cannabidiol. Future studies will include optimization of the intranasal drug formulation for enhanced rapid absorption.

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CANNABIDIOL INHIBITS THE EFFECTS OF PSYCHOTOMIMETIC DRUGS IN MICE

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Introduction: Cannabidiol (CBD), a compound isolated from *Cannabis sativa*, is a possible antipsychotic cannabinoid. It may inhibit some effects of Δ^9THC. Moreover, like Haloperidol, it inhibits stereotyped behaviour induced by Apomorphine in rats. The aim of this work was to test the hypothesis that CBD would attenuate the effects of psychotomimetic drugs in mice. Methods: Swiss mice (n=7) received ip injections of CBD (15, 30 or 60 mg/kg) or Vehicle (VEH) 20 minutes before Amphetamine (AMP, 5 mg/kg) or saline (SAL). In another experiment, CBD or VEH were injected 10 minutes before Ketamine (KET, 60 mg/kg) or SAL. 30 minutes after the CBD injection the locomotor activity was measured in an open field during 10 minutes. The data were analysed by ANOVA followed by the Duncan test. Results: AMP increased the distance moved in the open field. CBD did not induced a sedative effect and inhibited the effect of AMP in a dose-related fashion ($p<0.05$ for 30 mg/kg and 60 mg/kg) [Distance moved (mean ± sem): VEH+SAL = 2518±419 cm; CBD(60)+SAL = 2030±256; VEH+AMP = 5326±1160; CBD(15)+AMP = 4838±683; CBD(30)+AMP = 2689±383; CBD(60)+AMP = 1730±379]. There was a tendency for CBD 30 mg/kg to inhibit the effect of KET ($p<0.1$) [Distance moved (mean ± sem): VEH+SAL = 2581±273 cm; CBD+SAL = 1977±84; VEH+KET = 5154±235; CBD(15)+ KET = 4191±985; CBD(30)+ KET = 3253±506; CBD(60)+ KET = 4127±962]. Conclusion: CBD inhibits the effects of psychotomimetic drugs in mice. It is a possible antipsychotic cannabinoid.

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CALIFORNIA CANNABIS CONSULTANCY
PROPOSED MINIMUM PRACTICE STANDARD

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Background

1996 California voters approved the Compassionate Use Act of **1996** (H&S code sec 11362.5) indemnifying persons who obtained recommendation and or approval for the use of cannabis from a licensed California physician.

Because of vagueness in both the law and California medical practice standards, physicians who developed practices with significant numbers of cannabis self medicators became targets of investigation by local criminal justice authorities, California Medical Board and the California Attorney General.

As minimum practice standards were not defined, the California Cannabis Research Medical Group proposed a minimum practice standard (MPS) and presented it to the local medical association. While the Alameda - Contra Costa Medical Association did not support the resolution, but was referred to the California Medical Association where the MPS was modified into a mandate for CMA and CMB to develop a MPS.

Method

Persons who desired legal status are interviewed by California licensed physicians. A medical questionnaire is completed by the applicant and reviewed with the physician. A yearly follow up interview review another questionnaire. CCRMG MPS components of :

1. The initial examination is "face to face," in person, confidential, and live. (Follow up may be video, photographic, telephonic, or email.)
2. The examination is memorialized with elements of: Name, sex, birthdate, address, phone number, date of examination, codable diagnoses.
3. Documentation supporting the diagnoses.
   Compliance with HIPAA where required.

Results

A reasonable and inclusive medicolegal consultative record is created to facilitate long term and continuing research into the efficacy and costs of cannabis as well as provide information for administrative purposes.

Discussion

A specific, concise and well-defined standard of cannabis consultative practice is created in California for medical research and legal documentation.

ATROPHIE BLANCHE TREATED WITH CANNABIS AND/OR THC

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Introduction

Atrophie blanche / livedoid vasculitis, is a chronic non-palpable primary cutaneous purpuric clotting disorder with petechiae of autoimmune etiology. Clinically, there is recurrent breakdown of tissue with edema, pain, and secondary infection.

Method

L.H.R., 56 year old single caucasian accountant was evaluated for eligibility for the California Compassionate Use Act.

History

He contracted this rare condition after infection of his feet. While incarcerated in prison he abstained from using cannabis. The condition gradually worsened for the next 4 years until his release from a half way house. (A course of antihypertensive drugs may have also aggravated during the onset stages.) He underwent venous stripping and numerous courses of topical and systemic antibiotics without relief. The recurrent attacks of painful breakdown of skin and underlying tissue with edema caused him to be immobilized and house bound. He experienced significant reactive depression secondary to pain and immobility.

After release he resumed his use of cannabis begun at age 15. He noted significant relief of symptoms and decreased frequency of attacks using high grade cannabis smoked twice daily.

He is currently maintained on coumadin 5mg daily, digoxin 0.25 mg daily.

Findings

The feet exhibited some thinning, tightening, and rubor of the skin with mild atrophic changes. The patient walked gingerly but with essentially normal gait.

Results

Marinol 10 mg 1 – 2 Q6 PRN was begun. The patient reports excellent results using 10 mg BID with no relapses. His depression has gradually lifted- commensurate with his improved mobility.

Conclusion

THC and cannabis would appear to have analgesic immunomodulative and antidepressive properties.

CANNABIDIOL PRESERVES RETINAL NEURONS AND REDUCES VASCULAR PERMEABILITY IN EXPERIMENTAL DIABETES

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Glutamate-induced oxidative stress is likely responsible for the early clinical features of diabetic retinopathy, including increased vascular permeability and neuronal cell injury. We have previously demonstrated the causal effects of reactive oxygen species in stimulating the blood-retinal barrier breakdown in experimental diabetic rats and increasing retinal neuronal cell death in intravitreal N-methyl-D-aspartate (NMDA)-injected rats. We have also shown that the non-psychotropic cannabinoid cannabidiol (CBD) prevents NMDA-induced retinal neuronal cell death. Based on these, we test the hypothesis that CBD reduces vascular permeability and preserves retinal neuronal cells in experimental diabetes. Experimental diabetes was induced by streptozotocin injection in rats. Animals were treated with CBD (10 mg/kg of body weight) by intraperitoneal injections every two days. Control animals received vehicle injections. Vascular permeability was determined by albumin leakage analysis. Apoptosis was determined by terminal dUTP nick end-labeling-horseradish peroxidase (TUNEL-HRP) analysis in whole-mount retinas. Albumin leakage analysis showed a 4-fold increase in vascular permeability after 2 weeks of diabetes as compared to the non-diabetic control. TUNEL-HRP analysis showed a 7-fold increase in the number of apoptotic retinal cells after 4 weeks of diabetes as compared to the non-diabetic control. CBD treatment reduced vascular permeability by 50% and reduced the number of TUNEL-positive apoptotic cells by 60% as compared to the age-matched diabetic control. These results suggest that CBD could be a valuable new therapy for the treatment/prevention of diabetes' retinal complications.

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TO INVESTIGATE THE ANTI-EMETIC ACTION OF CANNABINOID EXTRACTS CONTAINING HIGH LEVELS OF CANNABIDIOL AND CANNABIDIOLIC ACID ON MOTION INDUCED EMESIS IN SUNCUS MURINUS

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A cannabinoid extract containing >6.3% cannabidiol (CBD) and <2% tetrahydrocannabinol (THC) was found to significantly attenuate motion induced emesis in Suncus murinus (Javid et al., FENS Abstr. A142.14. **2002**). It was further identified that the CBD content of the extract was composed of 7 parts cannabidiolic acid (CBDA) and 1 part CBD. The aim of the present work was to further investigate the antiemetic potential of CBD, CBDA and a botanical drug substance containing high levels (63.8%) of CBD (BDS (CBD)).

Adult Suncus murinus (61.4±1.6g) were administered i.p. with either CBD (0.5, 1 or 2 mg/kg), CBDA (0.02, 0.1 or 0.5 mg/kg), BDS (CBD) (0.25, 0.5 or 1 mg/kg) or vehicle. The animals were placed individually in transparent cages (100(W) X 150(L) X 150 (H) mm) of a motion stimulator and observed for 45 min for any behavioural changes before motion was applied, at a frequency of 1 Hz and amplitude of 40 mm for 10 min. The number of emetic episodes and latency of onset to the first episode (s) were recorded. Data were expressed as the mean ± S.E.M. of n=5-6 and analysed using the Students t-test with $P<0.05$ being taken as a significant difference between treatments. The number of emetic episodes was reduced by 67% (from 11.5±2.3 to 3.8±1.1 emetic episodes ($P<0.05$)) and 94% (from 12.0±2.2 to 0.8±0.8 emetic episodes ($P<0.001$)) following the i.p. injection of 0.1 and 0.5 mg/kg CBDA respectively, as compared to the vehicle treated animals. Administration of 0.1 and 0.5 mg/kg CBDA significantly ($P< 0.01$) increased the latency of onset to the first emetic episode from 115.3±23.2 to 400.2±80.3 s and 153±31.9 to 500.4±83.0 s respectively, as compared to the vehicle treated animals. Animals injected with 0.02 mg/kg CBDA showed a comparable emetic response to motion to the vehicle treated animals ($P>0.05$). In shrews challenged with 0.5, 1 and 2 mg/kg CBD the number of emetic episodes and latency of onset to the first emetic episode were comparable to those in the vehicle treated animals ($P>0.05$). Following the administration of 0.25, 0.5 and 1 mg/kg BDS (CBD) both the number of emetic episodes and latency of onset of emesis were comparable to values observed in the vehicle treated animals ($P>0.05$).

In summary, the administration of CBDA dose-dependently attenuated the emetic response to motion in S. murinus; CBD and BDS (CBD) were ineffective. The data indicates an antiemetic pharmacological activity of a cannabinoid carboxylic acid in Suncus murinus.

TO INVESTIGATE THE EFFECT OF CANNABINOID EXTRACTS CONTAINING HIGH LEVELS OF TETRAHYDROCANNABINOL AND TETRAHYDROCANNABINOLIC ACID ON MOTION INDUCED EMESIS IN *SUNCUS MURINUS*

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It is well established that tetrahydrocannabinol (THC) has anti-emetic actions against nausea and vomiting induced by cytotoxic-chemotherapy (Ward & Holmes, Drugs, 30, 127) and radiation therapy (Priestman et al., Clin Radiol, 38, 543). However, its effect on motion-induced emesis is unknown.

Adult *Suncus murinus* (59.7±1.4g) were administered i.p. with either THC (0.25, 0.5 or 1 mg/kg), tetrahydrocannabinolic acid (THCA) (0.25, 0.5 or 1 mg/kg), botanical drug substance containing 73% THC (BDS (THC)) (0.25, 0.5 or 1 mg/kg) or vehicle. The animals were placed individually in transparent cages (100(W) X 150(L) X 150 (H) mm) of a motion stimulator and observed for 45 min for any behavioural changes before motion was applied at a frequency of 1 Hz and amplitude of 40 mm for 10 min. The number of emetic episodes and latency of onset to the first episode (s) were recorded. Data were expressed as the mean ± S.E.M. of n=5-10 and analysed using the Students t-test with $P<0.05$ being taken as a significant difference between treatments. The number of motion-induced emetic episodes and latency of onset of emesis recorded following pre-treatment with 0.25, 0.5 and 1 mg/kg THC were comparable to the vehicle treated animals ($P>0.05$). In shrews challenged with 0.25, 0.5 and 1 mg/kg THCA the number of emetic episodes in response to motion were comparable to values observed in the vehicle treated animals ($P>0.05$). However, the latency of onset to the first emetic episode was reduced from 395.5±80.5 to 191.8±32.1 s ($P<0.05$) and from 393±63.9 to 125.2±29.9 s ($P<0.01$) following the i.p. administration of 0.25 and 0.5 mg/kg THCA respectively, as compared to the vehicle treated animals. Animals injected with 1 mg/kg THCA showed a comparable latency of onset to the first emetic episode as the vehicle treated animals ($P>0.5$). I.p. injection of 0.25, 0.5 and 1 mg/kg BDS (THC) did not affect motion-induced emesis in *S.murinus* compared to the vehicle treated animals ($P>0.05$).

In contrast to its antiemetic effect on drug-induced emesis, it appears that THC may not be anti-emetic to motion-induced emesis in *S.murinus* using the described parameters.

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