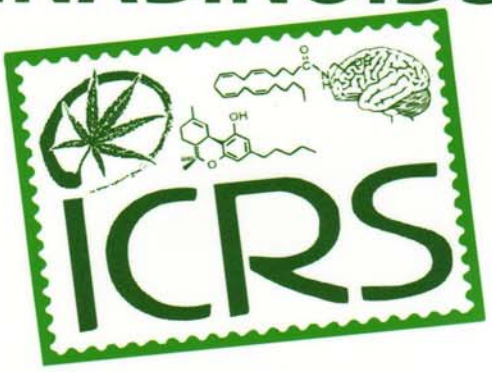
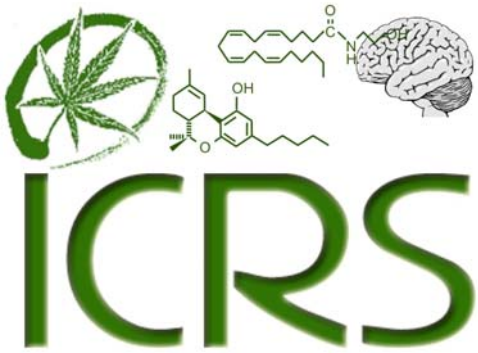
16TH ANNUAL SYMPOSIUM ON THE CANNABINOIDS



Hotel Club Tihany
Tihany, Hungary
June 24-28, 2006

PROGRAM AND ABSTRACTS



Program and Abstracts

16TH ANNUAL
SYMPOSIUM ON THE CANNABINOIDS

Hotel Club Tihany
Tihany, Hungary

JUNE 24 – 28, 2006

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University of Vermont
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2006 Symposium on the Cannabinoids

June 24-28, Club Tihany
Tihany, Hungary

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16 th Annual Symposium On The Cannabinoids

2006 Program

Registration June 24, 2006 (1600-2000)

Day 1
Sunday, June 25th

7.50 - 8.00	***OPENING REMARKS***		
Session 1. SAR Studies and New Synthetic Molecules ➢ Chairs*: Drs. Brian Thomas and Raj Razdan*			Page #
8.00	Yehoshua Maor, Ruth Gallily and Raphael Mechoulam	THE RELEVANCE OF THE STERIC FACTOR IN THE BIOLOGICAL ACTIVITY OF CBD DERIVATIVES – A TOOL IN IDENTIFYING NOVEL MOLECULAR TARGET FOR CANNABINOIDS	1
8.15	John W. Huffman, Lea W. Padgett, Matthew L. Isherwood, Jenny L. Wiley and Billy R. Martin	1-PENTYL-2-ARYL-4-(1-NAPHTHOYL)PYRROLES: A NEW CLASS OF HIGH AFFINITY LIGANDS FOR THE CB1 AND CB2 RECEPTORS	2
8.30	T.M. Fong, L.S. Lin, X-M Guan, D.J. Marsh, C-P Shen, J. Lao, D.S. Stribling, K.M. Rosko, H. Yu, Y. Feng, J.C. Xiao , L.H.T. Van der Ploeg, M.T. Goulet, W.K. Hagmann, T.J. Lanza, Jr., J.P. Jewell, P. Liu, S.K. Shah, H. Qi, X. Tong, J. Wang, and S. Xu, R. Gibson, B. Francis, S. Patel, A.M. Strack, D.E. MacIntyre and L.P. Shearman	DISCOVERY OF A NOVEL CB1R INVERSE AGONIST	3
8.45	Yanan Zhang, Marcus Brackeen, Anne Gilliam, Herbert Seltzman and Brian Thomas	CONFORMATIONALLY-CONSTRAINED ANALOGS OF SR141716	4
Session 2. Receptor Structure and Signal Transduction ➢ Chairs*: Drs. Ruth Ross and Allyn Howlett*			
9.00	Itai Bab, Joseph Tam, Orr Ofek, Catherine Ledent, Ester Fride, Yankel Gabet, Ralph Müller, Andreas Zimmer, Ken Mackie, Raphael Mechoulam and Esther Shohami	INVOLVEMENT OF CB1 SIGNALLING IN BONE REMODELLING	5
9.15	Sharon Anavi-Goffer, Daniel Fleischer, Dow P. Hurst, Diane L. Lynch, Judy Barnett-Norris, Shanping Shi, Deborah L. Lewis, Somnath Mukhopadhyay, Allyn C. Howlett, Patricia H. Reggio and Mary E. Abood	HELIX 8 LEUCINE OF CB1 CANNABINOID RECEPTOR PROMOTES SELECTIVE G PROTEIN COUPLING	6
9.30	Sergio Oddi, Monica Bari, Filomena Fezza, Valeria Gasperi, Paola Spagnuolo, Nicoletta Pasquariello, Alessandro Finazzi-Agrò and Mauro Maccarrone	LIPID RAFTS, CB2 RECEPTOR SIGNALING AND METABOLISM OF 2-ARACHIDONOYL-GLYCEROL IN HUMAN IMMUNE CELLS	7

9.45	Adèle Thomas, Gemma Baillie, Alexander Phillips, Ruth Ross and Roger Pertwee	CANNABIDIOL IS AN INVERSE AGONIST AND EXHIBITS UNEXPECTEDLY HIGH POTENCY AS AN ANTAGONIST AT MOUSE BRAIN CB1 AND HUMAN CB2 RECEPTORS	8
10.00	Ruth A. Ross, Gemma L. Baillie, Cherry Alexander, Emma Baillie, Lisa Gauson, Sarah Robertson, Lesley A. Stevenson, Kirsty Muirhead, Laurent Trembleau and Roger G. Pertwee	ALLOSTERIC MODULATION OF THE CANNABINOID CB1 RECEPTOR: NOVEL ALLOSTERIC MODULATORS	9
10.15	Allyn Howlett, Jenelle Jones and Derek Norford	CB1 CANNABINOID RECEPTORS MEDIATE TRANSLOCATION OF NO-SENSITIVE GUANYLYL CYCLASE IN NEURONAL CELLS	10
10.30 - 11.00	**Coffee Break**		
11.00	Christopher Kearn, Giovanna Cacciola, Tanya Daigle and Ken Mackie	RAPID RECEPTOR DESENSITIZATION MODULATES CANNABINOID RECEPTOR ACTIVATION OF P42/P44 MAP KINASE	11
11.15	Chris Breivogel and Vanita Puri	THE EFFECTS OF CHRONIC CANNABINOIDS IN BETA-ARRESTIN2 -/- MICE	12
11.30	Neta Rimmerman, Heather Bradshaw, David O'Dell and J. Michael Walker	CELLULAR SIGNALING PROPERTIES OF THE NEWLY IDENTIFIED FATTY ACID AMIDE N-PALMITOYL GLYCINE FOUND IN RAT CENTRAL NERVOUS SYSTEM AND SKIN	13
11.45	Patricia Reggio, Dow Hurst, Diane Lynch, Judy Norris, Sharon Anavi-Goffer and Mary Abood	CB1 L7.60I AND L7.60F MUTATIONS RESULT IN CONFORMATIONAL CHANGES IN HELIX 8 R7.56(400)-K7.58(402) ELBOW AND INTRACELLULAR FACE REGIONS OF CB1	14
12.00 - 12.15	Free Time		
12.15 - 13.45	**Lunch**		
Session 3. Bioactive Lipids & Analytical Lipidology ➢ Chairs*: Drs. Rao Rapaka and Heather Bradshaw*			
13.45	George Kunos	MECHANISMS IN THE BIOSYNTHESIS OF CRITICAL LIPIDS	
14.00	Raphael Mechoulam	UPDATE ON NEW ENDOGENOUS LIPID LIGANDS	
14.15	J. Michael Walker	LIPIDOMIC APPROACHES TO THE IDENTIFICATION OF NOVEL, ENDOGENOUS FATTY ACYL AMIDES	15
14.30	Mauro Maccarrone, Anna Fiori, Monica Bari, Filippo Granata, Valeria Gasperi, M. Egle De Stefano, Alessandro Finazzi-Agrò and Roberto Strom	ANANDAMIDE TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER	16
14.45	Alexandros Makriyannis	ANALYTICAL PROTEOMICS & FISHING FOR THE PHARMACOPHORE	
15.00	Vincenzo Di Marzo and Luciano De Petrocellis	"VANILLIPIDOMICS": AN UPDATE ON ENDOGENOUS LIPID LIGANDS OF VANILLOID RECEPTORS	17
15.15	Daniele Piomelli	LIPIDOMICS: THEN AND NOW	

15.30 - 17.30	**Coffee Break / Poster Session** **Sessions # 2 and 4**		
Session 4. Biosynthesis and Inactivation of Endocannabinoids and Related Lipids ➢ Chairs*: Drs. Sherrye Glaser and Nephi Stella*			
17.30	Jenny Wang, David Woodward, Alex Kharlamb, Benjamin Cravatt, Giorgio Ortar and Vincenzo Di Marzo	LACK OF EFFECT OF FATTY ACID AMIDE HYDROLASE (FAAH) INHIBITORS ON INTRAOCULAR PRESSURE IN "GLAUCOMATOUS" MONKEYS	18
17.45	Christopher Fowler and Lina Thors	DOES THE INITIAL CELLULAR UPTAKE OF ANANDAMIDE SHOW TEMPERATURE-DEPENDENCY AND SATURABILITY?	19
18.00	Nephi Stella	IDENTIFICATION OF A NOVEL MONOACYLGLYCEROL LIPASE (MGL)	20
18.15	Andrew J. Thorpe, Joel, Schlosburg, Benjamin F. Cravatt, Billy R. Martin, Laura J. Sim-Selley and Aron H. Lichtman	FAAH (-/-) MICE EXHIBIT NORMAL CB1 RECEPTOR FUNCTION FOLLOWING ACUTE OR REPEATED ADMINISTRATION OF CANNABINOIDS	21
18.30	Heather Bradshaw, Sherry Shu-Jung Hu, Neta Rimmerman, David O'Dell, Kim Masuda, Benjamin Cravatt and J Michael Walker	NOVEL METABOLISM OF N-ARACHIDONOYL GLYCINE BY FATTY ACID AMIDE HYDROLASE	22
18.45	Bo Tan, Heather B. Bradshaw, David K. O'Dell, M. Francesca Monn, Y. William Yu, Harini Srinivasan, Jocelyn F. Krey and J. Michael Walker	RAPID IDENTIFICATION OF NOVEL ENDOGENOUS FATTY ACYL AMIDES USING NANO-LC-MS/MS AND INFORMATION-DEPENDENT ACQUISITION	23
20.00	**Dinner**		

Notes:

Day 2
Monday, June 26th

Session 5. Neuronal Development			
➢ Chairs*: Drs. Tibor Harkany and Tiziana Rubino*			
8.00	Paul Berghuis, Michela Matteoli, Ken Mackie and Tibor Harkany	NEURONAL GROWTH CONE GUIDANCE BY ENDOCANNABINOIDS	24
8.15	María Gómez, Mariluz Hernández, Ruth Pazos, Rosa Tolón, Julián Romero and Javier Fernández-Ruiz	THE ACTIVATION OF CANNABINOID RECEPTORS DURING BRAIN DEVELOPMENT INFLUENCES CELL ADHESION MOLECULE L1 IN RATS	25
Session 6. Nervous Function and Neuropsychiatric Disorders			
➢ Chairs*: Drs. Tibor Harkany and Tiziana Rubino*			
8.30	Emmanuel Onaivi and Hiroki Ishiguro	FURTHER EVIDENCE FOR THE INVOLVEMENT OF CB2 CANNABINOID RECEPTORS IN DEPRESSION AND SUBSTANCE ABUSE	26
8.45	József Haller, Krisztina Soproni, Balázs Varga, Beáta Németh, Éva Mikics, Tamás F. Freund, Ferenc Mátyás and Norbert Hájos	CORRELATED SPECIES DIFFERENCES IN THE EFFECTS OF CANNABINOID LIGANDS ON ANXIETY AND ON GABAERGIC / GLUTAMATERGIC SYNAPTIC TRANSMISSION	27
9.00	Tiziana Rubino, Cinzia Guidali, Natalia Realini, Daniela Viganò, Chiara Castiglioni and Daniela Parolaro	NEUROANATOMICAL SITES AND BIOCHEMICAL CORRELATES UNDERLYING CANNABINOID MODULATION OF ANXIETY	28
9.15	Aldemar Degroot and George Nomikos	CB1 RECEPTOR ANTAGONISTS IN THE PHARMACOTHERAPY OF ACTIVE AVOIDANCE RELATED ANXIETY	29
9.30	Paola Fadda, Maria Scherma, Paola Salis, Paola Mascia, Liana Fattore and Walter Fratta	INVOLVEMENT OF THE 5-HT1A SEROTONERGIC RECEPTORS IN THE ANXIETY-LIKE EFFECTS INDUCED BY THE CB1 CANNABINOID RECEPTOR AGONIST WIN55,212-2	30
9.45	Leonora Long, Daniel Malone and David Taylor	CANNABIDIOL REVERSES MK-801-INDUCED SOCIAL WITHDRAWAL IN RATS	31
10.00	Richard Musty and Richard Deyo	A CANNABIGEROL EXTRACT ALTERS BEHAVIORAL DISPAIR IN AN ANIMAL MODEL OF DEPRESSION	32
10.15	Aron Lichtman, Pattipati Naidu, Stephen Varvel, Kyunghye Ahn, Benjamin Cravatt and Billy Martin	FAAH INHIBITION AUGMENTS ANANDAMIDE'S PHARMACOLOGICAL EFFECTS BUT IS DEVOID OF ANXIOLYTIC-LIKE OR ANTIDEPRESSANT-LIKE EFFECTS	33
10.30 - 11.00	**Coffee Break**		
Session 7. Nervous Function: Retrograde Signalling, Memory and Learning			
➢ Chairs*: Drs. Gregory Gerdeman and Krisztina Monory*			
11.00	Norbert Hajos, Judit Makara, Istvan Katona, Jan de Vente and Tamas Freund	INVOLVEMENT OF NITRIC OXIDE IN CB1 CANNABINOID RECEPTOR-MEDIATED RETROGRADE SIGNALING	34

11.15	Henry Yin and David Lovinger	D2 RECEPTORS MEDIATE FREQUENCY-DEPENDENT PRESYNAPTIC INHIBITION VIA RETROGRADE ENDOCANNABINOID SIGNALING AT CORTICOSTRIATAL SYNAPSES	35
11.30	Csaba Foldy, Axel Neu and Ivan Soltesz	ENDOCANNABINOID MODULATION OF GABA RELEASE AT THE CCK+ INTERNEURON TO PYRAMIDAL CELL SYNAPSE, A KEY SITE FOR THE REGULATION OF HIPPOCAMPAL NETWORK OUTPUT	36
11.45	Istvan Katona, Gabriella Urban, Matthew Wallace, Catherine Ledent, Kwang-Mook Jung, Daniele Piomelli, Ken Mackie and Tamas F Freund	THE ENDOCANNABINOID SYSTEM AT GLUTAMATERGIC SYNAPSES OF THE BRAIN	37
12.00	Gregory Gerdeman, Jason Schechter and Edward French	INHIBITION OF STIMULUS-RESPONSE (HABIT) LEARNING BY STRIATAL INJECTION OF THE CB1 ANTAGONIST RIMONABANT	38
12.15 - 13.45	**Lunch**		
Session 8. Pain and Inflammation ➢ Chairs*: Drs. Barbara Costa and Michael Walker*			
13.45	Takayuki Sugiura, Maiko Gokoh, Seishi Kishimoto and Saori Oka	PATHOPHYSIOLOGICAL ROLES OF 2-ARACHIDONOYLGLYCEROL AND THE CB2 RECEPTOR IN ALLERGIC INFLAMMATION	39
14.00	Barbara Costa, Francesca Comelli, Mariapia Colleoni and Gabriella Giagnoni	EFFECT OF CB1/TRPV1 AGONIST, ARVANIL, IN ACUTE INFLAMMATION AND IN PERSISTEN INFLAMMATORY AND NEUROPATHIC PAIN	40
14.15	Sreenivasulu Pattipati and Aron Lichtman	INHIBITION OF FAAH PRODUCES CB1 RECEPTOR MEDIATED ANTI-HYPERALGESIC EFFECTS IN COLLAGEN-INDUCED ARTHRITIC PAIN	41
14.30	Rossella Brusa, Marilena Campanella, Giorgia Quadrato, Nadia Bernardini, Chiara Foglia, Valentina Liporati, Silvia Conti, Rosalia Bertorelli, Angelo Reggiani and Massimiliano Beltramo	CB2 RECEPTOR AGONISTS PHARMACOLOGICAL PROFILING	42
14.45	Stephen Varvel, Jenny Wiley and Billy Martin	ACUTE INTERACTIONS BETWEEN CBD, CBN, AND THC FOLLOWING INTRAVENOUS OR INHALATION EXPOSURE IN THE MOUSE TETRAD	43
15.00 - 17.15	**Coffee Break / Poster Session** **Sessions # 1, 6, 7 and 8**		
Session 9. Food Intake and Energy Balance ➢ Chairs*: Drs. Ester Fride and Jenny Wiley*			
17.15	Ester Fride and Nikolai Gobshtis	UNDESIRABLE WEIGHT GAIN CAUSED BY PROLONGED USE OF ANTI-DEPRESSANT MEDICATION MAY BE PREVENTED WITH RIMONABANT WITHOUT LOSS OF ANTIDEPRESSANT EFFECTIVENESS	44

17.30	Frédéric Béquet, Mathieu Desbazeille, Pascal Ludwiczak, Mohamed Maftouh, Claudine Picard, Bernard Scatton and Gérard Le Fur	CB1 RECEPTORS CONTROL ENDOCANNABINOIDS RELEASE IN THE HYPOTHALAMUS OF FREELY MOVING RATS	45
17.45	Isabel Matias, Luciano De Petrocellis, Katarzyna Starowicz, Tiziana Bisogno, Luigia Cristino and Vincenzo Di Marzo	FUNCTIONAL ACTIVITY OF CANNABINOID RECEPTORS IN MODELS OF ADIPOCYTES AND BETA-PANCREATIC CELLS	46
18.00	M. Jerry Wright Jr., James J. Burston, Darnica C. Leggett, Raj K. Razdan and Jenny L. Wiley	THE INFLUENCE OF SELECTED ENDOCANNABINOID ANALOGS ON FOOD INTAKE AND LOCOMOTION	47
18.15 - 19.00	**Plenary Lecture** **Kang Tsou Memorial Lecture** **Dr. Miklós Palkovits** Semmelweis University **"**Neuroanatomical and Neurochemical Organization of the Central Regulation of Food Intake" Page P1		
20.00	**Boat Excursion with Dinner**		

Notes:

Day 3
Tuesday, June 27th

Session 10: Immune System

➢ Chairs: *Drs. David Baker and Mauro Maccarrone*

8.00	Fernando Correa, Fabian Docagne, Leyre Mestre, Diego Clemente, Frida Loría, Miriam Hernangómez, Christoph Becker and Carmen Guaza	ANANDAMIDE REGULATES IL-12P40 PRODUCTION BY ACTIVATING THE PROMOTER REPRESSOR ELEMENT GA-12	48
8.15	Douglas McHugh and Ruth A. Ross	ENDOCANNABINOID METABOLITES MODULATE HUMAN NEUTROPHIL MIGRATION	49
8.30	David Baker, J. Croxford Croxford, Gareth Pryce, Samuel M. Jackson, Catherine Ledent, Giovanni Marsicano, Beat Lutz, Gavin Giovannoni, Takashi Yamamura and Roger G Pertwee	CANNABINOID-MEDIATED IMMUNOSUPPRESSION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS	50
8.45	Michelle Roche, Michael Diamond, John P. Kelly and David P. Finn	CANNABINOID RECEPTOR-MEDIATED MODULATION OF LIPOPOLYSACCHARIDE-INDUCED ALTERATIONS IN PERIPHERAL CYTOKINE LEVELS, CIRCULATING LYMPHOCYTES AND HYPOTHALAMO-PITUITARY-ADRENAL AXIS ACTIVITY IN RATS	51
9.00	Evelyn Gaffal, Meliha Karsak, Regina Steuder, Andreas Zimmer and Thomas Tüting	ROLE OF THE ENDOGENOUS CANNABINOID SYSTEM IN EXPERIMENTAL CONTACT HYPERSENSITIVITY	52
9.15	Norbert Kaminski, Alison Springs and Barbara Kaplan	A PRELIMINARY IMMUNOLOGICAL CHARACTERIZATION OF CB1-/-/CB2-/- MICE AND THEIR SENSITIVITY TO IMMUNE MODULATION BY Δ^9-TETRAHYDROCANNABINOL	53

Session 11: Cardiovascular, Gastrointestinal and Other Peripheral Functions

➢ Chairs: *Drs. Somnath Mukhopadhyay and David Kendall*

9.30	Emma Robinson, Sue Pyne, Nigel Pyne, Ros Brett, Kathy Kane and Simon Kennedy	EFFECT OF CANNABINOIDS ON RAT CORONARY ARTERY REACTIVITY	54
9.45	Nissar Darmani, Jennifer Crim and Jano Janoyan	SYNTHETIC AND PLANT-DERIVED CANNABINOIDS PREVENT RADIATION-INDUCED VOMITING VIA CB1-RECEPTOR	55
10.00	Raj Makwana, Areles Molleman and Mike Parsons	THE RECEPTORS MEDIATING THE INHIBITORY EFFECTS OF CANNABINOIDS ON THE ELECTRICALLY STIMULATED RAT ISOLATED MYENTERIC PLEXUS LONGITUDINAL MUSCLE PREPARATION ARE DEPENDENT ON THE FREQUENCY OF STIMULATION	56
10.15	Yosefa Avraham, Olga Zolotarev, Yossi Dagon, Iddo Magen, Raphael Mechoulam, Yaron Ilan and Elliot Berry	ENDOCANNABINOIDS AFFECT LIVER FUNCTION IN THIOACETAMIDE INDUCED HEPATIC ENCEPHALOPATHY	57
10.30 - 11.00	**Coffee Break**		
11.00	Marnie Duncan, Quentin Pittman, Ken Mackie, Kamala Patel and Keith Sharkey	LPS TREATMENT UPREGULATES CB2 RECEPTORS IN THE ENTERIC NERVOUS SYSTEM OF THE RAT ILEUM	58

11.15	Saoirse O'Sullivan, Andrew Bennett, David Kendall and Michael Randal	CANNABINOID LIGANDS AS ACTIVATORS OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPAR GAMMA)	59
11.30	Fatima Teixeira-Clerc, Boris Julien, Pascale Grenard, Jeanne Tran Van Nhieu, Vanessa Deveaux, Lyling Li, Valérie Serriere-Lanneau, Catherine Ledent, Ariane Mallat and Sophie Lotersztajn	CB1 CANNABINOID RECEPTOR ANTAGONISM: A NOVEL STRATEGY FOR THE TREATMENT OF LIVER FIBROSIS	60
11.45	Somnath Mukhopadhyay	ANANDAMIDE-MEDIATED ANGIOGENESIS: INTERPLAY BETWEEN CB1 RECEPTOR AND NON-CB1/CB2 ANANDAMIDE RECEPTOR	61
12.00 -14.00	**Lunch** **NIDA InfoLuncheon –** European/American Funding Initiatives		
Session 12: Human and Clinical Studies ➢ Chairs*: Drs. Ethan Russo and Roger Pertwee*			
14.00	Ethan Russo	SATIVEX® CANNABIS BASED MEDICINE IMPROVES SLEEP QUALITY IN PATIENTS WITH MULTIPLE SCLEROSIS, NEUROPATHIC AND RHEUMATIC PAIN	62
14.15	Mary Lynch, Christine Short, Young Judee and Joanne Walker	REPORT ON A CASE SERIES OF PATIENTS USING A BUCCAL CANNABIS EXTRACT CONTAINING THC AND CBD FOR TREATMENT OF NEUROPATHIC PAIN AND SPASTICITY	63
14.30	Marta Duran, Sergio Abanades, Dolors Capellà and Seguivex Study Group	FOLLOW UP STUDY OF PATIENTS WITH NEUROPATHIC PAIN, SPASTICITY SECONDARY TO MULTIPLE SCLEROSIS AND ANOREXIA CAQUEXIA SYNDROME TREATED WITH A WHOLE PLANT CANNABIS EXTRACT	64
14.45	Donald Abrams, Hector Vizoso, Starley Shade, Cheryl Jay, Mary Ellen Kelly and Neal Benowitz	VAPORIZATION AS A SAFE AND EFFICIENT SMOKELESS CANNABIS DELIVERY SYSTEM	65
15.00	David Gorelick, Silvia Martins, Marc Copersino, Carl Soderstrom, Gordon Smith, Patricia Dischinger, David McDuff, J. Richard Hebel, Timothy Kerns, Shiu Ho and Kathleen Read	RISK OF CANNABIS DEPENDENCE IN CANNABIS USERS AMONG TRAUMA INPATIENTS	66
15.15	Matthew Hill, Greg Miller, Erica Carrier, Vanessa Ho, Boris Gorzalka and Cecilia Hillard	DYNAMIC REGULATION OF SERUM ENDOCANNABINOID CONTENT IN AFFECTIVE DISEASE AND FOLLOWING SOCIAL STRESS IN HUMANS	67
15.30 - 18.15	**Coffee Break / Poster Session** **Sessions # 9 - 12**		
17.45 - 18.15	**Hot and Controversial Issue Session #1:** *"Are There CB_2 Receptors in CNS Neurons?"* Chairs: Drs. Emmanuel Onaivi and Marnie Duncan		
18.15 - 19.00	**Plenary Lecture** **Dr. Angelo Izzo** "Cannabinoids and the Gut: Focus on Intestinal Motility and Secretion" **Young Investigator Award** Page P2		
20.00	**Dinner**		

Day 4
Wednesday, June 28th

Session 13: Reward and Abuse

➢ Chairs*: Drs. Daniela Parolaro and Thomas Lundqvist*

8.00	Leigh Panlilio, Marcello Solinas, Stephanie Matthews and Steven Goldberg	PREVIOUS EXPOSURE TO THC ALTERS THE REINFORCING EFFECTS AND ANXIETY-RELATED EFFECTS OF COCAINE IN RATS	68
8.15	Balapal Basavarajappa, Ninan Ipe, Thomas Cooper and Ottavio Arancio	ENDOCANNABINOIDS MEDIATE ETHANOL-INDUCED MODULATION OF EXCITATORY POSTSYNAPTIC CURRENTS IN CULTURED HIPPOCAMPAL NEURONS	69
8.30	Mariaelvina Sala, Chiara Castiglioni, Valeria Limonta, Chiara Guerini-Rocco, Alessia Zani, Simona Pegorini and Daniela Braida	INVOLVEMENT OF THE CB1 CANNABINOID RECEPTOR ON SALVINORIN-A-INDUCED REWARD	70
8.45	Ros Brett, Claire Allison and Judith Pratt	MAPPING CANNABIDIOL-INDUCED ALTERATIONS IN REGIONAL BRAIN ACTIVITY BY 2-DEOXYGLUCOSE IMAGING	71
9.00	Raffaella Tonini, Sonia Ciardo, Milica Cerovic, Tiziana Rubino, Daniela Parolaro, Michele Mazzanti and Renata Zippel	ERK-DEPENDENT MODULATION OF CEREBELLAR SYNAPTIC PLASTICITY FOLLOWING CHRONIC Δ^9-TETRAHYDROCANNABINOL EXPOSURE	72
9.15	Marco Pistis, Simona Perra, Giuliano Pillolla, Anna Lisa Muntoni and Miriam Melis	THE ENDOCANNABINOID SYSTEM IN THE EFFECTS OF ALCOHOL ON LIMBIC NEURONS: ELECTROPHYSIOLOGICAL EVIDENCE IN VIVO	73
9.30	Eliot Gardner, Zheng-Xiong Xi, Jeremy Gilbert, Arlene Pak, Xiao-Qing Peng and Xia Li	CB1 RECEPTOR ANTAGONIST AM251 INHIBITS COCAINE'S REWARDING EFFECTS AND COCAINE-PRIMED RELAPSE TO DRUG-SEEKING BEHAVIOR IN RATS BY A DA-INDEPENDENT MECHANISM	74
9.45	Sara Jane Ward, Frederick Henry, Ashmita Chatterjee, Tonya Gerald, Steven Franklin, Allyn Howlett and Linda Dykstra	CANNABINOID CB1 RECEPTOR DELETION AND CB1 RECEPTOR ANTAGONISM BOTH ENHANCE MORPHINE CONDITIONED PLACE PREFERENCE	75

Session 14: Cancer

➢ Chairs*: Drs. Raphael Mechoulam and Vincenzo Di Marzo*

10.00	Natalya Kogan, Michael Schlesinger, Ronen Beeri and Raphael Mechoulam	THE NOVEL ANTICANCER CANNABINOID QUINONE HU-331 IS MORE POTENT AND LESS CARDIOTOXIC THAN DOXORUBICIN - A COMPARATIVE IN-VIVO STUDY	76
10.15	Alessia Ligresti, Aniello Schiano Moriello, Isabel Matias, Katarzyna Starowicz, Simona Pisanti, Luciano De Petrocellis, Giuseppe Portella, Maurizio Bifulco and Vincenzo Di Marzo	ANTI-CANCER EFFECTS OF CANNABIDIOL IN HUMAN BREAST CARCINOMA: CELLULAR AND MOLECULAR MECHANISMS OF ACTION AND INHIBITION OF METASTATIC SPREADING	77
10.30 - 11.00	**Coffee Break**		

11.00	Simona Pisanti, Cristina Borselli, Olimpia Oliviero, Patrizia Gazzerro, Chiara Laezza, Claudia Grimaldi and Maurizio Bifulco	ANANDAMIDE INHIBITS ENDOTHELIAL CELL SPROUTING ANGIOGENESIS IN VITRO AND IN VIVO	78
11.15	Burkhard Hinz, Robert Ramer and Karin Eichele	INHIBITION OF COX-2 PROTECTS HUMAN CERVICAL CARCINOMA CELLS FROM R(+)-METHANANDAMIDE-INDUCED APOPTOTIC DEATH	79
Session 15: Neuroprotection ➢ Chairs*: Drs. Javier Fernandez-Ruiz and Julián Romero*			
11.30	Fabian Docagne, Vilma Muñeton, Frida Loría, Diego Clemente, Fernando Correa, Miriam Hernangómez, Leyre Mestre and Carmen Guaza	NEUROPROTECTIVE EFFECTS OF CANNABINOIDS INDUCE THERAPEUTIC PROPERTIES IN A VIRAL MODEL OF MULTIPLE SCLEROSIS	80
11.45	Joseph Tam, Alex Alexandrovich, Vincenzo Di Marzo, Stefania Petrosino, Victoria Trembovler, Andreas Zimmer, Raphael Mechoulam, Esther Shohami and Itai Bab	CB1, BUT NOT CB2, MEDIATES STIMULATION OF BONE FORMATION INDUCED BY TRAUMATIC BRAIN INJURY	81
12.00	Iddo Magen, Yosefa Avraham, Zvi Ackerman, Raphael Mechoulam and Elliot M. Berry	SR141716A IMPROVES COGNITIVE AND NEUROLOGICAL FUNCTION IN A MODEL OF SECONDARY BILIARY CIRRHOSIS IN THE RAT	82
12.15 - 13.45	**Lunch**		
13.45	Cristina Benito, Juan Pablo Romero, Diego Clemente, Fabian Docagne, Rosa María Tolón, Carmen Guaza and Julián Romero	IMMUNOHISTOCHEMICAL STUDY OF THE ENDOGENOUS CANNABINOID SYSTEM IN MULTIPLE SCLEROSIS POSTMORTEM HUMAN SAMPLES	83
14.00	David Fernández-López, Ignacio Lizasoain, Maria Angeles Moro, Pedro Lorenzo and Jose Antonio Martínez-Orgado	WIN 55,212-2 IMPROVES CEREBRAL TISSUE RECOVERY AFTER EXPERIMENTAL NEWBORN HYPOXIA-ISCHEMIA IN A CB1 AND CB2 RECEPTOR DEPENDENT MANNER	84
14.15	Yosef Sarne, Isabella Tselnicker, Roey Senn and Ora Keren	A SINGLE ULTRA-LOW DOSE OF THC INDUCES LONG-LASTING COGNITIVE DEFICITS IN MICE: IMPLICATIONS FOR CHRONIC EXPOSURE TO CANNABINOID DRUGS	85
14.30	Cindy Casteels, Dorien Vercammen, Karolien Goffin, Peter Vermaelen, Bert Vanbilloen, Guy Bormans and Koen Van Laere	CHRONIC EXPOSURE TO Δ^9-TETRAHYDROCANNABINOL INDUCES INCREASED DOPAMINE TRANSPORTER AVAILABILITY	86
14.45	Aoife Gowran	LYSOSOMAL DYNAMICS IN Δ^9-TETRAHYDROCANNABINOL-INDUCED APOPTOSIS IN RAT CORTICAL NEURONS	87
15.00	Moises Garcia-Arencibia, Sara Gonzalez, Eva de Lago, Jose Antonio Ramos, Raphael Mechoulam and Javier Fernandez-Ruiz	NEUROPROTECTION WITH CANNABINOIDS IN A RAT MODEL OF PARKINSON'S DISEASE	88
15.15 - 16.15	**Coffee Break & Business Meeting**		

Session 16: Non-Mammalian Organisms ➢ Chairs*: Drs. John McPartland and Maurice Elphick*			
16.15	John McPartland	SPECIES TREES AND GENE TREES AS METHODS FOR INFERRING COEVOLUTION	89
16.30	Maurice Elphick and Michaela Egertová	THE EVOLUTIONARY ORIGIN OF CANNABINOID RECEPTORS: EVIDENCE OF AN ANCIENT ROLE IN PRESYNAPTIC REGULATION OF NEURONAL SIGNALLING IN INVERTEBRATE CHORDATES	90
16.45	Athanasia Siafaka-Kapadai, Eleni Farmaki, Alexandra Theodoridou and Dimitrios Anagnostopoulos	MAGL (MONOACYLGLYCEROLIPASE) ACTIVITY IN TETRAHYMENA	91
17.00 - 19.15	**Poster Session** **Sessions # 13 - 16**		
18.45 – 19.15	**Hot and Controversial Issue Session #2:** *"Pharmacology of Cannabidiol:* *Cannabinoid Receptors and Beyond"* Chairs: Drs. Chris Fowler and Adèle Thomas		
20.00	**Dinner**		

Notes:

15.30 – 17.30	**Day 1 – Sunday, June 25th** **Poster Sessions # 2 & # 4**	
2. Receptor Structure and Signal Transduction		Page #
Eva V. Varga, Teodora Georgieva, Suneeta Tumati, Zdzislaw Salamon, William R. Roeske, Gordon Tollin, Henry I. Yamamura	PLASMON WAVEGUIDE RESONANCE (PWR) SPECTROSCOPY: A NOVEL TOOL TO EXAMINE CANNABINOID RECEPTOR SIGNALING	92
Lisa S. Middleton, Michael P. Cassidy, Dana E. Selley and Laura J. Sim-Selley	LIGAND-DEPENDENT ALTERATIONS IN CB1 RECEPTOR FUNCTION AND LOCALIZATION IN CB1 RECEPTOR-EXPRESSING CHO CELLS	93
E. Gkoumassi, M. J. Dröge, C.R.S. Elzinga, B. G. J. Dekkers, H. Meurs, J. Zaagsma and S. A. Nelemans	DUAL MODULATION OF ADENYLYL CYCLASE BY CANNABINOIDS IN HUMAN BRONCHIAL EPITHELIAL CELLS	94
Gábor Nyíri, Eszter Szabadits, Csaba Cserép, Javier Gracia-Llanes and Tamás F. Freund	THE NITRIC OXIDE SENSITIVE GUANYLYL CYCLASE ALPHA1 SUBUNIT IS ABUNDANT IN DIFFERENT TYPES OF INTERNEURONS BUT NOT IN PYRAMIDAL CELLS IN RAT HIPPOCAMPUS	95
Gemma L. Baillie, Lesley A. Stevenson, Adèle Thomas, Roger G. Pertwee and Ruth A. Ross	ALLOSTERIC MODULATION OF THE CANNABINOID CB1 RECEPTOR: AGONIST DEPENDENCE	96
Barbara Bosier, Emmanuel Hermans and Didier M.Lambert	DIFFERENTIAL REGULATION OF TYROSINE HYDROXYLASE EXPRESSION IN NEUROBLASTOMA CELLS BY DISTINCT CANNABINOID CB1 RECEPTOR AGONISTS	97
Irina A. Khasabova, Catherine Harding-Rose, Donald A. Simone and Virginia S. Seybold	ANANDAMIDE INHIBITS CALCIUM CHANNELS IN DORSAL ROOT GANGLION NEURONS INDEPENDENT OF CANNABINOID-1 RECEPTORS	98
Eleni Farmaki, Dimitrios Anagnostopoulos and Athanasia Siafaka-Kapadai	THE ENDOCANNABINOID SYSTEM IN RABBIT BRAIN. EVIDENCE FOR THE PRESENCE OF CB2 RECEPTORS	99
Federico Massa, Krisztina Monory, Matthias Eder, Gerhard Rammes, Maria R. Domenici, Shahnaz Azad, Walter Zieglgänsberger, Giovanni Marsicano and Beat Lutz	ROLE OF CB1 IN DIFFERENT NEURONAL SUBPOPULATION IN THE BRAIN	100
Meliha Karsak, Jennifer Rehnelt, Edda Erxlebe, Julia Essig, Itai Bab and Andreas Zimmer	EXPRESSION ANALYSES OF TWO HUMAN CB2 PROTEIN VARIANTS	101
Chris Henstridge, Neil McDonald, Chris Connolly and Andrew Irving	CELL SURFACE CB1 AND GPR55 ARE TARGETED TO DISTINCT CELLULAR DOMAINS WHEN EXPRESSED RECOMBINANTLY IN CULTURED HIPPOCAMPAL NEURONS	102
Lesley Ford and Andrew Irving	ANANDAMIDE MODULATION OF CALCIUM SIGNALLING IN CULTURED HIPPOCAMPAL NEURONS AND GLIA	103
Ankur Kapur, Daniel Fleischer, Dow P. Hurst, Patricia H. Reggio, Alexandros Makriyannis and Mary E. Abood	THE IMPORTANCE OF RESIDUES, S7.39 AND S2.60 IN MODULATING THE FUNCTION OF THE HUMAN CB1 CANNABINOID RECEPTOR	104

15.00 – 17.15	**Day 2 – Monday, June 26th** **Poster Sessions # 1, # 6 - 8**	
1. SAR Studies and New Synthetic Molecules		
Alicia L. S. Thompson, John W. Huffman, Jenny L. Wiley and Billy R. Martin	DEOXY BICYCLIC COMPOUNDS AS POTENTIAL CB2 SELECTIVE LIGANDS	122
Valerie J. Smith, John W. Huffman, Jenny L. Wiley and Billy R. Martin	EFFECTS OF HALOGEN SUBSTITUENTS UPON CB1 AND CB2 RECEPTOR AFFINITIES IN THE 1-ALKYL-3-(1-NAPTHOYL)INDOLE SERIES	123
Tapio Nevalainen, Teija Parkkari, Outi M.H. Salo, Kristiina M. Huttunen, Juha R. Savinainen, Jarmo T. Laitinenb, Antti Poso, and Tomi Järvinen	DIMETHYLHEPTYL DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL (2-AG) AND 2-ARACHIDONYL GLYCERYL ETHER (2-AGE): SYNTHESIS AND CB1 RECEPTOR ACTIVITY	124
Michael J. Dart, Jennifer M. Frost, Karin R. Tietje, Anthony V. Daza, George K. Grayson, Yihong Fan, Sutapa Mukherjee, Tiffany Runyan Garrison, Betty Bei Yao and Michael D. Meyer	1-ALKYL-3-KETO-INDOLES: IDENTIFICATION AND IN VITRO CHARACTERIZATION OF A SERIES OF POTENT CANNABINOID LIGANDS	125
M.G. Cascio, T. Bisogno, E. Palazzo, A. Thomas, A. Brizzi, V. de Novellis, R. Pertwee, V. Brizzi, S. Maione and V. Di Marzo	IN VITRO AND IN VIVO PHARMACOLOGY OF SYNTHETIC OLIVETOL-DERIVED CANNABINOID RECEPTOR LIGANDS	126
Herbert H. Seltzman, Michael J. Roche, Marcus F. Brackeen, Mary E. Abood, Daniel Fleischer, Deborah Lewis, Shangping Shi, Ester Fride, Nikolai Gobshtis, Dow P. Hurst and Patricia H. Reggio	STUDIES TOWARDS ENHANCED BINDING AND ACTIVITY OF NEUTRAL ANTAGONISTS FOR THE CB1 RECEPTOR	127
M. Paola Castelli, Clementina Manera, Veronica Benetti, Adriano Martinelli, A. Paola Piras, Giuseppe Saccomanni, Tiziano Tuccinardi and Alfredo Vannacci	DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW 1,8-NAPHTHYRIDIN-4(1H)-ON-3-CARBOXAMIDE AND QUINOLIN-4(1H)-ON-3-CARBOXAMIDE DERIVATIVES AS CB2 SELECTIVE AGONISTS	128
Katerina Antoniou, George Panagis, Kyriaki Thermos, Maria Papazoglou, Niki Mastrodimou, Alexia Polissidis, Styliani Vlachou, Victoria Nahmias, Michalis P. Kondylis, Dimitris Papahatjis, Zeta Daifoti-Papadopoulou and Christina Spyraki	PHARMACOLOGICAL PROFILE OF A NEW LIGAND FOR CB1 AND CB2 CANNABINOID RECEPTORS	129
Srinivas Gullapalli, Meyyappan Muthuppalaniappan, Abraham Thomas, Neelima Khairatkar-Joshi, Kanthikiran Varanasi, Swaroop Kumar V.S. Vakkalanka and Shridhar Narayanan	NOVEL ORALLY ACTIVE CB2 RECEPTOR AGONISTS WITH POTENTIAL ANTI-HYPERALGESIC EFFECTS	130
6. Nervous Function and Neuropsychiatric Disorders		
Lindsey Teasenfitz, Zoila Mora, Babatunde Akinshola and Emmanuel S. Onaivi	BEHAVIORAL EFFECTS OF CB2 CANNABINOID RECEPTOR LIGANDS	131
Nikolai Gobshtis and Ester Fride	CANNABINOIDS, SCHIZOPHRENIA AND GENDER PRENATAL EVENTS MODULATE CB1R AGONIST-INDUCED IMPAIRMENT OF PRE-PULSE INHIBITION (PPI) DIFFERENTLY IN MALES AND FEMALES	132
Daniela Braida, Valeria Limonta, Simona Pegorini, Alessia Zani and Mariaelvina Sala	INVOLVEMENT OF 5-HT1A SEROTONERGIC RECEPTOR ON Δ9-TETRAHYDROCANNABINOL (Δ^9-THC)-INDUCED EMOTIONAL RESPONSE IN RATS	133
Jenny L. Wiley, Seth H. Kendler, James J. Burston, Dana E. Selley, and Laura J. Sim-Selley	SEX DIFFERENCES IN SENSITIVITY TO ANTIPSYCHOTIC EFFECTS ON CANNABINOID CB1 RECEPTOR FUNCTION IN RATS	134

15.30 - 18.15	**Day 3 – Tuesday, June 27th** **Poster Sessions # 9 - 12**	
9. Food Intake and Energy Balance		
Dana Kevin Sindelar, John Schaus, Stephen Hitchcock, Amy Porter, Jennifer Allen, Winton Jones, Robert Stratford, Henry Havel, Eyassu Chernet, Lee Phebus, John Hart, Jesline Alexander-Chacko, Xia Li, Richard Davis, David McKinzie, Jeffrey Witkin and George Nomikos	NEUROPSYCHOPHARMACOLOGICAL CHARACTERIZATION OF A SULFONAMIDE COMPOUND [4-(3-CYCLOPENTYL-INDOLE-1-SULFONYL)-N-(TETRAHYDRO-PYRAN-4-YLMETHYL)-BENZAMIDE], AS A NOVEL, POTENT, SELECTIVE AND ORALLY ACTIVE CB1 CANNABINOID RECEPTOR ANTAGONIST	162
M. Maccarrone, B. Spoto, G. Parlongo, N. Battista, E. Sgro', A. Finazziagrò and C. Zoccali.	THE ENDOCANNABINOID SYSTEM IN HUMAN ADIPOSE TISSUE FROM HEALTHY SUBJECTS	163
Patrizia Gazzerro, Maria Gabriella Caruso, Maria Notarnicola, Giovanni Misciagna, Vito Guerra, Chiara Laezza and Maurizio Bifulco	ASSOCIATION BETWEEN CANNABINOID TYPE-1 RECEPTOR POLYMORPHISM AND BODY MASS INDEX IN A SOUTHERN ITALY POPULATION	164
Gitte Petersen, Camilla Sørensen, Patricia C. Schmid, Andreas Artmann, Mads Tang-Christensen, Steen H. Hansen, Philip J. Larsen, Harald H. O. Schmid and Harald S. Hansen	INTESTINAL LEVELS OF ANANDAMIDE AND OLEOYLETHANOLAMIDE IN FOOD-DEPRIVED RATS ARE REGULATED THROUGH THEIR PRECURSORS	165
Sevasti Karaliota, Alexandra Gountopoulou, Chrysanthi Gondinou, Athanasia Siafaka-Kapadai and Mary Mavri-Vavayanni	ANANDAMIDE INDUCES THE DIFFERENTIATION OF RAT PREADIPOCYTES THROUGH THE INDUCTION OF PPARγ	166
10. Immune System		
LaToya A. Griffin and Guy A. Cabral	CANNABINOIDS ALTER NFκB BINDING IN MICROGLIA	167
Manop Buranapramest and Nancy E. Buckley	EFFECTS OF WIN 55,212-2 ON CD4+ T-CELL PROLIFERATION AND CYTOKINE SECRETION AT NANOMOLAR CONCENTRATIONS	168
Anna Maria Malfitano, Gokce Altay Toruner, Chiara Laezza, Seema Husain, Patrizia Gazzerro, Davide Eletto, Pierangelo Orlando, Luciano De Petrocellis, Alexandra Terskiy, Marvin Schwalb, Emilia Vitale and Maurizio Bifulco	ARVANIL AND ANANDAMIDE UP-REGULATE CD36 EXPRESSION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS	169
11. Cardiovascular, Gastrointestinal and Other Peripheral Functions		
Javier Fernández Solari, Juan Pablo Prestifilippo, Paula Vissio, Samuel M. McCann, Valeria Rettori and Juan Carlos Elverdin	THE ACTIVATION OF CENTRAL CANNABINOID RECEPTORS INHIBITS SALIVARY SECRETION	170
Juan Pablo Prestifilippo, Javier Fernández Solari, Juan Carlos Elverdin, Angela Suburo, Samuel M. McCann and Valeria Rettori	LIPOPOLYSACCHARIDE (LPS) ACTIVATES THE ENDOCANNABINOID SYSTEM IN THE SUBMANDIBULARY GLAND OF THE RAT	171
Nina L. Cluny, Farideh A. Javid, Brian Whittle and Robert J. Naylor	RELAXATION OF THE GASTROINTESTINAL TRACT OF SUNCUS MURINUS INDUCED BY CANNABIDIOLIC ACID (CBDA) AND CANNABIDIOL (CBD)	172

17.00 - 19.15	**Day 4 – Wednesday, June 28th** **Poster Sessions # 13 - 16**	

13. Reward and Abuse

Plenary Lecture

18.15 - 19.00
Monday, June 26th, 2006

Kang Tsou Memorial Lecture

"NEUROANATOMICAL AND NEUROCHEMICAL ORGANIZATION OF THE CENTRAL REGULATION OF FOOD INTAKE"

Miklós Palkovits

Semmelweis University

The central regulation of the appetite and food intake is organized by simultaneous actions of the different humoral and neuronal agents. A complex neural circuitry is involved in this mechanism including afferent neuronal inputs to the hypothalamus, obligatory processing in hypothalamic orexigenic and anorexigenic neuronal circuits, and descending commands through vagal and spinal neurons to the body. Circulating substances (leptin, ghrelin, glucose, insulin) carrying signals connected to changes in body food homeostasis and energy balance enter both the hypothalamus and the dorsomedial medulla oblongata. Receptors sensitive to glucose metabolism, body fat reserves, distension of the stomach have been identified and localized in both brain areas. In addition, a number of neuropeptides, peptid and cannabinoid receptors are involved in the regulation of feeding. Evidence has been accumulated over the last years to indicate that endocannabinoids act as orexigenic signals via cannabinoid CB1 receptors in the central nervous system. Recent data are compatible with the CART peptide being a downstream mediator of the CB1-mediated orexigenic effect of endonegnous anandamide. Hypothalamic peptidergic neurons (CART peptide, CRH, orexin, melanin-concentrating hormone, NPY) give rise to projection to the nucleus accumbens subserving the control of feeding behavior, and to autonomic centers in the brainstem and the spinal cord with potential for stimulation or inhibition of food intake, energy balance and appetite.

Plenary Lecture

18.15 - 19.00
Tuesday, June 27th, 2006

Young Investigator Lecture

"CANNABINOIDS AND THE GUT: FOCUS ON INTESTINAL MOTILITY AND SECRETION"

Angelo A Izzo

Department of Experimental Pharmacology, University of Naples Federico II, via D Montesano 49, 8031 Naples Italy

The presence of cannabinoid receptors in the gastrointestinal tract has been demonstrated by anatomical and functional evidence. Cannabinoid CB_1 receptors are located on enteric nerve terminals where they exert inhibitory action on neurotransmission to reduce intestinal motility and secretion. In the guinea-pig myenteric plexus, sensory, interneuronal and motoneuronal cell bodies and nerve fibres expresses CB_1 receptors. CB_1 immunoreactivity co-localizes with the marker of cholinergic neurones cholineacetyl transferase (ChAT) as well as with a small population of substance P-immunoreactive neurones. CB_1 receptors co-localize with vasoactive intestinal peptide (non-cholinergic) and neuropeptide Y (cholinergic) secretomotorneurones and are also present on extrinsic primary afferent terminals innervating the submucousal plexus.
Cannabinoid agonists act on prejunctional CB_1 receptors to reduce smooth muscle contractility and peristalsis in different regions of the gastrointestinal tract, an effect mainly due to reduction of acetylcholine release from myenteric nerves. CB_1 receptor activation reduces transit in rodents in vivo, both in physiological and in pathophysiological states. There is also preliminary evidence that cannabinoids may reduce motility through activation of CB_2 in the inflamed gut. Interestingly, both CB_1 (hyper-expressed during inflammation) and CB_2 receptors might mediate intestinal antinflammatory effects.

Cannabinoids may affect electrolyte movements though activation of CB_1 receptors located on submucosal neurones and extrinsic primary afferents in the submucosa. Pharmacological studies have shown that endogenous anandamide exerts an antisecretory (antidiarrhoeal) role in animals treated with cholera toxin through activation of hyper-expressed CB_1 receptors.

Overall, the inhibitory effect of cannabinoids on intestinal secretion and motility are relevant in the light of their possible clinical use for the treatment of irritable bowel syndrome and inflammatory bowel disease.

THE RELEVANCE OF THE STERIC FACTOR IN THE BIOLOGICAL ACTIVITY OF CBD DERIVATIVES - A TOOL IN IDENTIFYING NOVEL MOLECULAR TARGET FOR CANNABINOIDS

Yehoshua Maor[1], Ruth Gallily[2] and Raphael Mechoulam[1]

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[2]The Lautenberg Center for General and Tumor Immunology, The Hebrew University of Jerusalem, Ein Kerem Campus, 91120 Jerusalem, Israel

Over the last decade extensive chemical and biological studies have contributed to the identification of novel molecular targets and receptor agonists for the endocannabinoid system. Cannabidiol (CBD) is the major non-psychotropic phytocannabinoid and does not bind to the known cannabinoid receptors. However its (+) unnatural enantiomer binds to both CB1 and CB2 as well as TRPV1 receptors. In view of this interesting structure-activity relationship, we synthesized the (+) enantiomers of Abnormal-cannabidiol (Abn-CBD), an agonist of an additional cannabinoid receptor which has not been cloned to date.

We evaluated the enantiomers of Abn-CBD and CBD for their ability to inhibit the generation of reactive oxygen species (ROS) and nitric oxide (NO) by RAW 247.3 cells and murine peritoneal macrophages respectively. Both enantiomers of Abn-CBD and CBD inhibited the formation of ROI and NO by murine macrophages in a dose-dependent and $CB_{1/2}$ independent manner. Their activity was neither inhibited by capsazepine nor GW 9662 (a PPAR-γ antagonist).

These results suggest that in macrophages (+) and (-) Abn-CBD share a molecular target which differs from the pertussis toxin-sensitive Abn-CBD receptor, expressed in the endothelial cells of some vascular beds and from TRPV1 receptors. These effects may be modulated by a non-stereoselective ionic channel or may be receptor-independent. Further development of the subject can be used as a tool in cannabinoid-based drug design.

1-PENTYL-2-ARYL-4-(1-NAPHTHOYL)PYRROLES: A NEW CLASS OF HIGH AFFINITY LIGANDS FOR THE CB_1 AND CB_2 RECEPTORS

John W. Huffman[1], Lea W. Padgett[1], Matthew L. Isherwood[1], Jenny L. Wiley[2] and Billy R. Martin[2].

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[2]Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298-0613, U.S.A.

Several years ago our group reported the synthesis and pharmacology of a series of cannabimimetic 1-alkyl-3-(1-naphthoyl)pyrroles (Lainton *et al. Tetrahedron Lett.* **1995**, *36*, 1401). One of these compounds, JWH-030, 1-pentyl-3-(1-naphthoyl)pyrrole has moderate affinity for the CB_1 receptor with $K_i = 87 \pm 3$ nM and little affinity for the CB_2 receptor ($K_i = 320 \pm 27$ nM). The compound was approximately equipotent to Δ^9-THC in the *in vivo* mouse model of cannabinoid activity. These pyrroles were designed based upon a common pharmacophore for traditional cannabinoids and cannabimimetic indoles, however subsequent studies indicated that the indoles and dibenzopyran based ligands interacted with the CB_1 receptor in a considerably different manner.

Since there is a body of evidence that cannabimimetic indoles interact with the CB_1 receptor primarily by aromatic stacking, it was felt that an additional aromatic substituent on pyrroles similar to JWH-030 would lead to ligands with greater receptor affinity. A series of 1-alkyl-2-phenyl-4-(1-naphthoyl)pyrroles was synthesized and their affinities for the CB_1 and CB_2 receptors were determined. The 1-pentyl through 1-heptyl analogs have CB_1 receptor affinities from 11 to 21 nM. To further explore the structure-activity relationships (SAR) for this series of cannabimimetic pyrroles 24 additional 1-pentyl-2-aryl-4-(1-naphthoyl)pyrroles were synthesized and their receptor affinities (CB_1 and CB_2) were determined.

In general, those pyrroles with a small *ortho*-substituted phenyl group have high affinity for both receptors, while the *para*-isomers have considerably lower CB_1 affinities. The *meta*-substituted compounds are intermediate in their affinities for the CB_1 receptor. Those compounds with the highest affinity for the CB_1 receptor are the *ortho*-methyl (JWH-370), *ortho*-fluoro (JWH-307) and *ortho*-chloro (JWH-369) compounds, with $K_i = 5.6 \pm 0.4$, 7.7 ± 1.8 and 7.9 ± 0.4 nM, respectively. The CB_2 receptor affinities range from 3.4 ± 0.2 nM for JWH-370 to 104 ± 18 nM for the *para*-methoxy compound (JWH-243).

The method of synthesis of these compounds and their SAR will be discussed.

Acknowledgements: Supported by grants DA03590, DA15340, DA15579 and DA03672 all from the National Institute on Drug Abuse.

DISCOVERY OF A NOVEL CB1R INVERSE AGONIST

T.M. Fong, L.S. Lin, X-M Guan, D.J. Marsh, C-P Shen, J. Lao, D.S. Stribling, K.M. Rosko, H. Yu, Y. Feng, J.C. Xiao , L.H.T. Van der Ploeg*, M.T. Goulet*, W.K. Hagmann, T.J. Lanza, Jr., J.P. Jewell, P. Liu, S.K. Shah, H. Qi, X. Tong, J. Wang, and S. Xu, R. Gibson #, B. Francis #, S. Patel #, A.M. Strack, D.E. MacIntyre, L.P. Shearman

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CB1R has been implicated in the control of energy balance. To further explore the pharmacological utility of CB1R inhibition, we evaluated a novel CB1R inverse agonist in a variety of biological assays. The newly discovered inverse agonist is structurally distinct from all previously reported CB1R ligands, and inhibits the binding and functional activity of both synthetic agonists and endocannabinoids. In vivo, the inverse agonist inhibits food intake and weight gain in mice and rats. Acute efficacy is CB1R-mediated as demonstrated by the lack of efficacy in CB1R-deficient mice. Partial occupancy of CB1R by the inverse agonist is sufficient to cause food intake reduction and weight loss. These data indicated that the novel lead is a highly potent and selective CB1R inverse agonist and is orally active in rodent obesity models.

CONFORMATIONALLY-CONSTRAINED ANALOGS OF SR141716

Yanan Zhang, Marcus Brackeen, Herb H. Seltzman, Anne F. Gilliam and Brian F Thomas
Research Triangle Institute, Research Triangle Park, NC 27709

SR141716 is a cannabinoid receptor (CBR1) antagonist that has attracted great interest due to its potential in the treatment of obesity, alcoholism, addiction, and other therapeutic indications. Numerous derivatives of SR141716 have been synthesized and tested, and additional CBR1 antagonists with novel structures continue to be reported. We are particularly interested in conformationally constrained analogs of SR141716, based on the assumption that a limited set of relatively low energy conformers represent active conformations. In an effort to determine the bioactive conformation(s) of SR141716, and characterize the affect of its conformational constraint on CBR1 receptor affinity, we synthesized and tested a series of rotationally constrained analogs of SR141716. Conformational properties of these compounds were studied by NMR, X-ray analysis, and molecular modeling, and their affinity and selectivity were determined in radioligand binding assays using [^{3}H]CP55940 and [^{3}H]SR141716. In some instances, such as when the pyrazole and the parachlorophenyl ring is bridged with four ethylene units, the rotational freedom of the parachlorophenyl ring system is reduced to such an extent that theoretically, it should be possible to isolate two unique, mirror image conformers. That is, with this particular analog, the interconversion energy barrier between low energy conformations, as calculated by Spartan (WaveFunction Inc.), is greater than 20 kcal/mol. Thus, with this molecule, two non-superimposable mirror image conformations are expected. Indeed, NMR analysis demonstrated that the synthetic product is conformationally rigid at room temperature, as evidenced by the appearance of nonequivalent geminal protons. No coalescence was observed when the compound was heated to over 100^{o}C. Finally, recrystallization in ethanol with (+)-α-methylbenzylamine was used to attempt to crystallize one conformer as a salt. X-ray analysis of the resulting crystals showed that a single family of conformers was present, where the 4-carbon bridge shows some degree of freedom, but the energy barrier prevents the bridge from rotating the ring systems to the other conformer at room temperature. The conformational properties, receptor affinity and selectivity of these novel, rotationally constrained molecules will be further described and discussed.

INVOLVEMENT OF CB1 SIGNALLING IN BONE REMODELING

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Recent publications point to both CB1 and CB2 as important regulators of bone remodelling and bone mass. The CB1 mutant phenotype, reported in $CD1^{CB1-/-}$ mice is that of high bone mass (HBM). However, our experiments in $C57^{CB1-/-}$ mice pointed to a low bone mass (LBM) phenotype occurring in the absence of functional CB1 receptors. These studies were carried out using different methods to analyze the bone. To solve this critical discrepancy, we analyzed the expression of CB1 in bone and compared the skeletons of the $C57^{CB1-/-}$ and $CD1^{CB1-/-}$ mouse lines using identical methods, equipment and expertise. The expression of CB1 in bone was analyzed by RT-PCR in primary bone marrow-derived osteoblast (the bone forming cell) and osteoclast (the bone resorbing cell) cultures and osteoblast- and osteoclast-like cell lines and by *in vivo* immunohistochemistry. The skeletal phenotype of 9 to 12-week-old, female and male $C57^{CB1-/-}$ and $CD1^{CB1-/-}$ mice was characterized by qualitative and quantitative micro-computed tomography (μCT). Where applicable, femoral bone formation and resorption parameters were determined by histomorphometry. Expression of CB1 was found in osteoclasts. In addition, it was expressed in bone sympathetic, thyrosine hydroxylase positive neurons, in particular in trabecular bone. These nerve endings are in close proximity to osteoblasts, which do not stain for CB1. Both female and male $C57^{CB1-/-}$ mice have a marked LBM phenotype, characterized by decreased radial bone growth as well as low trabecular bone volume density and trabecular number. The mechanism leading to this LBM involves decreased bone formation and increased bone resorption. By contrast, male $CD1^{CB1-/-}$ mice exhibited an exceptionally HBM phenotype, whereas females had a normal trabecular bone mass and structure. In spite of the phenotypic differences between the $C57^{CB1-/-}$ and $CD1^{CB1-/-}$, our findings assign an important role for CB1 signalling in the regulation of bone formation and bone mass. While the expression of CB1 in osteoclasts suggests a direct effect of cannabinoid ligands on this cell, it is likely that the effect on osteoblasts is mediated through norepinephrine secretion by the sympathetic nervous system. Reportedly, such sympathetic agonists inhibit bone formation by activating osteoblastic β2-adrenergic receptors. Several phenotypic discrepancies have been reported between $C57^{CB1-/-}$ and $CD1^{CB1-/-}$ mice, and thus the dissimilar skeletal phenotype in the latter is not entirely surprising. More intriguing are the gender differences within the $CD1^{CB1-/-}$ line, which may suggest a relationship between endocannabinoid and gonadal hormones in the regulation of bone mass.

HELIX 8 LEUCINE OF CB1 CANNABINOID RECEPTOR PROMOTES SELECTIVE G PROTEIN COUPLING

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The juxtamembrane carboxyl terminus region of G-protein coupled receptors has been shown to be an intracellular helical domain, the helix 8 (H8) domain. The corresponding domain of the CB1 cannabinoid receptor has been shown to directly activate $G\alpha_o$ and $G\alpha_{i3}$, but not $G\alpha_{i1/2}$ proteins. More specifically, CB1 lacks the $NPXXY(X)_{5,6}F$ motif, having a Leu instead of a Phe. Molecular modeling studies which introduced the $NPXXY(X)_{5,6}F$ motif to the CB1 receptor (see abstract by P. Reggio) suggests that the intracellular face of the CB1 helix 8 (H8) is altered. We hypothesized that these alterations to H8 structure may be important for differential coupling to G-proteins.

The $NPXXY(X)_{5,6}F$ motif was introduced into CB1 via a L7.60(404)F mutation. A bulkier mutant L7.60(404)I which lacks aromaticity at 7.60 was also constructed. The mutant receptors were introduced into HEK cells. Saturation binding analyses showed similar Bmax and Kd of both mutations to wild type CB1. Both mutations significantly reduced the maximal stimulation levels (Emax) of [^{35}S]GTPγS binding. The L7.60(404)I mutation significantly reduced the Emax for CP-55,940>HU-210>(+)WIN-55,212-2, while rimonabant failed to inhibit [^{35}S]GTPγS binding. Immunoprecipitation studies revealed that both mutants couple to $G\alpha_{i1}$ and $G\alpha_{i2}$, but not to $G\alpha_{i3}$. The mutant receptors internalized more rapidly than WT CB1. Electrophysiological recordings showed alterations in the ability of rimonabant to activate Ca^{2+} current.

In conclusion, the leucine of the helix 8 in the CB1 receptor is required for association with Gi_3 and is also required for maximal efficacy. These results provide additional support for differential coupling to G-proteins by structurally different cannabinoids and demonstrate for the first time that this selective coupling affects CB1 maximal activation. The reduction in the rate of CB1 receptor internalization may disclose molecular mechanisms involved in tolerance towards cannabinoids.

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LIPID RAFTS, CB2 RECEPTOR SIGNALING AND METABOLISM OF 2-ARACHIDONOYL-GLYCEROL IN HUMAN IMMUNE CELLS

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Recently we have shown that treatment of rat C6 glioma cells with the membrane cholesterol depletor and raft disruptor methyl-β-cyclodextrin (MCD) doubles the binding of anandamide (AEA) to type-1 cannabinoid receptors (CB1R), followed by CB1R-dependent signaling via adenylate cyclase (AC) and p42/p44 mitogen-activated protein kinase (MAPK) activity (Bari, M., Battista, N., Fezza, F., Finazzi-Agrò, A. and Maccarrone, M. (2005) *J. Biol. Chem*. 280, 12212-12220). Here, we investigated whether also type-2 cannabinoid receptors (CB2R), widely expressed in immune cells, are modulated by MCD. We show that treatment of human DAUDI leukemia cells with MCD does not affect AEA binding to CB2R. The activation of CB2R by AEA triggers a similar [^{35}S]GTPγS binding in MCD-treated and control cells, and thus a similar effect on AC and MAPK activity, and on MAPK-dependent protection against apoptosis. The other AEA-binding receptor TRPV1, the AEA synthetase NAPE-PLD and the AEA hydrolase FAAH were not affected by MCD, whereas the activity of the AEA membrane transporter AMT was reduced to ~55% of the controls. Furthermore, neither diacylglycerol lipase nor monoacylglycerol lipase, which respectively synthesize and degrade 2-arachidonoylglycerol, were affected by MCD, whereas the transport of 2-arachidonoylglycerol was reduced to ~50%. Instead, membrane cholesterol enrichment almost doubled the uptake of 2-arachidonoylglycerol and AEA. Transient expression of CB1R and CB2R in human immune cells ruled out that the different effect of raft disruption on the two receptor subtypes might be due to the gross cellular environment.
Altogether, the present data demonstrate that lipid rafts control the activity of CB1R, but not that of CB2R, and endocannabinoid transport across the plasma membranes.

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CANNABIDIOL IS AN INVERSE AGONIST AND EXHIBITS UNEXPECTEDLY HIGH POTENCY AS AN ANTAGONIST AT MOUSE BRAIN CB1 AND HUMAN CB2 RECEPTORS

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We have reported previously that the non-psychotropic phytocannabinoid, cannabidiol (CBD), opposes the ability of cannabinoid receptor agonists to inhibit electrically-evoked contractions of the mouse isolated vas deferens with a potency greater than expected from its affinity for CB_1 or CB_2 receptors (Pertwee *et al.*, 2002). We have also found that, by itself, CBD can enhance the amplitude of electrically-evoked contractions of this tissue. The present investigation was directed at exploring the ability of CBD to behave as an antagonist/inverse agonist at mouse brain CB_1 receptors and human CB_2 receptors. The methods we used are detailed elsewhere (Thomas *et al.*, 2005).

Both 1 μM CBD and 10 nM SR141716A produced a rightward shift in the log concentration-response curve of the established CB_1/CB_2 receptor agonist, CP55940, in mouse brain membranes when the measured response was stimulation of [^{35}S]GTPγS binding. However, while the apparent K_B of SR141716A (0.09 nM) was only slightly less than its CB_1 K_i value (2.2 nM) for [^{3}H]CP55940 displacement from mouse brain membranes, the apparent K_B of CBD (78.8 nM) was well below its CB_1 K_i value (4.9 μM). By itself, CBD at 1 and 10 μM but not at 1 or 100 nM produced significant inhibition of [^{35}S]GTPγS binding to these membranes. The inhibitory effect of 1 μM CBD matched that of 1 μM SR141716A, whereas the inhibitory effect of 10 μM CBD greatly exceeded that of 10 μM SR141716A. In human CB_2-CHO cell membranes, 1 μM CBD produced a downward as well as a rightward shift in the log concentration-response curve of CP55940 for stimulation of [^{35}S]GTPγS binding. The apparent K_B of CBD (65.1 nM) was 64.5 times less than its CB_2 K_i value (4.2 μM). SR144528 also produced a downward as well as a rightward displacement of the log concentration response curve of CP55940 in this bioassay and its apparent K_B (0.49 nM) was 15 times less than its CB_2 K_i value (7.5 nM). By themselves, CBD and SR144528 each inhibited [^{35}S]GTPγS binding to CB_2-CHO cell membranes. Although the inhibitory efficacy of CBD matched that of SR144528, CBD was about 1000 times less potent. The downward displacement of the CP55940 log concentration-response curve in CB_2-CHO cell membranes that CBD and SR144528 produced may explain why their apparent K_B values for CP55940 antagonism at CB_2 receptors were so far below their CB_2 Ki values.

In conclusion we have discovered that CBD behaves as an inverse agonist and as an antagonist of CP55940 both in mouse brain membranes and at the human CB_2 receptor. The potency exhibited by CBD as a CP55940 antagonist was significantly greater than the potency it exhibited in [^{3}H]CP55940 displacement assays.

Pertwee, R.G. *et al.* (2002) *Eur. J. Pharmacol.*, **456**, 99-106.
Thomas, A. *et al.* (2005) *Br. J. Pharmacol.*, **146**, 917-926.

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ALLOSTERIC MODULATION OF THE CANNABINOID CB_1 RECEPTOR: NOVEL ALLOSTERIC MODULATORS

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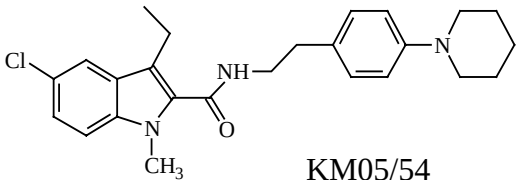
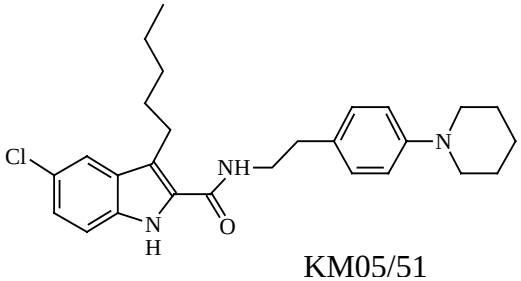
In a recent publication (Price et al, 2005) we identified three novel allosteric modulators of the cannabinoid CB_1 receptor. Interestingly, these compounds are allosteric *enhancers* of agonist binding *affinity*, but allosteric *inhibitors* of agonist signalling *efficacy*. To date, this is the most striking example in the GPCR field of dissimilitude between modulator effects on orthosteric ligand affinity versus efficacy, and provides us with a unique opportunity to dissect the mechanistic basis of this effect. The compounds that we have identified display a number of characteristics commonly associated with allosteric modulators, including (a) enhancement of the binding of the orthosteric ligand binding of [^{3}H]CP55950 to mouse brain membranes, (b) a slowing of the dissociation rate constant(s) for [^{3}H]CPP55940 from the occupied CB_1 receptor and (c) a non-competitive inhibition of orthosteric agonist efficacy, as demonstrated, for example, by the effect of the compounds on stimulation of [^{35}S]GTPγS binding to mouse brain membranes and inhibition of electrically-evoked contractions of the mouse vas deferens by CB_1 receptor agonists. We have extended our study by synthesising two novel structural analogues of these compounds (see below):



KM05/54



KM05/51

In equilibrium binding assays in mouse brain membranes, KM05/51 significantly increased the binding of the CB_1 receptor agonist, [^{3}H]CP55940, indicative of a positively cooperative allosteric effect. In contrast, KM05/54 produced an incomplete inhibition of the binding of [^{3}H]CP55940, indicative of negative allosteric modulation. [^{3}H]CP55940 dissociation kinetic studies also validated the allosteric nature of the KM compounds, since both significantly decreased radioligand dissociation from mouse brain membranes. In functional assays, the compounds both behaved as insurmountable antagonists of receptor activation; in the mouse vas deferens assay and [^{35}S]GTPγS binding assay, they elicited a significant reduction in the E_{max} value for the CB_1 receptor agonists WIN55212-2 and CP55940. The data presented confirm that the cannabinoid CB_1 receptor contains an allosteric binding site that can be recognized by synthetic small molecule ligands.

References: Price M.R. et al (2005) Mol Pharmacol 68: 1485-1495

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CB_1 CANNABINOID RECEPTORS MEDIATE TRANSLOCATION OF NO-SENSITIVE GUANYLYL CYCLASE IN NEURONAL CELLS

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NO (nitric oxide)-sensitive guanylyl cyclase (GC) is a heterodimeric protein that mediates the down-stream effects of NO. NO binding to NO-sensitive GC catalyzes the conversion of GTP to second messenger cGMP, which mediates various physiological processes. NO production could be stimulated by cannabinoid receptor agonists in N18TG2 neuroblastoma cells (Mukhopadhyay et al., 2002; Norford et al., 2002). After 1 h incubation with cannabinoid agonists CP55940 and WIN55212-2, $GC\beta_1$ in the cytosol was significantly reduced, whereas $GC\beta_1$ in the membranes was increased. Translocation of $GC\beta_1$ from the cytosol was blocked by the CB_1 antagonist rimonabant (SR141716) and by the Gi/o inactivator pertussis toxin, indicating that the CB_1 receptor and Gi/o proteins are required for the response. Long-term treatment (48 h) with CB_1 receptor agonists reversed the response that had been observed acutely, indicating that the response could be desensitized. N18TG2 cells are known to produce endocannabinoids including anandamide and 2-arachidonoylglycerol (Di Marzo et al., 1996a; Di Marzo et al., 1996b), and thus, the receptors may be experiencing tonic, low-level stimulation as the endocannabinoids accumulate in the media. Treatment with the antagonist rimonabant or with pertussis toxin reduced the amount of $GC\beta_1$ in the cytosol, suggesting that endocannabinoids synthesized by these cells may produce a tonic maintenance of the enzyme levels in the cells. It is possible that the NO-sensitive $GC\alpha_2$ subunit is able to localize to synaptic membranes by binding to post-synaptic density-95 (PSD-95), PSD-93, SAP97 or SAP102 due to its PDZ domain binding capability. The functional significance of the CB_1 receptor-mediated translocation of NO-sensitive GC to the membrane may be to allow spacial and temporal coordination of signaling rather than activation of the enzyme.

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RAPID RECEPTOR DESENSITIZATION MODULATES CANNABINOID RECEPTOR ACTIVATION OF P42/P44 MAP KINASE

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Mechanisms mediating the development of tolerance to cannabinoids remain poorly understood. On the cellular level, CB1 receptors are phosphorylated by G protein receptor kinases (GRK's) and bind β-arrestin following activation by agonists. Additionally, CB1 receptors are rapidly internalized from the cell surface following agonist stimulation. To determine the balance of these two processes in regulating CB1 receptor signal transduction during sustained receptor stimulation, we evaluated the parameters affecting p42/p44 MAP kinase activation in HEK cells stably expressing CB1 receptors.

CB1 receptor agonists mediate a transient activation of p42/p44 MAP kinase with a distinct structure-activity relationship relative to peak activation. CP55,940 and WIN55,212-2 are full agonists, 2-AG and THC are partial agonists and anandamide is a weak partial agonist.

To determine if receptor desensitization or internalization was responsible for the termination of CB1 receptor signal transduction, activation of p42/p44 MAP kinase was evaluated in cells expressing CB1 receptors with the putative GRK phosphorylation sites (S426A/S430A) mutated. From earlier studies, it is known that mutation of these residues attenuates some forms of CB1 receptor desensitization, without affecting CB1 receptor internalization. In HEK cells, CB1 (S426A/S430A) receptors activated p42/44 MAP kinase with the expected efficacy but the duration of the response was markedly prolonged relative to wild-type receptors.

These data indicate that the duration of p42/p44 MAP kinase activation by CB1 receptors is regulated by receptor desensitization, likely by phosphorylation of S426 and/or S430, and underscore the importance of receptor uncoupling in the regulation of CB1 receptor signaling.

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THE EFFECTS OF CHRONIC CANNABINOIDS IN BETA-ARRESTIN2 -/- MICE

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Previously studies have shown that the effects of acute Δ^9-tetrahydrocannabinol (THC), but not other cannabinoid agonists (CP55940, methanandamide, JWH-073 and O-1812), were greater in beta-arrestin2 -/- mice than in their wild type counterparts. The present study examines the role of beta-arrestin2 on the effects of chronically administered cannabinoids.

In separate groups of mice, THC, CP55940 or vehicle (1:2:37, ethanol:emulphor:ddH20) were administered ip once per day. Prior to the 1st and after each subsequent injection, tail withdrawal latency and rectal temperature were measured. THC and CP55940, but not vehicle, produced increased tail withdrawal latencies and decreased rectal temperature. At 25, 37.5 and 50 mg/kg, THC had greater antinociceptive and/or hypothermic effects in beta-arrestin2-/- than in wild-type mice on the 1st and/or 2nd days of treatment, but these differences disappeared with further treatment. In contrast, there were no differences between wild-type and beta-arrestin2-/- mice at 0.5 or 1 mg/kg CP55940 at any time point. Mice developed rapid tolerance to THC and CP55940 at rates that did not appear to differ between wild-type and beta-arrestin2-/- mice.

Following treatment with 0.5 mg/kg CP55940, 50 mg/kg THC or vehicle, mice were observed before and after injections of 10 mg/kg of the CB_1 antagonist, SR141716A, to quantify signs of spontaneous and antagonist-precipitated withdrawal. The predominant behaviors for all groups prior to antagonist were rearing and to a lesser extent grooming, accompanied by small amounts of jumping or scratching. Wriggling and head-shaking appeared only after antagonist and only in agonist-treated groups.

Following antagonist injection, vehicle-treated groups exhibited increased scratching and grooming and decreased rearing, with no significant differences between wild-type and beta-arrestin2-/- mice. The groups treated with CP55940 showed small increases in jumping and scratching and decreases in rearing, but the beta-arrestin2-/- group exhibited significantly more wriggling than the wild-type group (46 ± 9 vs. 18 ± 2, $p < 0.01$). Both groups treated with THC showed similar increases in wriggling, scratching and grooming, however the wild-type and beta-arrestin2-/- differed in the other behaviors. Wild-type mice exhibited significantly more head-shaking (3.1 ± 0.7 vs. 0.3 ± 0.1, $p = 0.001$), jumping (3.5 ± 0.8 vs. none) and rearing (62 ± 8 vs. 10 ± 3, $p < 0.0001$) than beta-arrestin2-/- mice.

These studies demonstrate that beta-arrestin2 is not involved in the development of tolerance to THC or CP55940, even though beta-arrestin2-/- mice are more sensitive to acute THC. In contrast, the presence of beta-arrestin2 (in wild-type mice) appears to increase the severity of antagonist-precipitated withdrawal from CP55940 or THC. The withdrawal profiles differed, since CP55940 withdrawal was characterized by frequent wriggling that differed across genotypes, while THC withdrawal produced head-shaking and smaller amounts of wriggling.

CELLULAR SIGNALING PROPERTIES OF THE NEWLY IDENTIFIED FATTY ACID AMIDE N-PALMITOYL GLYCINE FOUND IN RAT CENTRAL NERVOUS SYSTEM AND SKIN

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Following the identification of twelve endogenous fatty acid amides in rat brain (Bradshaw et al, 2005; ICRS abstracts online, 120) we further investigated the distribution and cellular signaling properties of N-palmitoyl glycine (PalGly), N-stearoyl glycine (StrGly), N-oleoyl glycine (OlGly), N-linoleoyl glycine (LinGly), and N-docosahexaenoyl glycine (DHGly). The levels of these compounds were quantified in rat central nervous system, visceral organs, and skin, and their release from F-11 DRG X Neuroblastoma cell line and the HaCat human epithelial cell line was examined. In addition, these five acyl glycines were synthesized, and the mechanisms by which they induce calcium mobilization in the F11 cells was studied. *Method:* levels in body tissues and from cellular release assays were analyzed using LC/MS/MS. Single cell calcium mobilization was measured in Fura-2/AM loaded cells alternately excited at 340 and 380nm. *Results*: Each of the acyl glycines was found in all tissues measured with the highest levels in skin, spinal cord, and lung (e.g. PalGly skin levels ~1.5 nmol/g tissue). F11 and HaCat cells showed release of compounds into incubation media, PalGly, StrGly, and OlGly being the most abundant. Single cell calcium imaging experiments revealed a dose-dependent (EC_{50} ~5μM), immediate and robust intracellular calcium mobilization following application of PalGly. StrGly gave a weaker response followed by OlGly and no response to LinGly and DHGly. N-palmitoyl alanine (differs from PalGly by one carbon) was inactive. The effect of PalGly was not blocked by SR141716A, SR144528, or MK801. Calcium mobilization was dependent on the presence of extracellular calcium and was blocked by the transient potential (TRP) channel blocker Ruthenium red (10μM) but not by the TRPV1 antagonist 5'-iodoresiniferatoxin I-RTX (35nM). In addition, PalGly-induced calcium mobilization was attenuated by the receptor operated or voltage-gated calcium channel inhibitor SK&F 96365 (> 10μM). N-palmitoyl glycine was further tested on TRPV1, TRPV3, and TRPV4 transfected HEK293 cells yielding no significant increases in calcium mobilization. *Discussion:* N-palmitoyl glycine is a newly discovered endogenous compound widely distributed in skin, lung and spinal cord and released in cellular media further investigation are underway to determine activity at other TRP channels. *Conclusions*: The newly discovered endogenous family of acyl glycine compounds is prevalent throughout the body and at least one member, PalGly, induces calcium mobilization in a dorsal root ganglion neuronal cell line. The potency of PalGly together with the structure-activity data indicates that PalGly likely acts through a receptor, possibly a TRP channel.

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CB1 L7.60I AND L7.60F MUTATIONS RESULT IN CONFORMATIONAL CHANGES IN HELIX 8 R7.56(400)-K7.58(402) ELBOW AND INTRACELLULAR FACE REGIONS OF CB1

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The cannabinoid CB1 receptor is a member of the Class A (rhodopsin-like) G protein coupled receptor (GPCR) superfamily. One of the critical sequence divergences between the cannabinoid CB1 receptor and rhodopsin is in the intracellular extension of transmembrane helix 7 (TMH7), also called Helix 8. The rhodopsin sequence contains the NPXXY$(X)_{5,6}$F motif in the TMH7-Hx8 region. This motif links through aromatic stacking, F7.60 in Helix 8 with Y7.52 in TMH7. The CB1 receptor is not capable of a similar interaction, as sequence position 7.60(404) in CB1 is a Leu. We performed two *in silico* mutations at sequence position 7.60(404) in our model of the CB1 inactive state to investigate the impact of the introduction of aromaticity at position 7.60 (L7.60(404)F mutation) or the introduction of additional bulk at 7.60 (L7.60(404)I mutation). Each resultant model was positioned in a pre-equilibrated 1-palmitoyl-2-oleoyl- *sn*-glycero-3-phosphocholine (POPC) bilayer, in order to examine the effects of each mutation on Hx8 in the presence of a membrane bilayer environment. The system contained 150 POPC molecules, 6286 water molecules, and the CB_1 receptor. The CHARMM27 parameter set was applied to the CB_1 model, which resulted in all ionizable residues being charged. Conjugate gradient energy minimization (to a gradient of less than 0.05 kcal/mol) was used to remove unfavorable contacts. The protein was then restrained and the system was heated to 310K in 50K increments of 50ps. This allowed for some adjustment of the phospholipid and water to the presence of the receptor. Then the protein, as well as the lipid and water molecules that were within 10 Å of the protein were energy minimized.

Simulation results for the L7.60(404)F and L7.60(404)I mutants, as well as for WT CB1 showed that the region from D7.59(403) to P7.69(413) forms an amphipathic helix which is situated parallel to the membrane plane and lies in the phosphate/glycerol region of the bilayer. Two important changes were noted in the mutants vs. WT. First, the conformation of the elbow segment linking TMH7 and Hx8, R7.56(400)- K7.58(402), is different between WT and L7.60(404)F and L7.60(404)I mutants. Second, the pattern of polar and non-polar residues on the intracellular face of each receptor in the region of Hx8 was different, with residues exposed in the mutants that were buried in WT CB1. Since the intracellular face of CB1 is the face presented to G protein, these results suggested that the L7.60(404)F and L7.60(404)I mutants may differ from WT CB1 in G protein recognition.

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LIPIDOMIC APPROACHES TO THE IDENTIFICATION OF NOVEL, ENDOGENOUS FATTY ACYL AMIDES

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The discovery of endogenous fatty acyl amides such as N-arachidonoyl ethanolamine (anandamide), N-oleoyl ethanolamine (OEA), oleamide and N-arachidonoyl dopamine (NADA) as signalling molecules in the central and peripheral nervous system led us to pursue other unidentified signalling molecules. Until recently, technical challenges, particularly those associated with lipid purification and chemical analysis, substantially hampered the identification of low abundance signalling lipids. Improvements in chromatography and mass spectrometry (MS) such as miniaturization of HPLC components, hybridization of multistage mass spectrometers and time of flight technology, the development of electrospray ionization (ESI), and information dependent acquisition, now permit rapid identification of novel, low abundance signalling lipids. Novel compounds from this line of research along with data on their bioactivity will be discussed.

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ANANDAMIDE TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER

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The endocannabinoid anandamide (AEA) has many neurovascular activities. However, it is not yet clear how AEA can be metabolized at the neurovascular interface, and how it can move through the vascular and the cerebral compartments. The results reported in this article show that isolated bovine brain microvessels, an *ex vivo* model of the blood-brain barrier, have detectable levels of endogenous AEA and possess the biochemical machinery to bind and metabolize it, *i.e.* type-1 and type-2 cannabinoid receptors (CB1R and CB2R), a selective AEA membrane transporter (AMT), an AEA-degrading fatty acid amide hydrolase, and the AEA-synthesizing enzymes *N*-acyltransferase and *N*-acyl-phosphatidylethanolamines-specific phospholipase D. We also show that activation of CB1R enhances AMT activity, through increased nitric oxide synthase (NOS) activity and subsequent increase of NO production. AMT activity is instead reduced by activation of CB2R, that inhibits NOS and NO release. In addition, binding experiments and immunoelectronmicroscopy demonstrate that different endothelial cells differ in the expression of CB1R and CB2R on the luminal and/or abluminal sides. The different localization of CBRs can lead to a different effect on AMT activity on the luminal and abluminal membranes, suggesting that the distribution of these receptors may drive AEA directional transport through the blood-brain barrier and other endothelial cells.

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"VANILLIPIDOMICS": AN UPDATE ON ENDOGENOUS LIPID LIGANDS OF VANILLOID RECEPTORS

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Endovanilloids are defined as endogenous ligands of transient receptor potential vanilloid type 1 (TRPV1) channels (Di Marzo et al., Trends Pharmacol. Sci, 2001; Curr. Opin. Neurobiol., 2002). TRPV1 channels represent a compendium of all possible ways the activity of a protein can be regulated by lipids. All endovanilloids identified to date are lipid derivatives of arachidonic acid (see Ross et al., Br. J. Pharmacol., 2003; van der Stelt & Di Marzo, Eur. J. Biochem., 2004 for reviews), and the most studied examples include 12-hydroperoxyeicosatetraenoic acid (Hwang et al., Proc. Natl. Acad. Sci. USA, 2000), leukotriene B_4 (McVey & Vigna, Gastroenterology, 2005; McHugh et al., J. Pharmacol. Exp. Ther. 2006), the endocannabinoids anandamide (Zygmunt et al., Nature, 1999) and N-arachidonoyldopamine (NADA) (Huang et al., Proc. Natl. Acad. Sci. USA, 2002), and unsaturated long chain N-acylethanolamines and N-acyldopamines (Chu et al.; J. Biol. Chem., 2003; Movahed et al., J. Biol. Chem., 2005).

Recently we have shown that anandamide can act as an intracellular messenger in intact cells and sensory neurons by contributing, via TRPV1 activation, to store-operated Ca^{2+} entry (van der Stelt et al., EMBO J., 2005). This presentation will focus on some more recent developments in endovanilloid research obtained in our and other laboratories, in particular on the capability of anandamide and NADA to act at both TRPV1 and CB_1 receptors under physiological or pathological conditions. Examples will include: 1) the capability of NADA to exert both stimulatory and inhibitory effects on glutamate release from neurons innervating dopaminergic neurons in the substantia nigra compacta, via TRPV1 and CB_1 receptors, respectively (Marinelli et al., submitted); 2) evidence that anandamide can produce inhibition of locomotion via TRPV1 in rodents (Tsavara et al., Biol. Psych., 2006); 3) the finding that elevated anandamide levels in the rat ventrolateral periaqueductal grey matter cause both anti-nociceptive and pro-nociceptive effects via TRPV1 and CB_1 receptors, respectively (Maione et al., J. Pharmacol. Exp. Ther., 2006); 4) the observation that anandamide causes phosphorylation-dependent desensitization of TRPV1 in skeletal muscle arterioles and in CHO-TRPV1 cells, thereby transforming the ligand-gated TRPV1 into a phosphorylation-gated channel (Lizanecz et al., Mol. Pharmacol., 2006), with possible therapeutic implications; and 5) the capability of both NADA and anandamide to stimulate intracellular Ca^{2+} mobilization in rat insulinoma cells by simultaneously activating TRPV1 and CB_1 receptors (De Petrocellis et al., in preparation). These recent findings will be presented in the light of our immunohistochemical results pointing to the co-expression of CB_1 and TRPV1 receptors in several brain neurons (Cristino et al., Neuroscience, 2006) and peripheral cells. Finally, the possible therapeutic potential of "hybrid" drugs targeting TRPV1 receptors as well as other signalling pathways (Appendino et al., FEBS Lett., 2006; Marquez et al., Mol. Pharmacol., 2006; Maione et al., in preparation) will be briefly discussed.

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LACK OF EFFECT OF FATTY ACID AMIDE HYDROLASE (FAAH) INHIBITORS ON INTRAOCULAR PRESSURE IN "GLAUCOMATOUS" MONKEYS

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Introduction: Anandamide, a natural mammalian cannabinoid, has been found present in mouse and human eyes. Several studies have demonstrated that exogenously administered cannabimimetics lower intraocular pressure (IOP), raising the possibility that endogenous anandamide may regulate IOP. The purpose of this study was to investigate whether increasing endogenous levels of anandamide would result in a similar effect on IOP. This was accomplished by using potent inhibitors of Fatty Acid Amide Hydrolase, (FAAH), a key enzyme regulating tissue anandamide levels.

Methods: Intraocular pressure studies were performed in laser-induced, ocular hypertensive cynomolgus monkeys. IOP was measured in conscious animals trained to accept pneumatonometry.

Results: Exogenously administered anandamide (0.1%) and its stable analog S1-methanandamide (0.1%) significantly lowered IOP in ocular hypertensive primates. In marked contrast, the FAAH inhibitors OL-135 (0.3%) and URB-597 (0.5%) produced an effect that was indistinguishable from that of control treatment with saline.

Conclusion: Although the reversible FAAH inhibitor OL-135 has been reported to produce analgesia after single dose administration, a therapeutic effect on IOP was not apparent. These data indicate that FAAH plays no role in regulating IOP in experimental glaucoma.

DOES THE INITIAL CELLULAR UPTAKE OF ANANDAMIDE SHOW TEMPERATURE-DEPENDENCY AND SATURABILITY?

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The cellular uptake of anandamide (AEA) is generally regarded as being temperature-dependent, and this dependency has often been used to define the "specific" uptake of AEA. However, such a temperature-dependency may reflect differences in the concentration of AEA available for uptake rather than differences in the rates of transport. One way of investigating this possibility is to use BSA in the assays, and to calculate unbound AEA concentrations on the basis of the temperature-dependent binding of AEA to BSA (Bojesen & Hansen, *J Lipid Res* 44 [2003], 1790-4). Using this approach, it was shown that the apparently non-saturable initial uptake of AEA into RBL2H3 cells became saturable when the unbound concentrations, rather than the added concentrations, of AEA were used (Kaczocha *et al., J Biol Chem*, in press). However, it is unclear whether the unbound concentrations of AEA are a valid representation of the AEA concentrations available for uptake.

Three separate experiments indicated that calculation of unbound AEA concentrations is not a valid measure in this regard: a) the reduction of AEA uptake by wells seeded with ND7/23 cells (or by wells alone) produced by increasing concentrations of BSA was less pronounced than would be predicted on the basis of a reduction in the unbound AEA concentration; b) the rapid (~1 min) uptake of a fixed calculated unbound concentration of AEA (0.4, 1 or 3 nM) to either wells alone or wells seeded with RBL2H3 cells increased, rather than decreased, as the assay temperature decreased from 37 to 10 °C; c) the rate of uptake into ND7/23 cells over a range of AEA concentrations was lower at 4 °C than at 37 °C when the data was plotted as added [AEA], but higher when the data was replotted as unbound [AEA] due to a pronounce leftward shift of the data at 4 °C relative to the data at 37 °C.

An alternative measure of the AEA concentration available for uptake is the use of the binding to the wells alone in the presence of BSA, since this represents a high capacity, low affinity site. The binding to the wells was found to be linear with the added AEA concentration, and the slope at 37 °C was approximately double that at 4 °C. When the initial uptake of AEA into RBL2H3 cells at 10, 23 and 37 °C was plotted against the observed binding to the wells, the points all fitted on a straight line passing through the origin. Finally, when the uptake into wells seeded with ND7/23 cells at 4 °C and 37 °C were replotted compensating for the temperature-dependent difference in available AEA (from the well data described above), the rates of uptake were essentially superimposable. Further experiments undertaken using several incubation time points at 37 °C and wells seeded with ND7/23 cells indicated that the initial rate of uptake was not saturable, whereas the time-dependent uptake showed saturability. Furthermore the time-dependent uptake of AEA was sensitive to AM404, whereas the time-dependent uptake of oleic acid was not. It is concluded that the binding of AEA to wells in the presence of BSA is a useful measure of the concentration of AEA available for uptake, and that under the assay conditions used, the apparent temperature-dependency of AEA uptake primarily reflects differences in the concentration of AEA available for the uptake process.

IDENTIFICATION OF A NOVEL MONOACYLGLYCEROL LIPASE (MGL)

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Two-step processes inactivate endocannabinoids: uptake into cells followed by intracellular hydrolysis. Strong pharmacological and genetic evidence show that FAAH hydrolyzes anandamide and MGL hydrolyzes 2-AG. Additional enzymes, including diacylglycerol lipase (DGL), cyclooxygenase and lipoxygenase, have been shown to also hydrolyze endocannabinoids under certain conditions. We found that the microglial cell line BV-2 does not express MGL mRNA and yet expresses a [^{3}H]-2-AG-hydrolyzing activity. This activity is pH-dependent (maximal activity between pH 6 and 8) and inactivated by boiling. Experiments using excess of 2-AG and anandamide show that 2-AG constitutes a better substrate. This activity is not affected by URB597, RHC80267, indomethacin and NADG, ruling out the involvement of FAAH, DGL, cyclooxygenase and lipoxygenase. Thus, we propose to refer to this novel [^{3}H]-2-AG-hydrolyzing activity as MGL2. In collaboration with Didier Lambert and Maria Luz Lopez Rodriguez, we have identified several compounds that inhibit MGL2 with IC_{50} in the micromolar range. Our results show that an additional enzymatic entity capable of hydrolyzing 2-AG exists and remains to be identified at the molecular level.

FAAH (-/-) MICE EXHIBIT NORMAL CB1 RECEPTOR FUNCTION FOLLOWING ACUTE OR REPEATED ADMINISTRATION OF CANNABINOIDS

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Mice lacking fatty acid amide hydrolase (FAAH(-/-)), the principle enzyme responsible for the degradation of anandamide (AEA) and other fatty acid amides, possess elevated levels of these endogenous signaling molecules, but display normal CB1 receptor function. Additionally, FAAH (-/-) mice develop an equivalent magnitude of tolerance to the pharmacological effects of Δ^9-THC as wild type (+/+) mice and also develop tolerance to exogenously administered AEA. The goals of the present study were to examine whether FAAH (-/-) mice would display different degree of CB1 receptor desensitization in brain and a decrease in withdrawal related behavior compared with (+/+) mice following repeated administration (twice daily for 5.5 days) of Δ^9-THC (50mg/kg) or AEA (50 mg/kg). To precipitate withdrawal, SR141716 was administered 30 min after the final injection of Δ^9-THC in both FAAH (-/-) and (+/+) mice. SR141716 precipitated an equivalent magnitude of withdrawal related behaviors in both genotypes. WIN55,212-2 stimulated guanosine 5-0-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding was assessed in hippocampus, striatum, cerebellum, substantia nigra, globus pallidus, and periaquiductal gray (PAG) to evaluate CB1 receptor desensitization following each dosing regimen. After repeated Δ^9-THC administration, [^{35}S]GTPγS binding was significantly reduced in all analyzed regions regardless of genotype. On the other hand, AEA, did not reduce [^{35}S]GTPγS binding in any analyzed region of (+/+) mice, but was significantly reduced in all analyzed regions of (-/-) mice except the cerebellum. These data indicate that FAAH (-/-) mice have normal CB1 functioning and undergo the same degree of withdrawal as (+/+) mice despite possessing constitutively elevated levels of AEA. We are currently examining whether the resistance of cerebellar CB1 receptors to desensitize following repeated administration of AEA, but not THC, is due to pharmacokinetic or pharmacodynamic factors. Nonetheless, repeated doses of large doses of THC produce identical degrees of behavioral tolerance, dependence, and CB1 receptor desensitization as observed in (+/+) mice. The observations that repeated cannabinoid administration results in CB1 desensitization in PAG striatum and globus pallidus is consistent with the behavioral tolerance to analgesic and motor effects of cannabinoids. Collectively, these findings suggest that FAAH (-/-) mice exhibit normal CB1 receptor function to the acute or subchronic effects of cannabinoids.

NOVEL METABOLISM OF N-ARACHIDONOYL GLYCINE BY FATTY ACID AMIDE HYDROLASE

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Metabolism of the fatty acid amide, anandamide, by fatty acid amide hydrolase (FAAH) has been well-established. Another endogenous fatty acid amide, N-arachidonoyl glycine (NAGly) has been shown to inhibit anandamide degradation and therefore, was hypothesized to be acting as a competitive hydrolytic substrate for FAAH. There is no direct evidence to date, however, that FAAH hydrolyzes NAGly.

Here, we examine the role of FAAH in NAGly metabolism using LC/MS/MS after solid phase extraction of brain tissue on reversed phase cartridges. We measured the effect of the FAAH inhibitor, URB 597, on brain levels of NAGly and anandamide. In a dose-dependent manner, the levels of NAGly significantly decreased while levels of anandamide increased after injections of URB 597.

Due to concerns about the specificity of URB 597, we measured the amounts of NAGly and anandamide in FAAH knockout and wild-type mice. Consistent with the effects of the enzyme-inhibitor, the levels of NAGly were significantly lower in FAAH knockout mice compared to wild type mice, while levels of anandamide were significantly higher in FAAH knockout mice compared to wild-type mice.

We then reexamined the biosynthesis of NAGly, originally conceived to occur by direct condensation of glycine with arachidonic acid. We incubated RAW 264.7 cells (a macrophage cell line that has been shown to express FAAH mRNA) with either [$^2H_8$]-anandamide or [$^2H_8$]-NAGly for one hour. Significant amounts of [$^2H_8$]-NAGly were measured after [$^2H_8$]-anandamide treatment, however, no [$^2H_8$]-anandamide was measured after [$^2H_8$]-NAGly treatment. Incubation of [$^2H_8$]-arachidonic acid with RAW cells likewise, did not produce any [$^2H_8$]-NAGly or [$^2H_8$]-anandamide.

These experiments provide evidence that support the hypothesis that FAAH plays a role in NAGly metabolism; however, that role appears to be one of anabolism. Additionally, these data suggest that anandamide may serve as a precursor for NAGly, which differs from anandamide only by the oxidation state of the carbon β to the amido nitrogen.

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RAPID IDENTIFICATION OF NOVEL ENDOGENOUS FATTY ACYL AMIDES USING NANO-LC-MS/MS AND INFORMATION-DEPENDENT ACQUISITION

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In recent years a variety of novel lipids related to endocannabinoids have been discovered and found to modulate basic bodily functions such as appetite, cardiovascular tone, sleep, memory, reproduction, and immune function. These studies have indicated the presence in brain and other mammalian tissues of large families of novel acyl amides that appear to act in many cases as signalling molecules. The ongoing identification of new endogenous lipids promises to provide new insights into molecular signalling.

Rodent brains were homogenized in cold methanol, centrifuged, and the supernatants were loaded on three solid phase extraction columns sequentially (ion exchange, C18 reversed phase, and SI normal phase columns; Varian Bond-Elute series). Fractions were concentrated and injected onto a nano LC/hybrid quadrupole/time-of-flight mass spectrometer using nano-electrospray ionization (Applied Biosystems/MDS Sciex QStar, Foster City, CA). Data were acquired with information dependent acquisition (IDA) in Analyst Software. The data were analyzed with a computer program constructed in house, which compared spectral peaks from the MS with theoretical masses based on the formulas combinatorially of 15 fatty acids and 23 amino acids and other small endogenous molecules capable of forming amides. An initial match required the spectra from the IDA experiment to contain the mass of the molecular ion and a fragment mass corresponding to the amino acid. Subsequent inspection of matches was carried out to confirm the presence of other predicted fragments. The MS/MS spectra of brain extract were also compared to a small library of synthetic compounds (~100). The results obtained from the computer program were consistent with those obtained from manual inspection of the mass spectra. One run of an IDA experiment generated approximately 1000 mass spectra from which more than 40 acyl amino acids were identified. Of these, more than thirty were novel compounds and had structures similar to previously identified fatty acyl amides. It is possible that these novel endogenous compounds have the functions that are also similar to those of endocannabinoids. The structures identified in these experiments, too many to list in this abstract, will be provided.

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NEURONAL GROWTH CONE GUIDANCE BY ENDOCANNABINOIDS

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In utero exposure to Δ^9-THC, the active component from marijuana, induces cognitive deficits enduring into adulthood. Although changes in synaptic structure and plasticity may underlie Δ^9-THC-induced cognitive impairments, the neuronal basis of Δ^9-THC-related developmental deficits remains unknown. Recently, the existence of CB_1Rs in developing cortical neurons has been demonstrated. We have previously provided evidence that CB_1Rs control neurotrophin-induced differentiation of interneuron progenitors by the src-kinase dependent transactivation of the TrkB receptor and the coincident assembly of CB_1R/TrkB hetero-complexes. Here, we define the functional significance of CB_1R on axonal growth cones *in vivo* and in sorted CB_1R+ cortical interneurons. CB_1Rs were found selectively enriched in growth cones of a subset of GABAergic interneurons engaged in intracortical or intrahippocampal migration between embryonic days 18 and postnatal day 2. CB_1Rs were functional in growth cone preparations, formed heterodimers with TrkB receptors, and coupled to downstream ERK activation. In axonal growth cones of cultured CB_1R^+ interneurons, agonist stimulation of the CB_1R suppressed KCl-induced calcium influx. Notably, CB_1R signaling was only evident in developed growth cones with multiple philopodial extensions. Locally applied CB_1R agonist gradients resulted in short-term growth arrest and antagonism of BDNF-induced growth cone elongation and turning. These data, together with the increase in the density of hippocampal CCK^+ interneurons after prenatal Δ^9-THC treatment reveals that prenatal activation of CB_1Rs conveys fundamental differentiation signals in the developing CNS and determine postnatal neuronal positioning and connectivity patterns. We conclude that interfering with CB_1R signaling during embryogenesis can profoundly modify the proper patterning and specification of cortical neuronal networks.

THE ACTIVATION OF CANNABINOID RECEPTORS DURING BRAIN DEVELOPMENT INFLUENCES CELL ADHESION MOLECULE L1 IN RATS

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Cannabinoid CB1 receptors and their ligands emerge early in brain development and are abundantly expressed in certain brain regions that play key roles in processes related to cell proliferation and migration, neuritic elongation and guidance, and synaptogenesis. This would support the notion that the cannabinoid system might play a modulatory role in the regulation of these processes. We have recently presented preliminary in vivo evidence showing that this modulatory action might be exerted, among others, through regulating the synthesis of cell adhesion molecules, such as L1, although this study focused only in fetal ages (Gómez et al., Dev. Brain Res. 147, 201-207, 2003). Now, we conducted a similar study focused on early postnatal ages (PND1, PND5 and PND12) and found that cannabinoid receptor activation also affected the levels of L1 transcripts but only in PND1, these effects disappearing during further days. We observed a reduction in L1-mRNA levels in the cerebral cortex, septum nuclei, striatum, dentate gyrus and CA3 subfield of the Ammon's horn. White matter areas and subventricular zones were, however, more resistant to cannabinoid receptor activation. We also tried to elucidate whether CB1 receptors and L1 colocalize in those regions where activation of these receptors influences L1 synthesis during fetal ages (Gómez et al., Dev. Brain Res. 147, 201-207, 2003). Comparative analyses with antibodies for the CB1 receptor and L1 revealed an equivalent immunostaining for both proteins in the corpus callosum, thus indicating a possible colocalization. In addition, CB1 receptors also colocalize with GAP43, a marker of growth cones, but not with synaptophysin. On the other hand, using cultures of fetal cortical nerve cells, we found that L1 is present mainly in neurons but not in glial cells, and, importantly, that the activation of CB1 receptors in these cells modified L1 levels. In summary, the cannabinoid system seems to modulate the levels of L1 in several brain structures during specific periods of development (late gestation and very early postnatal days), which correlates with the periods of most atypical expression of CB1 receptors in the developing brain. Possibly, this function is the consequence of colocalization of this cell adhesion molecule with CB1 receptors in several brain regions during brain development. Considering the role played by L1 in different events related to neural development, our observations might support the occurrence of a physiological mechanism by which the cannabinoid system might regulate processes such as cell proliferation and migration, neuritic elongation and guidance, and synaptogenesis.

FURTHER EVIDENCE FOR THE INVOLVEMENT OF CB2 CANNABINOID RECEPTORS IN DEPRESSION AND SUBSTANCE ABUSE

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Depression and substance abuse are mental health problems associated with stressful events in life with high relapse and reoccurrence even after treatment. The neuronal expression of cannabinoid CB2 receptors in the brain had been ambiguous and controversial and its role in depression and substance abuse is unknown. We tested the hypothesis that genetic variants of CB2 gene might be associated with depression in a human population and that alteration in CB2 gene expression may be involved in the effects of abused substances including opiates, cocaine and ethanol in rodents. Here we demonstrate that a high incidence of Q63R but not H316Y polymorphism in the CB2 gene was found in Japanese depressed subjects. CB2 cannabinoid receptors and their gene transcripts are expressed in the brains of naïve mice and are modulated following exposure to stressors and administration of abused drugs. Mice that developed alcohol preference had reduced CB2 gene expression and chronic treatment with JWH015 a putative CB2 cannabinoid receptor agonist, enhanced alcohol consumption in stressed but not in control mice. The direct intracerebroventricular microinjection of CB2 anti-sense oligonucleotide into the mouse brain reduced mouse aversions in the plus-maze test, indicating the functional presence of CB2 cannabinoid receptors in the brain that modifies behavior. Furthermore, we report for the first time using transmission electron microscopy the ultra structural and sub cellular localization of CB2 cannabinoid receptors that are mainly on post-synaptic terminals in mouse brain. Our data demonstrate the functional expression of CB2 cannabinoid receptors in brain that may provide novel targets for the effects of cannabinoids in depression and substance abuse disorders beyond neuro-immunocannabinoid activity.

CORRELATED SPECIES DIFFERENCES IN THE EFFECTS OF CANNABINOID LIGANDS ON ANXIETY AND ON GABAERGIC/GLUTAMATERGIC SYNAPTIC TRANSMISSION

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We showed earlier that cannabinoid signaling affected anxiety in mice *via*: (i) a CB1 receptor-dependent mechanism that was absent in CB1- KOs, was inhibited by the CB1 antagonist AM-251, and mediated anxiolytic effects; (ii) a mechanism that was functional in CB1-KOs, was insensitive to AM-251, but was sensitive to SR-141716A, and mediated anxiogenic effects. As discrepant findings were reported in mice and rats, here we compared the effects of cannabinoids on anxiety in the two species. As shown earlier, the cannabinoid agonist WIN-55,212 decreased, whereas AM-251 increased anxiety in mice. AM-251 abolished the effects of WIN-55,212. As earlier, WIN-55,212 increased anxiety in rats. Surprisingly, AM-251 potentiated the effects of WIN-55,212. Cannabinoids affect both GABAergic and glutamatergic functions, which play opposite roles in anxiety. We hypothesized that discrepant findings resulted from species differences in the relative responsiveness of the two transmitter systems to cannabinoids. We investigated this hypothesis by assessing the effects of WIN-55,212 on evoked hippocampal inhibitory and excitatory postsynaptic currents (I/EPSC). IPSCs were one order of magnitude more sensitive to WIN-55,212 in mice than in rats. In mice (in contrast to rats), IPSCs were more sensitive to WIN-55,212 than EPSCs. Thus, WIN-55,212 likely reduced anxiety in mice *via* GABAergic mechanisms that were inhibited by AM-251, whereas in rats, WIN-55,212 increased anxiety *via* a dominant action on glutamatergic mechanisms. AM-251 potentiated this effect in rats by inhibiting the "anxiolytic" GABAergic mechanism. We suggest that the anxiety-related effects of cannabinoids depend on the relative cannabinoid responsiveness of GABAergic and glutamatergic neurotransmission.

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NEUROANATOMICAL SITES AND BIOCHEMICAL CORRELATES UNDERLYING CANNABINOID MODULATION OF ANXIETY

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Cannabinoids have long been known to affect anxiety, however the direction of this effect has been controversial. The studies performed in these last few years suggest a biphasic effect of cannabinoids on anxiety, with low doses being anxiolytic and high doses anxiogenic. In order to elucidate the neuroanatomical substrates underlying the anxiolytic effect of peripheral injections of low doses of delta-9-tetrahydrocannabinol (THC, 0.75 mg/kg ip) we investigated Fos expression in anxiety-related brain regions rich in CB1 receptor (prefrontal cortex, nucleus accumbens, amygdala and hippocampus) of rats exposed to the elevated plus-maze (EPM). Assessment of Fos expression is the most widely used functional anatomical mapping tool to identify cells and extended circuitries that become activated in response to various stimuli including anxiogenic environment. THC treatment significantly decreased cFos amounts in the prefrontal cortex and amygdala of rats exposed to the EPM without affecting the other cerebral areas investigated. When the CB1 receptor antagonist AM251 was injected before THC, the observed decrease was fully reversed, suggesting the CB1-dependence of this effect. Since increasing evidence indicates that CREB function can regulate anxiety-like behavior in rats we then checked the level of phosphorylated CREB (pCREB) in the nuclear extracts from the prefrontal cortex, nucleus accumbens, amygdala and hippocampus of rats exposed to EPM. Among all the brain regions checked, a significant increase in the level of pCREB was observed only in the prefrontal cortex and hippocampus of rats treated with THC. The pretreatment with AM251 fully reversed the increase, suggesting again the CB1 receptor-dependence of this effect.

The second step of this work was to further investigate the cerebral areas where CB1 stimulation is mostly involved in the anxiogenic/anxiolytic effect of cannabinoids. To this aim we injected THC in specific rat brain regions that are rich in CB1 cannabinoid receptors and particularly relevant for emotional behavior, such as the amygdala, prefrontal cortex and hippocampus. The elevated plus-maze test was then performed in these animals to measure their anxiety state.

THC injection in the prefrontal cortex and in the hippocampus significantly increased the percentage of entries and time spent on the open arm and the number of head dips, suggesting an anxiolytic effect of the cannabinoid compound. When THC was injected in the amygdala, it elicited an anxiogenic response, as demonstrated by the decrease of entries and time spent on the open arms of the elevated plus-maze and the reduced number of head dips. The effect in the prefrontal cortex and amigdala was CB1-dependent since it was reversed by AM251, whereas it was not affected by the antagonist pre-treatment in the hippocampus. At a cellular level, the anxiolytic/anxiogenic effect of THC was paralleled by alteration in the phosphorylated amounts of CREB. Specifically, in the prefrontal cortex and hippocampus, where an anxiolytic response to THC was observed, pCREB significantly increased; in the amygdala, where THC produced an anxiogenic effect, a significant decrease in pCREB was detected. The CB1-dependence of this effect was similar to the one observed for behavioural parameters.

The picture that emerges from these results contributes to a better understanding of the role that the cannabinoid system plays in the modulation of emotional state as well as the neuronal substrates and neurochemical mechanisms underlying the complex and contrasting scenario induced by cannabinoids on anxiety-related behavior.

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CB_1 RECEPTOR ANTAGONISTS IN THE PHARMACOTHERAPY OF ACTIVE AVOIDANCE RELATED ANXIETY

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Increased or decreased neurotransmission at the cannabinoid CB_1 receptor has been shown to have a variety of therapeutic applications, which include the treatment of anxiety disorders. Although the exact role CB_1 receptors play in anxiety modulation remains unclear, it has recently been aided through the development of specific CB_1 receptor agonists/antagonists and genetically engineered animals. Still, blocked endocannabinoid neurotransmission through the CB_1 receptors have been shown to induce both anxiolytic and anxiogenic effects. More consistent results may be obtained in stressful situations (e.g., performance anxiety), where anxiety can be evaded through active avoidance versus passive avoidance. For instance, we demonstrated that genetic deletion as well as pharmacological blockade of CB_1 receptors decreased active avoidance, but did not affect passive avoidance in a 15 min shock-probe burying test. A lack of effect on passive avoidance is similar to what has been found in other studies. Inconsistent data may also be due to exposure time or previous habituation to the stressful environment. In fact, using microdialysis, we found that genetic deletion of CB_1 receptors decreases ACh levels during the first 15 min of a 60 min predatory odor stress test, but increases ACh efflux over the final 45 min, even though increased and decreased ACh efflux have diametrically opposite actions on anxiety modulation. Similarly, the importance of environmental context has been demonstrated in other studies.

In line with the preclinical research, clinical data indicate that a facilitation of cannabinoid neurotransmission can either increase or decrease anxiety depending on previous exposure, environment, mood, or dose. However, generally, high acute doses induce anxiety and panic attacks. An acute administration of a CB_1 receptor antagonist may reduce types of anxiety, which can be actively avoided, such as performance anxiety. Thus, CB_1 receptor antagonists may provide an alternative therapeutic method to beta blockers that are commonly used as medicinal aids in performance anxiety in people suffering from asthma, heart complications, or diabetes. The purported favorable effects of CB_1 receptor antagonism in attentional and mnemonic processes may also contribute to a better clinical profile, in this regard.

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INVOLVEMENT OF THE 5-HT_{1A} SEROTONERGIC RECEPTORS IN THE ANXIETY-LIKE EFFECTS INDUCED BY THE CB_1 CANNABINOID RECEPTOR AGONIST WIN 55,212-2

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Convergent data from genetic, pharmacological and behavioural studies have provided evidence that the endocannabinoid system play a role in the pathophysiology of anxiety disorders. Administration of Δ^9-THC or synthetic CB_1 receptor agonists induces modifications of the behavioural state causing anxiolytic or anxiogenic response in both humans and rats. Within the CNS the serotonergic system is considered one of the neurotransmitter systems mainly involved in anxiety and represents a successful therapeutic target for most forms of this disorder. Although experimental data have pointed out a behavioral and biochemical interactions between serotonergic and endocannabinoid systems, reliable results have not shown to date specific interplay between them in anxiety response. The main objective of the present study was to elucidate the relationship between cannabinoid and serotonergic systems in anxiety-like response in rats. To this aim we evaluated:

Experiment 1: the effect of intravenous (iv) CB_1 receptor agonist WIN 55,212-2 administration on extracellular serotonin (5-HT) levels in the shell part of the nucleus accumbens (NAcc), basolateral amygdala and medial prefrontal cortex, by mean of the "in vivo" microdialysis technique in rats.

Experiment 2: the effect of iv WIN 55,212-2 either alone or in combination with the CB_1 receptor antagonist Rimonabant (ip) or 5-HT_{1A} receptor antagonist WAY 100635 (sc) on anxiety–like behaviour in rats using the elevated plus maze test (EPM).

Experiments 3: the effect of direct WIN 55,212-2 microinfusion into the shell part of the NAcc either alone or in co-administration with the upper mentioned specific antagonists (systemically injected) in the EPM.

Results showed that acute iv administration of low (0.15 mg/kg) or high (0.3 mg/kg) doses of WIN 55,212-2 induced opposite effects on 5-HT release in the rat NAcc with respect to basal values, i.e increased and decreased extracellular levels, respectively. These modifications were reversed by the CB1 receptor antagonist Rimonabant (3 mg/kg ip) that did not modify 5-HT release *per se***.** The two doses of WIN 55,212-2 tested induced only a weak effect if any in the medial prefrontal cortex and basolateral amygdala. The dose of 0.3 mg/kg i.v. of WIN 55,212-2 produced an anxiogenic-like response with decreased open arms time and entries. Rimonabant (3mg/kg ip) and WAY 100635 (0.3 mg/kg sc) co-treatment, which was only partially blocked the WIN 55,212-2 induced anxiogenic response. Direct WIN 55,212-2 microinfusion (0.1 µg/area) in the shell part of the NAcc induced a marked anxiogenic-like effect only partially reversed by Rimonabant while totally antagonized by WAY 100635

Administration of each drug alone as well as their combinations did not modify locomotor activity .

These findings seem to confirm an involvement of the serotonergic system in cannabinoids induced anxiety-like response in rats specifically mediated by the shell part of the NAcc.

CANNABIDIOL REVERSES MK-801-INDUCED SOCIAL WITHDRAWAL IN RATS

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The cannabis constituent cannabidiol has been suggested to have therapeutic potential in the treatment of psychosis. Cannabidiol is an agonist for the vanilloid 1 receptor in the transient receptor potential family (TRPV1) and may also alter endocannabinoid activity by inhibiting fatty acid amide hydrolase (FAAH). Previous work in our laboratory has demonstrated that cannabidiol is able to reverse deficits in sensorimotor gating induced by the non-competitive NMDA receptor antagonist MK-801. This effect suggested that cannabidiol may be able to reverse abnormal behaviour associated with positive symptoms of schizophrenia. The present study aimed to investigate the effect of cannabidiol on MK-801-induced social withdrawal, an animal model of the negative symptoms of schizophrenia.

Separate groups of rats were treated with MK-801 (0.3 mg/kg i.p.) following pre-treatment with cannabidiol (5 mg/kg i.p.) or clozapine (3 mg/kg i.p.). Rats were placed in an open arena and videotaped for 10 min. Social behaviours such as sniffing and climbing and more aggressive interactions such as biting were assessed in addition to the measurement of locomotor activity. MK-801 produced social withdrawal, characterised by a decrease in social behaviours such as investigation, following and climbing over. Cannabidiol reversed this social withdrawal. Clozapine did not reverse social withdrawal, possibly due to the hypomotility also caused by this atypical antipsychotic. The present results support the evidence for the antipsychotic properties of cannabidiol. Further studies will aim to elucidate the central receptors involved in this effect.

A CANNABIGEROL EXTRACT ALTERS BEHAVIORAL DISPAIR IN AN ANIMAL MODEL OF DEPRESSION

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Introduction. We have previously reported that Cannabidiol (CBD) and Cannabi-chromene (CBC) extracts from the whole *Cannabis Sativa* plant have antidepressant activity,

The tail suspension test (TST) has been validated as a model of human depression which can be effectively reversed by antidepressants currently approved for human use (Steru *et al.* Psychopharmacology, 1985)

Materials and Methods. Mice were suspended from a bar by the tail. The total number of movements (struggling) and the total amount of time spent immobile were recorded. In addition, exertion was measured by strong struggling during the TST.

Animal and Drugs. Groups of 7-8 C57 Bl6J mice were tested with 0, 5, 10, 20, 40, 60 and 80 mg/kg ip, of CBG extracts were tested along with a standard dose of imipramine, 30 mg/kg ip.

Results. At the 40 mg/kg dose of CBG extract dose struggling were significantly increased compared with control (vehicle animals). Immediately following the TST, all mice were tested in an open field to evaluate the potential stimulation effects of CGG in activity (locomotion), fear (fecal boli excreted), curiousity (grooming) and exploratory behavior. No significant effects were found.

Discussion. This is the first report that a CBG extract has an antidepressive effect. Comparatively, CBD and CBC extracts are more potent than CBG in the TST. Future work will examine the potential interactions of CBG extracts with CBD and CBC to test for synergistic effects. Since CBD has also been shown to have anti-psychotic effects it is important to test CBC and CBG in animal models of Schizophrenia.

Acknowledgements: We wish to thank GW Pharmaceuticals for providing the extracts for the research.

FAAH INHIBITION AUGMENTS ANANDAMIDE'S PHARMACOLOGICAL EFFECTS BUT IS DEVOID OF ANXIOLYTIC-LIKE OR ANTIDEPRESSANT-LIKE EFFECTS

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Mice lacking fatty acid amide hydrolase (FAAH), the primary enzyme responsible for degradation of the endocannabinoid anandamide, or mice given selective FAAH inhibitors (URB597 and OL-135) display reduced responses to pain and inflammation. As URB597 has also been demonstrated to elicit anxiolytic-like and antidepressant-like effects in rodent models of emotional reactivity, the primary objective of the present study was to determine whether FAAH (-/-) mice would possess similar phenotypes. To this end, FAAH (-/-) mice and URB597-treated mice were evaluated in behavioral animal models of depression (i.e., tail suspension and forced swim tests) and anxiety (i.e., elevated plus maze). In initial studies, URB597 and OL-135 were screened for their ability to augment anandamide's pharmacological effects (i.e., analgesia, catalepsy, and hypothermia). Anandamide produced profound cannabinoid effects in FAAH (-/-) mice and mice treated with URB597. In contrast, OL-135 was at least 30 fold less potent than URB597 in enhancing anandamide's pharmacological actions. However, FAAH (-/-) mice treated with vehicle or anandamide as well as URB597-treated wild type mice failed to exhibit antidepressant-like or anxiolytic-like effects in the tests of emotional reactivity, even though the antidepressant desipramine and the anxiolytic agent midazolam were active in each appropriate assay. While growing evidence suggests that FAAH is a promising target to treat pain and inflammation, the results of the present study fail to demonstrate that inhibition of this enzyme produces effects in standard mouse models of depression or anxiety.

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INVOLVEMENT OF NITRIC OXIDE IN CB_1 CANNABINOID RECEPTOR-MEDIATED RETROGRADE SIGNALING

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Transient reduction of GABA release after depolarization of hippocampal pyramidal cells (depolarization-induced suppression of inhibition; DSI) is mediated by CB_1 cannabinoid receptors. In DSI, endocannabinoids might be released from the postsynaptic neuron and activate CB_1 receptors located on presynaptic terminals. Nitric oxide (NO), a well-known intercellular signaling molecule, can also be released from neurons in an activity- and Ca^{2+}-dependent manner. In this study, we investigated the possible interaction of the cannabinoid and NO signaling pathways in DSI.

Whole-cell patch-clamp recordings were made in CA1 pyramidal cells of hippocampal slices. Inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated in the presence of kynurenic acid. Carbachol was included in the bath solution to enhance spontaneous, action potential-dependent IPSCs. Evoked IPSCs were elicited by electrical stimulation of fibers at the border of the stratum radiatum/pyramidale. DSI was induced by depolarization of the postsynaptic neuron from –60 mV to 0 mV for 1 s.

In line with previous results, DSI of spontaneous IPSCs could be blocked by AM251, a CB_1 receptor antagonist, and was absent in CB_1 knockout mice. In addition, DSI was also prevented by inhibitors of NO synthase (L-NAME and 7-nitroindazole, 0.1 mM) and by extracellular or intracellular (postsynaptic) application of a membrane-impermeable NO scavenger (carboxy-PTIO, 0.5 and 1 mM). Treatment with a soluble guanylyl cyclase inhibitor (ODQ, 0.01 mM) also reduced DSI, whereas a cGMP analogue (8-Br-cGMP, 0.1 mM) or an NO donor (SNP, 0.2 mM) inhibited IPSCs in a CB_1-dependent manner, and partially occluded DSI. Similarly to DSI of spontanoues IPSCs, evoked IPSCs were also inhibited by AM251 and ODQ in the absence of carbachol. Immunocytochemical analysis revealed that NO-dependent cGMP accumulation was spatially confined to CB_1-immunopositive axon terminals targeting pyramidal neurons and was absent from the boutons of the parvalbumin-positive interneurons. Investigation of DSI in neuronal NOS (NOS1) knockout mice showed that its appearance and sensitivity to AM251 was comparable to that measured in wild types, but could not be blocked by ODQ.

Our data suggest that a NO-dependent pathway upstream of CB_1 receptors controls short-term plasticity at hippocampal GABAergic synapses, but impairing this mechanism could upregulate an alternative, NO-independent molecular machinery.

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D2 RECEPTORS MEDIATE FREQUENCY-DEPENDENT PRESYNAPTIC INHIBITION VIA RETROGRADE ENDOCANNABINOID SIGNALING AT CORTICOSTRIATAL SYNAPSES

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D_2 –like receptors (D_2Rs) have crucial roles in striatal function, but the mechanisms underlying their modulation of corticostriatal synaptic transmission have been controversial. A recent study suggested that D_2Rs inhibit glutamate release at this synapse, but only during high frequency synaptic activation. Since the release of postsynaptic endocannabinoids, which can act as retrograde messengers to inhibit presynaptic glutamate release, can be triggered by D_2R activation and intense synaptic activation, it is possible that such a mechanism mediates dopaminergic modulation of corticostriatal transmission. In this study, we show that D_2R activation reversibly reduces excitatory transmission onto striatal medium spiny neurons at a stimulation frequency of 20 Hz, but not at 1 Hz. This form of inhibition requires CB_1 receptor activation, as it is blocked by AM251, a CB_1 antagonist; and is absent in CB_1 knockout mice. It is also blocked by postsynaptic intracellular loading of Ca^{2+} chelators, thapsigargin (an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases), U73122 (a phospholipase C inhibitor); and by bath application of the $mGluR_1$ antagonist CPCCOEt. These results demonstrate dependent inhibition of glutamate release by the activation of striatal D_2Rs.

ENDOCANNABINOID MODULATION OF GABA RELEASE AT THE CCK+ INTERNEURON TO PYRAMIDAL CELL SYNAPSE, A KEY SITE FOR THE REGULATION OF HIPPOCAMPAL NETWORK OUTPUT

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Perisomatic inhibitory input from GABAergic basket cells has a major influence on action potential generation and spike timing of CA1 pyramidal cells. A particular feature of cholecystokinin- (CCK) positive basket cell input is that it can be selectively silenced by presynaptic cannabinoid receptors, both by the activity-dependent release of endocannabinoids from postsynaptic cells (depolarization-induced suppression of inhibition or DSI), and by the constitutive activity of the CB1 receptors. Importantly, developmental seizures persistently increase both the number of CB1 receptors and the extent of DSI. Paired patch clamp recordings were used to investigate the mechanisms that control GABA release at synapses between immunocytochemically identified CCK+ basket cells and CA1 pyramidal cells. Action potentials in the presynaptic basket cells evoked large IPSCs with fast kinetics in CA1 pyramidal cells that were abolished by depolarization of the postsynaptic membrane (DSI) and by the application of the CB1 receptor agonist WIN 55,212-2 (5μM) or the GABAB receptor agonist baclofen (100μM). Application of the muscarinic agonist carbachol (25μM) or the metabotropic glutamate receptor agonist DHPG (25μM) inhibited synaptic transmission at this synapse indirectly via the release of cannabinoids from postsynaptic cells. Interestingly, application of the CB1 receptor agonist AM251 (10μM) revealed a persistent suppression of synaptic transmission that was abolished by reducing postsynaptic calcium levels in the pyramidal cells. Our data obtained from identified CCK+ synapses show a persistent basal release of cannabinoids from the postsynaptic cells that can be increased pharmacologically or by depolarization. We propose that changes in this modulation of perisomatic inhibition might be critically involved in hyperexcitable states such as epilepsy. Supported by the NINDS (NS38580 to IS) and the DFG (NE1185/1-1 to AN).

THE ENDOCANNABINOID SYSTEM AT GLUTAMATERGIC SYNAPSES OF THE BRAIN

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Endocannabinoids are retrograde signals at a wide variety of synapses throughout the CNS. Here we show that principal cell populations in most brain areas express high levels of diacylglycerol lipase alpha (DGL-α), the enzyme involved in generation of the endocannabinoid 2-arachidonoyl-glycerol (2-AG). Immunostaining with two independent antibodies against DGL-α revealed that this lipase was concentrated in the head of dendritic spines in several principal cell types, eg. pyramidal neurons of the cerebral cortex. On the opposite side of the synapse, the axon terminals forming these excitatory contacts were found to be equipped with presynaptic CB_1 cannabinoid receptors. This precise molecular and anatomical architecture suggests that 2-AG produced by DGL-α on spine heads may be involved in retrograde synaptic signaling at glutamatergic synapses, whereas CB_1 receptors located on the afferent terminals are in an ideal position to bind 2-AG and thereby adjust presynaptic glutamate release as a function of postsynaptic activity. We suggest that this molecular composition of the endocannabinoid system may be a general feature of most glutamatergic synapses throughout the brain.

INHIBITION OF STIMULUS-RESPONSE (HABIT) LEARNING BY STRIATAL INJECTION OF THE CB_1 ANTAGONIST RIMONABANT

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The rodent striatum is a large forebrain area that receives extensive convergent excitatory inputs from the cerebral cortex, limbic system and thalamus. As the primary neuroanatomical entryway into the basal ganglia, the striatum is critical for many aspects of proper motor function, and is also necessary for particular processes of nondeclarative learning and memory. These are referred to as procedural or stimulus-response (S-R) learning, terms that describe the psychological formation of behavioral habits.

The endocannabinoid (eCB) system is implicated in various functions of learning and memory, involving mechanisms of synaptic plasticity in multiple brain networks. In the striatum, eCBs are believed to act as retrograde messengers mediating the induction of long-term depression (LTD) at excitatory corticostriatal synapses. Thus, the CB_1 antagonist/inverse agonist rimonabant has been found to prevent the induction of LTD in brain slice preparations of either the ventral or dorsolateral striatum (DLS). Both LTD and long-term potentiation (LTP) can be elicited in rodent striatum. Yet whereas these mechanisms of synaptic plasticity are generally thought to represent cellular substrates of learning and memory, there is currently little direct evidence linking these phenomena to striatal mnemonic functions, such as S-R learning.

We therefore tested the linked hypotheses that blockade of striatal LTD *in vivo*, by direct injection of rimonabant into the rat DLS, would either impair or facilitate the learning of S-R foraging strategies on a T-maze. Male Sprague-Dawley rats were bilaterally implanted with injection cannulae into the DLS. Following a recovery period, animals were maintained on a food-restricted diet and subsequently habituated to obtaining a food reward on a radial arm maze, located in a dimly lit room with no extramaze visual cues. Subjects were given intrastriatal injections of either rimonabant (5 nmol in 1 μL DMSO per hemisphere) or vehicle prior to testing. For each learning trial, the maze was arranged in a T-formation, with the central arm as the start position (determined randomly for each trial to negate the formation of relevant spatial cues). Subjects were required to make a consistent, fixed response (right or left turn, determined randomly) in order to find the reward. A criterion learning threshold was set at 9 correct responses in 10 consecutive trials, and learning curves were constructed to assess acquisition of correct responses over 75 total trials. Rimonabant treated rats were significantly impaired in learning the S-R behavioral strategy. Only 1 of 4 rats treated with rimonabant reached criterion responding in 75 trials (59), whereas all vehicle treated rats reached criterion (44±6.2, n=6). Learning curves demonstrated that rimonabant treated rats learned the task less effectively. We propose that eCB-mediated synaptic plasticity within the DLS is an important cellular mechanism for the learning of behavioral habits, consistent with a role of the eCB system in the etiology and/or treatment of mental disorders related to habitual behaviors, such as Tourette's Syndrome, obsessive compulsive disorder and addictions.

PATHOPHYSIOLOGICAL ROLES OF 2-ARACHIDONOYLGLYCEROL AND THE CB_2 RECEPTOR IN ALLERGIC INFLAMMATION

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2-Arachidonoylglycerol (2-AG) is an endogenous ligand for the cannabinoid receptors (Sugiura *et al*., (1995) *Biochem. Biophys. Res. Commun*. **215**, 89; Mechoulam *et al*., (1995) *Biochem. Pharmacol*. **50**, 83). We have proposed that 2-AG, rather than anandamide, is the true natural ligand for the cannabinoid receptors and investigated physiological and pathophysiological significance of 2-AG in various mammalian tissues including that in the immune system. Recently, we found that 2-AG induces enhanced production of chemokines such as IL-8 and MCP-1 in HL-60 cells, migration of HL-60 cells differentiated into macrophage-like cells, human peripheral blood monocytes, natural killer cells and eosinophils, morphological changes of differentiated HL-60 cells and enhanced adhesion of differentiated HL-60 cells to fibronectin and VCAM-1. We also found that the level of 2-AG was markedly elevated in TPA-induced acute inflammation in mouse ear and that 2-AG induced ear swelling in a CB_2 receptor-dependent manner. These results suggest that 2-AG plays important stimulative roles in inflammatory reactions. In this study, we investigated in detail possible pathophysiological roles of 2-AG and the CB_2 receptor in allergic inflammation. We first examined the level of 2-AG in the ear of mice with contact dermatitis. We found that the level of 2-AG was markedly elevated following challenge of the ear with oxazolone in sensitized mice. The levels of various other molecular species of 2-monoacylglycerol also increased in inflamed mouse ear. In contrast, the level of anandamide as well as those of other molecular species of *N*-acylethanolamines remained unchanged. Notably, topical application of SR144528 to mouse ear markedly reduced both ear swelling and the infiltration of leukocytes, suggesting that 2-AG and the CB_2 receptor are closely involved in the inflammation after challenge. Interestingly, the administration of SR144528 on mouse abdomen together with oxazolone upon sensitization also reduced ear swelling following challenge (five days after sensitization), suggesting that 2-AG and the CB_2 receptor are involved not only in inflammation following challenge but also in the process of sensitization. We further examined the effects of SR144528 on severe contact dermatitis, induced by repeated challenge with oxazolone, which is a model of allergic dermatitis. We found that the administration of SR144528 markedly reduced allergic responses (ear swelling and the infiltration of eosinophils). These results strongly suggest that 2-AG and the CB_2 receptor play crucial roles in the pathogenesis of allergic inflammation.

EFFECT OF CB1/TRPV1 AGONIST, ARVANIL, IN ACUTE INFLAMMATION AND IN PERSISTEN INFLAMMATORY AND NEUROPATHIC PAIN

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Both cannabinoids and vanilloids have been reported to be effective in the control of pain transmission. On these basis we tested the option that the development of "hybrid" compounds, with a dual capability to activate both cannabinoid and vanilloid receptors, might be very useful in the treatment of inflammatory diseases and chronic pain. One interesting compound is Arvanil that possesses direct agonist activity at the cannabinoid CB1 and vanilloid TRPV1 receptors and that is also able to block the membrane endocannabinoid transporter thus causing an increase of endocannabinoid level that may contribute to alleviate inflammation and pain. First we tested the efficacy of this compound in a model of acute inflammation such as that induced by an intraplantar injection of carrageenan in the mouse paw. Arvanil was able to prevent the development of oedema and, when administered in a therapeutic regimen, to abolish the established inflammation. The role of two key regulators of genes involved in the inflammatory/immune response, namely NF-kB and NF-AT, has been investigated in relation to the effect of Arvanil. The activation of NF-kB following the inflammatory stimulus was unaffected by Arvanil treatment, whereas the activation of NF-AT was completely prevented by the treatment with the compound. When repeatedly administered to rats in which a chronic inflammatory disease was induced by the injection of the complete Freund's adjuvant, Arvanil was able to significantly reduced the oedema associated to this kind of arthritic disease and, much more important, to relieve both thermal and mechanical hyperalgesia as well as allodynia. Surprisingly, both CB1 and CB2 receptors rather than TRPV1 receptor seem mediate the Arvanil-induced effect. Although it has been clearly demonstrated that the sensitivity and the expression of TRPV1 receptors is dramatically increased under inflammatory conditions, very few data exist concerning the modulation of these receptors during neuropathic pain. Such characterization is important since, for example, it has been shown that a down-regulation of opioid receptors is responsible for the lack of effect of the potent analgesic, morphine, on neuropathic pain. We try to characterize the pattern of expression of TRPV1 receptors under neuropathic pain disease induced in rats by a chronic constriction injury of the sciatic nerve. Particularly we have employed immunohistochemical, western immunoblotting and RT-PCR techniques to evaluate the expression of TRPV1 receptors in the injured sciatic nerve. Preliminary results indicate that an increased expression of TRPV1 receptors occurs in the sciatic nerve of unhealthy animals. On these bases and in the light of the enhanced expression of CB1 receptors in neuropathic pain models as previously shown, the ability of Arvanil to alleviate neuropathic pain other than the inflammatory pain is under investigation.
These findings provide new evidence for the biological activities of N-alkylvanillamides and strongly support the usefulness of the development of cannabinoid/vanilloid hybrid compounds as analgesic and anti-inflammatory agents.

INHIBITION OF FAAH PRODUCES CB1 RECEPTOR MEDIATED ANTI-HYPERALGESIC EFFECTS IN COLLAGEN-INDUCED ARTHRITIC PAIN

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There is increasing evidence that cannabinoid agonists alleviate increased pain sensitivity in animal models of neuropathic and inflammatory pain. Conversely, cannabinoids produce a variety of motor and psychotropic side effects that limit their clinical utility. Mice deficient of fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of anandamide, display analgesic and antiinflammatory phenotypes in the absence of motor deficits. However, little is known about whether inhibition of this enzyme will produce beneficial effects in animal models of arthritic pain. In the present study we investigated whether the FAAH inhibitor, URB597 has therapeutic efficacy in the collagen-induced arthritis (CIA) model of polyarthritis, which has many histopathological features in common with those observed in humans. CIA was induced by immunizing male DBA/1J mice with an emulsion of type II collagen (CII) in complete Freund's adjuvant. Following a booster injection, the mice gradually presented with clinical signs of arthritis in their limbs and exhibited a significant degree of hyperalgesia in the hotplate and tail immersion tests. Systemic administration of URB597 (10 mg/kg) reduced the thermal hyperalgesia in collagen-treated mice. These anti-hyperalgesic effects of URB597 were significantly reduced by pretreatment with the cannabinoid CB(1) receptor antagonist SR 141716A (3 mg/kg), but not the CB(2) receptor antagonist SR144528 (3 mg/kg). Neither antagonist produced any significant nociceptive effects when given alone. These findings suggest that the FAAH inhibitor URB597 produces anti-hyperalgesic effects in arthritic mice through a CB1 receptor mechanism of action. In conclusion, the results of the present study suggest that FAAH represents a promising target for the treatment of inflammatory pain disorders.

CB_2 RECEPTOR AGONISTS PHARMACOLOGICAL PROFILING

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The cannabinoid system is known to play a crucial role in the mechanism of nociception. Molecules which activate CB_1 receptors induce severe psychotropic adverse effects, which have prevented the development of CB_1 agonists as drugs. In this scenario, the CB_2 receptor has received increasing attention as a new target for neuropathic pain therapy. Since the CB_2 receptor is expressed in the periphery and only in some districts of the CNS, CB_2 selective agonists should elicit analgesic effects without displaying psychotropic side effects typical of the CB_1 ligands. In order to further validate the potential of CB_2 as analgesic target we profiled two cannabinoid agonists, (+)-AM1241 and JWH133, both *in vivo* and *in vitro*.

The antinociceptive effects of (+)-AM1241 and JWH133 were studied in the formalin and L5-L6 spinal nerves ligation (SNL) models. In the rat formalin test (+)-AM1241 (6 mpk, iv) and JWH 133 (6 mpk, iv) significantly reduced the licking behaviour during the second phase induced by the formalin injection. In the neuropathic pain model, the tactile allodynia was completely reverted by both (+)-AM1241 (6 mpk, iv) and JWH 133 (6 mpk, iv).

To complete the pharmacological characterization of the CB_2 agonists, we have generated CHO cells stably expressing rat CB_2 receptors. On this cell line we have evaluated the effect of non-selective (CB_1/CB_2) and selective CB_2 agonists in a functional assay based on the reduction of forskolin-induced release of cAMP. The rank order of potencies obtained with agonists was HU210 > JWH 015 = CP 55,940 > JWH 133 > noladin ether > 2-AG = anandamide, and all these compounds exhibited a full agonist profile. On the other hand, in this heterologous expression system, (+)-AM1241 was inactive. To elucidate if this compound was a protean agonist we studied its effect on cAMP in a native system: rat primary microglia cells. In microglia, (+)-AM1241 behaved as an agonist by decreasing forskolin-induced cAMP level, confirming the protean nature of this molecule. Moreover, *in vivo* administration of SR144528, a selective CB_2 inverse agonist, was able to block the analgesic effect induced by (+)-AM1241, confirming that, *in vivo*, this compound acts as an agonist.

Thus, (+)-AM1241 and JWH133 showed a similar antinociceptive activity *in vivo*, confirming the interest of CB_2 agonists as analgesic agents. In addition, the use of recombinant and native CB_2 receptor expressing systems allowed to unveil the protean nature of (+)-AM1241.

ACUTE INTERACTIONS BETWEEN CBD, CBN, AND THC FOLLOWING INTRAVENOUS OR INHALATION EXPOSURE IN THE MOUSE TETRAD

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Potential interactions between THC and other marijuana constituents may have important implications for understanding the long-term health consequences of chronic marijuana use as well as for attempts to develop therapeutic uses for THC and other CB_1 agonists. As part of a larger effort to systematically investigate these interactions, we evaluated whether cannabidiol (CBD) or cannabinol (CBN) could modulate the pharmacological effects of intravenously administered THC or inhaled marijuana smoke in ICR mice in the tetrad (hypoactivity, antinociception, catalepsy, and hypothermia), a series of well characterized models of CB_1 receptor-mediated activity. Intravenously administered CBD possessed very little activity on its own, and at a dose equal to a maximally effective dose of THC (3 mg/kg) failed to alter THC's effects on any measure. However, higher doses of CBD (ED_{50} = 7.4 mg/kg) dose-dependently potentiated the antinociceptive effects of a low dose of THC (0.3 mg/kg). Pretreatment with 30 mg/kg, but not 3 mg/kg, CBD significantly elevated THC blood and brain levels. No interactions between THC and CBD were observed in several variations of a marijuana smoke-exposure model, whether quantities of CBD were applied directly to marijuana, CBD and THC were both applied to placebo plant material, or mice were pretreated intravenously with 30 mg/kg CBD before being exposed to marijuana smoke. Intravenously administered CBN produced agonist effects which were approximately 10 times less potent than THC (antinociceptive ED_{50} = 40 mg/kg). No interactions were observed between equal doses of THC and CBN whether administered intravenously, nor when CBD was applied to marijuana and inhaled in smoke (approximately 1:1 THC:CBN ratio). Since the amount of CBD and CBN found in most marijuana strains is usually much less than that of THC, these results suggest that CBD and CBN exert very little, if any, modulatory effects on the CB_1 receptor-mediated pharmacological effects of most marijuana strains. However, our data suggest that there may be some therapeutic benefit of adding high doses of CBD to THC for pain management.

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UNDESIRABLE WEIGHT GAIN CAUSED BY PROLONGED USE OF ANTI-DEPRESSANT MEDICATION MAY BE PREVENTED WITH RIMONABANT WITHOUT LOSS OF ANTIDEPRESSANT EFFECTIVENESS

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Pharmacological antidepressant treatment has been used for decades. After short-term weight loss, antidepressant medication when administered for prolonged periods, often induces weight gain which in turn, puts the patient at risk for conditions such as coronary heart disease and noncompliance. Co-administration with pharmaceutical agents is compromised by their psychoactive and addictive potential and/or absence of a known weight loss mechanism. Marihuana, its active component THC and exogenous or endogenous cannabinoids, enhance food intake. Conversely, cannabinoid CB_1 receptor antagonists induce weight loss without undesirable side effects and may also regulate mood. Here, we explored co-treatment of antidepressants with the CB_1 receptor antagonist rimonabant (SR141716). Aims: to investigate 1. the effects of acute co-treatment of fluoxetine (‘prozac’) or desipramine (DMI) with rimonabant in the ‘forced swim test' (FST) for ‘depression’ and on motor activity 2. effects of chronic injections (3 months) of DMI and/or rimonabant on weight gain. 3. Effects of chronic injections of DMI and/or rimonabant on FST and in the Plus-maze test for anxiety.

Methods: 1. Female (Sabra) mice received rimonabant (5 mg/kg) prior to fluoxetine (20 mg/kg) or DMI (15 mg/kg), after which they were tested for FST and motor activity. 2. Chronically, rimonabant (2 mg/kg) and DMI (5 mg/kg) were injected daily for 3 months. Body weight, FST (9 min) and Plus-maze behavior (5 min) were assessed periodically during and after treatment.

Results: 1. Rimonabant did not interfere with the ‘anti-depressant' effects of DMI or fluoxetine in FST. Since rimonabant combined with either antidepressant, decreased motor activity, the ‘antidepressant’ effects of the combined treatment could not be caused by general hyperactivity. 2. The initial weight loss in DMI-treated mice was reversed after several weeks of treatment, developing into significantly greater weight gain compared to controls. Rimonabant induced weight loss which persisted even after cessation of treatment. Combined treatment with DMI prevented the weight gain induced by DMI alone. 3. FST testing throughout the treatment period indicated that the antidepressant effect of DMI and of the combined DMI+rimonabant treatment was preserved. Anxiety in the Plus maze was transiently increased by the combined DMI+rimonabant regimen.

Conclusions: A. In mice, like men, DMI induces weight increase upon prolonged treatment. B. Rimonabant does not interfere with the effects of anti-depressants. C. We have demonstrated for the first time, that rimonabant may be used as an adjunct medication to anti-depressants to prevent weight gain.

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CB1 RECEPTORS CONTROL ENDOCANNABINOIDS RELEASE IN THE HYPOTHALAMUS OF FREELY MOVING RATS

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There is now ample evidence that endogenous cannabinoids and CB1 receptors located in brain regions (e.g. the hypothalamus and nucleus accumbens) and in peripheral tissues (e.g. fat and liver) play a key role in the control of food intake and body weight. Blockade by rimonabant of CB1 receptors in those locations accounts for its pharmacological effects in obesity and related metabolic disorders. Moreover, substantial levels of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) have been measured in brain tissues suggesting that they are synthesized and released locally. However, the release of these transmitters in brain regions has barely been approached *in vivo* due to the lack of a sufficiently sensitive and specific analytical method to detect endocannabinoids in the extracellular compartment.

We have recently developed a new method, combining online brain microdialysis with liquid chromatography/tandem mass spectrometry, which allowed the detection of all endocannabinoids in the extracellular fluid in different rat brain areas. In the present study, we have examined the occurrence and regulation by CB1 receptors of endocannabinoids release in the hypothalamus of freely moving rats.

Detectable amounts of AEA and 2-AG were measured in microdialysates of the rat hypothalamus (3.7 ± 0.4 and 19.7 ± 1.9 fmoles / 30 min fraction, respectively). Neuronal depolarization with KCl (60-90 mM), infused via the dialysis probe, induced a moderate increase in extracellular levels of both AEA and 2-AG (maximal effect + 134.0 ± 6.8 % and + 125.3 ± 7.7 %, respectively). Similarly, perfusion with high calcium concentrations (10 mM) led to a substantial enhancement of extracellular levels of these endocannabinoids. Moreover, local infusion via the dialysis probe of a purported anandamide uptake inhibitor (AM404, 0.25 mM), induced a substantial increase of AEA with no increase in 2-AG extracellular levels. Similarly, systemic administration of the FAAH inhibitor URB597 (0.5 mg/kg ip) caused a marked increase in the extracellular content of AEA, but not 2-AG, in the hypothalamus. These data show that neural activity or inhibition of AEA inactivation processes stimulate the outflow of AEA in the rat hypothalamus.

Systemic administration of rimonabant (10 mg/kg ip) caused a marked increase in AEA outflow (maximal effect + 209.1 ± 22.1 %) and, in contrast, reduced 2-AG release (maximal decrease – 58.6 ± 5.2 %) in the rat hypothalamus. Conversely, the CB1 agonist WIN55,212-2 (2.5 mg/kg ip), gave a mirror image, decreasing AEA and increasing 2-AG extracellular levels in this region. Altogether, these results demonstrate that CB1 receptors are able to control the local release of endocannabinoids in the hypothalamus. Interestingly, AEA and 2-AG release is differentially regulated by CB1 receptor, consistent with the presumed distinct physiological role of these two endocannabinoids.

FUNCTIONAL ACTIVITY OF CANNABINOID RECEPTORS IN MODELS OF ADIPOCYTES AND β-PANCREATIC CELLS

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We have previously reported (Matias et al., ICRS 2005) that models of adipocytes (3T3F442A cells) and pancreatic islet insulin-secreting βcells (RINm5F insulinoma cells) contain all the known elements of the endocannabinoid system (i.e. the endocannabinoids, anandamide and 2-arachidonoylglycerol, the cannabinoid CB_1 and CB_2 receptors, and endocannabinoid biosynthesising and degrading enzymes), and that, in these cells, conditions mimicking hyperglycemia and hyperinsulinemia cause a dramatic up-regulation of endocannabinoid levels. We found that endocannabinoid levels are up-regulated during obesity and/or permanent hyperglycemia also in vivo, in the pancreas and adipose tissue of mice with dietary obesity. In the present study, we investigated the mechanisms underlying the previously described elevation of lipid levels and insulin release by the cannabinoid receptor agonist, HU210, in our models of adipocytes and pancreatic β-cells, respectively (Matias et al., ICRS 2005). Since stimulation of adenylyl cyclase activity and subsequent cAMP formation are usually coupled to lipolysis and inhibition of lipogenesis in adipocytes, we studied if the stimulatory effect of HU210 on lipid levels was due to inhibition of adenylyl cyclase by assessing its effect on forskolin-induced cAMP formation in mature 3T3F442A adipocytes. Since in β-cells insulin release is induced by elevation of intracellular Ca^{2+}, we studied the effect of HU210 on $[Ca^{2+}]i$ in RINm5F β cells using fluorimetric analysis and Fluo-4- or Fura-2-loaded intact cells. We also examined by immunofluorescence the presence and the localisation of CB_1 and CB_2 receptors in RINm5F cells. Finally, immunohistochemical studies were carried out in rat and mouse pancreatic sections to determine the presence of cannabinoid receptors and their possible co-localization with pancreatic islet β and α cell markers, i.e. insulin and glucagon respectively.

In 3T3F442A adipocytes we found that HU210 dose-dependently inhibits forskolin-induced cAMP formation (EC_{50}=145 nM) in a way that is significantly attenuated by a CB_1, but not by a CB_2, receptor antagonist. We also found that HU210 dose-dependently (EC_{50}=260 nM) elevates $[Ca^{2+}]i$ in RINm5F β cells in a way that is attenuated by both CB_1 and CB_2 antagonists. HU-210 enhanced $[Ca^{2+}]i$ with similar potency also in the absence of extracellular Ca^{2+}, suggesting an effect on intracellular Ca^{2+} mobilization. The potency of HU210 in these two tests corresponded with the previously reported potency at elevating lipid levels in adipocytes and insulin release from RINm5F β cells (Matias et al., ICRS 2005). Immunohistochemistry showed that a very small, albeit significant, number of insulin-expressing cells in rat and mouse pancreatic islets expresses CB_1 receptors, and that, however, most of these β-cells express instead CB_2 receptors. This study demonstrates that both the adipocyte and pancreatic islet β cell models used here express functional cannabinoid receptors coupled to intracellular events that are likely responsible for either increased lipid levels (inhibition of cAMP formation) or insulin release (stimulation of $[Ca^{2+}]i$), respectively.

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THE INFLUENCE OF SELECTED ENDOCANNABINOID ANALOGS ON FOOD INTAKE AND LOCOMOTION

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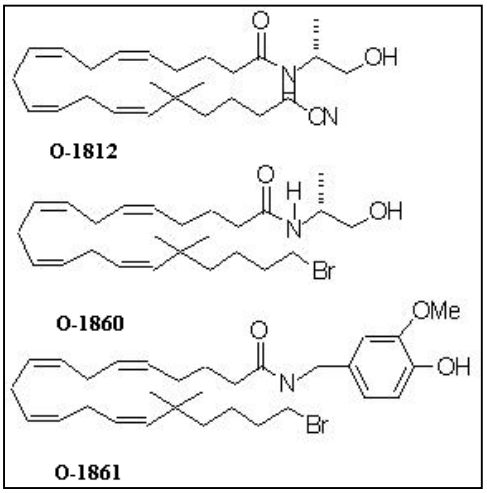
The endogenous cannabinoid anandamide (AEA) acts as an agonist at brain cannabinoid (CB_1) receptors, at transient receptor potential vanilloid type-1 (TRPV1) receptors and appears to be active at other receptors as well. This broad binding profile presents a challenge when attempting to characterize the pharmacology of anandamide mechanistically. One strategy to distinguish between the CB_1- and TRPV1-mediated actions of AEA is to develop novel, metabolically stable analogs that can differentially interact with these targets and then tease apart the resulting pharmacological actions. In these studies, we examined the influence of several such analogs (i.e., O-1812, O-1860, and O-1861) on food intake and locomotion. For comparison purposes, we also tested AEA and the exogenous cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) in the same assays. ICR mice were food-restricted for 24 hours, administered a randomized dose of each drug, and then allowed to feed on their regular chow for 1 hour. Locomotor inhibition was measured in separate mice that were not food restricted. Both AEA (3 and 10 mg/kg) and Δ^9-THC (1 and 3 mg/kg) stimulated food intake without significantly affecting locomotion. The highly selective and potent CB_1 agonist O-1812 stimulated food intake significantly at a dose that did not affect locomotion (0.1 mg/kg) and reduced both locomotion and food intake at higher doses (1 and 3 mg/kg). A similar compound, O-1860 proved to be less potent than O-1812, but did stimulate food intake at 5.6 mg/kg, a dose that produced no significant effect on locomotion. The "hybrid" CB_1/TRPV1 agonist O-1861 inhibited locomotion and failed to stimulate feeding at all doses tested (0.3 – 30 mg/kg). These results help validate the approach of using novel, metabolically-stable and differentially selective endocannabinoid analogs to discern possible mechanisms for the disparate effects of AEA. The hyperphagic properties of the compounds tested here are generally proportional to their CB_1 affinity. Moreover, the compound with high TRPV1 affinity (i.e., O-1861) did not significantly influence food intake, but did inhibit locomotion, suggesting that activity at TRPV1 receptors is not associated with increased feeding in food-deprived animals.



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ANANDAMIDE REGULATES IL-12P40 PRODUCTION BY ACTIVATING THE PROMOTER REPRESSOR ELEMENT GA-12

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There is a growing amount of evidence that the endocannabinoid system may exert benefical effects in different models of Central Nervous System (CNS) inflammation. IL-12p40 related cytokines such as IL-12 (p35/p40) and IL-23 (p19/p40) are potents regulators of adaptive immune responses and Th1 T cell differentiation. Microglial cells are the main source of these cytokines in the CNS.

Dysregulation of IL-12p40 gene has been associated with autoimmune processes. Using murine microglial primary cultures and macrophage cell line RAW264.7 stimulated with LPS/IFNγ, we demonstrated that anandamide (AEA) inhibits the secretion of IL-12p40 from activated microglia by a mechanism that is partially dependent on CB2 receptor. In an attemp to elucidate the molecular mechanisms involved in AEA actions we performed luciferase reporter gene assays for IL-12p40 promoter activity in RAW264.7 cells. We found that in activated cells, AEA downregulates IL-12p40 promoter activity by a mechanism independent on both vanilloid and cannabinoid receptors. Then we studied the responsive elements contained in the IL-12p40 promoter sequence. Site-directed mutagenesis of the C/EBPβ, NF-κB and ETS sites of the IL-12p40 promoter were unable to reverse the inhibitory effects of AEA on the reporter activity. However, when the GA-12 motif was mutated, AEA lost its inhibitory effects, suggesting that the mechanism of action of AEA involves the activation of the GA-12 represssor site on the IL-12p40 promoter. Decoy oligonucleotide assays confirmed these observations.

Interestingly, this repressor site is also regulated by prostaglandin-E_2. Prostamide E_2 (prostaglandin-E_2 ethanolamide) was recently recognized as a putative metabolite of AEA by COX-2 action. We demonstrate that prostamide E_2 is able to reverse the induction of the activity of IL-12p40 promoter by LPS/IFNγ which suggests that this compound may contribute to the AEA actions on IL-12p40 gene regulation.

ENDOCANNABINOID METABOLITES MODULATE HUMAN NEUTROPHIL MIGRATION

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The endocannabinoid anandamide binds to cannabinoid CB_1 and CB_2 receptors and has both analgesic and anti-inflammatory actions (Rice et al, 2002). Neutrophils play a critical role in the development of inflammation. The aim of this study was to investigate the effect of endocannabinoids on the migration of human peripheral polymorphonuclear neutrophils (PMNs).

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.

We have previously demonstrated that anandamide inhibits human 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. We investigated the effect of a series of enzyme inhibitors on the effects of anandamide. The inhibition of human neutrophil migration by anandamide (100nM) was unaffected by either the fatty acid amide hydrolase inhibitor, PMSF (100μM) or the cyclooxygenase inhibitor, indomethacin (10μM). However, in the presence of anandamide (100nM) with the 12 and 15 lipoxygnease inhibitor, eicosatetraynoic acid (ETYA, 10μM) the fMLP-induced migration was 79 +/- 2 % (n = 9) as compared to anandamide with vehicle (0.1% DMSO) of 43 +/- 4 %. In the presence of ETYA (10μM) alone, fMLP-induced migration was 100 +/- 1 % (n = 9).

These data indicate that a 12- or 15- lipoxygenase metabolite of anandamide may be responsible for inhibition of human neutrophil migration. It is notable that Edgemond et al., 1998 have previously demonstrated that human neutrophils metabolise anandamide to 12(S)- and 15(S)-hydroxy-arachidonylethanolamide.

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CANNABINOID-MEDIATED IMMUNOSUPPRESSION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Scientific evidence, initially demonstrated in experimental allergic encephalomyelitis (EAE) models, now supports previously anecdotal evidence that cannabinoids may be beneficial in symptom control in multiple sclerosis. Natural cannabinoids such as tetrahydrocannabinol or synthetic cannabinoid (CB) receptor agonists can also induce immunosuppression of EAE. However, the underlying mechanisms and receptor location and subtype involved in cannabinoid-induced immunosuppression remains unclear. We demonstrate that amelioration of EAE by cannabinoids is associated with the suppression of antigen-induced T cell proliferation, pro-inflammatory Th1 (interferon gamma and tumour necrosis factor) cytokine production and inhibition of mononuclear cell infiltration of the central nervous system. Secondly, although leucocytes express CB_1 and notably CB_2 receptors, cannabinoid-mediated immunomodulation is largely, if not exclusively CB_1 receptor-dependent. This was shown using selective agonists and by the loss of cannabinoid activity in CB_1-deficient animals. Thirdly, using conditional CB_1 knockout mice, we demonstrate that the major pathway of immunosuppression is not due to CB_1 receptor stimulation on T cells but more likely the indirect stimulation of CB_1 in brain centres. Finally, we demonstrate an interesting dichotomy between high and low dose cannabinoid therapy whereby immunosuppression occurred only with high doses of cannabinoids that induced stress responses and sedative physiological effects, which may not be clinically achievable or relevant. However, lower, non-immunosuppressive doses of cannabinoids that failed to inhibit the development of relapsing disease, slowed the accumulation of disability due to inflammatory attack and further highlights the neuroprotective potential of cannabinoids to slow the progression of MS.

CANNABINOID RECEPTOR-MEDIATED MODULATION OF LIPOPOLYSACCHARIDE-INDUCED ALTERATIONS IN PERIPHERAL CYTOKINE LEVELS, CIRCULATING LYMPHOCYTES AND HYPOTHALAMO-PITUITARY-ADRENAL AXIS ACTIVITY IN RATS

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The endogenous cannabinoid system represents a potential therapeutic target for neuroinflammatory disorders as it plays an important role in regulating both the nervous and immune systems. Several studies have reported the immunosuppressive effects of cannabinoids on peripheral, circulating cytokines (Smith et al., 2000, J Pharmacol Exp Ther.; Croci et al., 2003, Br J Pharmacol.). The aim of the present study was to expand this field by investigating the effects of systemic administration of the potent cannabinoid receptor agonist, HU210, the CB_1 receptor antagonist, SR141716A, and the CB_2 receptor antagonist, SR144528, on lipopolysaccharide (LPS)-induced elevations in rat plasma levels of a number of cytokines at two different timepoints. A further aim was to correlate alterations in plasma cytokine levels with alterations in circulating lymphocyte numbers and corticosterone levels. Male Sprague-Dawley rats (220-260 g, n = 6-9/group) were housed singly and habituated to handling and i.p. injection for 3 days. On the experimental day, rats received an acute i.p. injection of HU210 (100 µg/kg), SR141716A (3 mg/kg), SR144528 (3 mg/kg), HU210 (100 µg/kg) + SR141716A (3 mg/kg), HU210 (100 µg/kg) + SR144528 (3 mg/kg) or vehicle (ethanol: tween 80: saline at 1:1:18) 30 minutes prior to the i.p. administration of LPS (100 µg/kg) or saline. Blood was taken following cardiac puncture at 2 or 4 hr post-LPS injection. Levels of interleukin (IL)-1β, tumour necrosis factor α (TNFα), IL-6, IL-10 and interferon (IFN)-γ were measured in blood plasma using ELISA. LPS induced a significant increase in plasma TNFα levels 2 hours, IL-1β and IL-6 levels 2 and 4 hours and IFN-γ levels 4 hours post injection when compared with saline-treated controls. HU210 fully attenuated the LPS-induced increase in the levels of IL-1β, TNFα and IL-6 at the 2 hour time point and IFN-γ at 4 hours. SR141716A significantly attenuated the HU210 reduction of LPS-induced IL-1β and IL-6 levels at the 2 hour time point. SR144528 did not prevent the inhibitory effect of HU210 on LPS-induced plasma cytokine increases. Administration of SR141716A alone attenuated the LPS-induced IL-1β and TNFα levels at the 2 hour time point. SR144528 alone attenuated the LPS induction of plasma IL-1β, TNFα and IL-6 at the 2 hour time point and IFN-γ at the 4 hour time. Reduced circulating lymphocyte numbers and increased plasma corticosterone levels accompanied changes in cytokine levels in response to acute administration of LPS and/or cannabinoid drugs. These data demonstrate that the immunosupressive effects of HU210 on peripheral IL-1β and IL-6, are mediated, at least in part, by CB_1 receptors. In contrast, neither of the antagonists altered the HU210 effect on plasma TNFα levels or IFNγ levels, suggesting the potential involvement of novel cannabinoid receptor subtypes in regulating peripheral levels of these cytokines. Furthermore, the inhibitory effects of the selective CB_1 and CB_2 receptor antagonists on peripheral cytokine levels may indicate partial agonist-like activity of these compounds and/or complex modulation of neuro-immuno-endocrine interactions by the endogenous cannabinoid signalling system. *Work supported by HRB and NUIG Millennium Fund.*

ROLE OF THE ENDOGENOUS CANNABINOID SYSTEM IN EXPERIMENTAL CONTACT HYPERSENSITIVITY

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Introduction: Cannabis preparations have been used in traditional medicine for the treatment of inflammatory diseases. The major active constituent of the plant *Cannabis sativa* is Δ^9-THC (tetrahydrocannabinol). Two specific receptors mediate the effects of cannabinoids (CB_1- and CB_2-receptor). The discovery of endogenous CB-receptor ligands prooved the existence of an endogenous CB-system with many physiological functions. A participation of CB-receptors in the downregulation of inflammatory processes was demonstrated in experimental models for atherosclerosis or colitis. Therefore, we investigated the role of the CB-system in experimental contact hypersensitivity (CHS).

Methods: CHS was induced in C57Bl/6, C57Bl/6-$CB1^{-/-}$, -$CB2^{-/-}$ and -$CB1/2^{-/-}$ mice by application of 0,2 % DNFB on the shaved abdomen and elicited by painting the ears with 0,3 % DNFB. Ear swelling was measured after 24h, 48h and 72h. Additionally, wildtype mice were treated s.c. with the CB-antagonists SR141716 and SR144528 or the CB-agonist Δ^9-THC. Histopathological analyses were performed on inflamed skin.

Results: Contact allergy in mice lacking the CB-receptors was increased more than 100% in comparison to wildtype mice. Pharmacological blockade of CB-receptors with SR141716 and SR144528 also induced an increased ear swelling in wildtype mice. Histological analysis demonstrated elevated numbers of infiltrating $Gr\text{-}1^+$- and $MHC\text{-}II^+$-cells in CB-receptor deficient and SR141716- or SR144528-treated mice. Δ^9-THC reduced ear swelling and tissue infiltration of inflammatory cells.

Conclusions: Our results demonstrated that the endogenous CB-system is involved in downregulating cutaneous hypersensitivity responses. Future experiments will have to address how cannabinoids participate in this regulation. Furthermore, novel CB-receptor ligands may be developed for the treatment of inflammatory skin diseases.

A PRELIMINARY IMMUNOLOGICAL CHARACTERIZATION OF CB1$^{-/-}$/CB2$^{-/-}$ MICE AND THEIR SENSITIVITY TO IMMUNE MODULATION BY Δ^9-TETRAHYDROCANNABINOL

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The role of CB1 and CB2 in immune modulation by cannabinoids is poorly understood. Selective antagonists for CB1 and CB2 have provided some important insights but have been found to possess their own biological activity including off target effects, which can putatively confound the interpretation of results. In the present study the role of CB1 and CB2 in immune modulation by cannabinoids was analyzed in genetically engineered CB1/CB2 null mice. Utilizing CB1/CB2 null mice and wild-type counterparts, C57Bl/6J, the percentage of splenocyte subpopulations were determined by flow cytometry. No significant differences were detected in $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell subpopulations, nor were there any differences in $CD3^+$, $CD19^+$ or $F4/80^+$ cell populations between wild type and CB1/CB2 null mice. For a generalized analysis of immune status, in vivo and in vitro antibody forming cell (AFC) response assays were performed. Oral administration of Δ^9-THC suppressed the in vivo T cell-dependent IgM AFC response against sheep erythrocytes (sRBC) in the wild type mice but not in the CB1/CB2 null mice. Interestingly, following in vivo sensitization, the control anti-sRBC IgM AFC response in CB1/CB2 null mice was consistently greater than that in wild type mice. No differences in the in vitro IgM AFC response between wild type and CB1/CB2 null mice was observed to the polyclonal B cell activator, lipopolysaccharide (LPS). Moreover, the polyclonal IgM AFC response was not altered by Δ^9-THC treatment using splenocytes derived from either wild type or CB1/CB2 null mice at concentrations up to 15 µM Δ^9-THC. In a one-way mixed lymphocyte reaction, Δ^9-THC at 10 µM or 15 µM equally suppressed the response by CB1/CB2 null and wild type splenocytes. Similarly, the Δ^9-THC-mediated suppression of proliferation induced by LPS and PMA/Io appeared to be independent of CB1/CB2. In addition, IL-2 and IFNγ expression were both suppressed by Δ^9-THC by activated splenocytes derived from wild type and CB1/CB2 null mice. These results clearly indicates that CB1 and/or CB2 are involved in Δ^9-THC-mediated suppression of the in vivo anti-sRBC IgM AFC response while neither CB1 nor CB2 appear to be involved in the Δ^9-THC-mediated suppression of the mixed lymphocyte reaction, mitogen-stimulated B and T cell proliferation and IL-2 and IFNγ expression. (This work was supported in part by NIDA grants RO1 DA07908 and DA12740. In addition we thank Dr. Andreas Zimmer, University of Bonn for graciously providing CB1/CB2 null mice).

EFFECT OF CANNABINOIDS ON RAT CORONARY ARTERY REACTIVITY

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Introduction: Pharmacologically active compounds extracted from the cannabis plant *Cannabis sativa* (cannabinoids) have important effects in the cardiovascular system. Cannabinoids are vasodilators in a variety of vascular beds but the mechanisms involved remain unclear. Recent studies have suggested that cannabinoid signalling is mediated via a sphingosine messenger (Mo *et al*., 2004). The aim of this study was to examine the effect of cannabinoids and the sphingosine metabolite (S-1-P) on isolated rat coronary artery tone and identify potential mechanisms of action.

Methods: Coronary artery rings (2mm) from male Sprague-Dawley rats were set up in a 5ml myograph (37^oC) containing Krebs' solution and aerated with 95% O_2 and 5% CO_2. Arteries were precontracted with the thromboxame mimetic U46619 ($3x10^{-7}$ M) prior to cumulative additions of anandamide, HU210 ((6aR)-trans-3- (1,1-Dimethylheptyl)-6a, 7, 10, 10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b, d]pyran-9-methanol) and S-1-P ($1x10^{-9}$M – $3x10^{-6}$M).

Results: Anandamide caused a concentration-dependent relaxation in U46619 precontracted coronary arteries which reached a maximum of 40.5 ± 9.9% (n=7). It was found that pre-treatment of vessels with the anandamide breakdown inhibitor, phenylmethylsulphonyl fluoride (PMSF; $2x10^{-4}$M) did not significantly alter the vasorelaxant response to anandamide (48.3 ± 9.4%, n=7). The nitric oxide inhibitor, N^G-nitro-L-arginine-methylester (L-NAME; 100 µM) did not significantly alter the vasorelaxant response to anandamide (42.8 ± 8.3%; n=6) and endothelial denudation had no effect on anandamide responses (33.8 ± 1.0%; n=4). Pre-incubation with indomethacin (10 µM) significantly inhibited anandamide-induced vasorelaxation (22.7 ± 4.7%; P=0.0006; n=7) while the selective CB_1 antagonist, AM251 (1 µM) did not attenuate anandamide vasorelaxation (45.3 ± 7.4%; n=6). The stable synthetic cannabinoid, HU210, also caused a concentration-dependent relaxation in U46619 precontracted coronary arteries (41.1 ± 10.3%; n=6) and pre-incubation of arteries with the sphingosine kinase inhibitor N,N-dimethylsphingosine (DMS) significantly attenuated this vasorelaxant response (1.51 ± 8.0%; n=4). In the isolated rat coronary artery, S-1-P (1 µM) induced a vasorelaxant response (36.1 ± 4.44%; n=4) which was inhibited by pre-incubation with indomethacin (10 µM) (25.1 ± 12.2%; P<0.05; n=4).

Conclusions: Overall anandamide causes PMSF insensitive vasorelaxation in isolated rat coronary arteries that is not mediated via nitric oxide or CB_1 receptor activation but appears to be mediated via cyclooxygenase/prostanoid products. HU210 induces relaxation in the coronary artery that is abolished by the sphingosine kinase inhibitor DMS. Similar to anandamide, S-1-P causes vasorelaxation that is attenuated by indomethacin, again implicating prostanoid involvement, lending further support to the proposed link between the cannabinoid and sphingosine/sphingosine-1-phosphate pathways.

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SYNTHETIC AND PLANT-DERIVED CANNABINOIDS PREVENT RADIATION-INDUCED VOMITING VIA CB_1-RECEPTOR

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Several published clinical studies have indicated that Δ^9-THC has antiemetic potential against chemotherapy-induced emesis. Recent animal research indicate that cannabinoid CB_1/CB_2-receptor agonists prevent emesis via cannabinoid CB_1-receptors and appear to be broad-spectrum antiemetics since they prevent vomiting produced by diverse emetic stimuli including: cisplatin, morphine, serotonin 5-HT_3-, dopamine D_2/D_3- and neurokinin NK_1-receptor agonists. Currently, only scant clinical data is available and no basic research exists on the antiemetic potential of cannabinoids against radiotherapy-induced vomiting. The purpose of this study was to evaluate whether: 1) structurally diverse cannabinoids can prevent radiation-induced emesis in the least shrew (Cryptotis parva), and 2) the antiemetic activity of cannabinoids against radiation-induced emesis is CB_1-receptor mediated. Total body exposure to radiation (0, 5, 7.5 and 10 Gy) caused a robust frequency of emesis in a dose-dependent manner (Ed_{50} = 7.46 ± Gy) and all exposed shrews vomited at 10 Gy (Ed_{50} = 3.8 ± Gy). To investigate whether structurally diverse cannabinoids [CP55,940 (0, 0.025, 0.1 and 0.3 mg/kg), Δ^8-THC (0, 2.5, 5 and 10 mg/kg), Δ^9-THC (0, 1, 5, 10 and 20 mg/kg) and WIN55,212-2 (0, 1, 2.5, 5 and 10 mg/kg) can prevent radiation-induced emesis, the cited doses of CB_1/CB_2 agonists were administered to different groups of shrews 30 min prior to 10 Gy radiation. The frequency of vomiting and the number of shrews vomited were recorded for 30 min post-radiation. Both vomiting parameters were blocked in a potent and dose-dependent manner by the cited cannabinoids with the following ID_{50} potency order: CP55,940 (0.11- 0.12 mg/kg) > Δ^8-THC (2.13 - 4.36 mg/kg) = WIN55,212-2 (2.54 - 3.65 mg/kg) > Δ^9-THC (3.1 - 6.76 mg/kg).

To determine whether CB_1- and/or CB_2- receptors are responsible for the induced emesis, varying does of their respective antagonists [SR141716A (0, 1, 5 and 10 mg/kg) or SR144528 (0, 10 mg/kg)] were injected S.C. to different groups of shrews 30 min prior to an I.P. injection of an effective antiemetic dose of Δ^9-THC (20 mg/kg) or CP55,940 (0.3 mg/kg). Thirty minutes later, treated shrews received 10 Gy radiation and the emesis parameters were recorded for the next 30 min. The antiemetic capacity of both Δ^9-THC and CP55,940 was reversed in a dose-dependent manner by SR141716A but not by SR144528. The results confirm the antiemetic potential of cannabinoid CB_1/CB_2-receptor agonists against radiotherapy-induced emesis and the induced emetic effect occurs via the stimulation of cannabinoid-CB_1 receptor.

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THE RECEPTORS MEDIATING THE INHIBITORY EFFECTS OF CANNABINOIDS ON THE ELECTRICALLY STIMULATED RAT ISOLATED MYENTERIC PLEXUS LONGITUDINAL MUSCLE PREPARATION ARE DEPENDENT ON THE FREQUENCY OF STIMULATION

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Our objective was to establish whether the ability of representatives of the four classes of cannabinoid receptor agonists (CP 55,940, WIN 55,212-2, anandamide and Δ^9-THC) to inhibit electrically evoked contractions of various isolated nerve smooth muscle preparations extended to the myenteric plexus longitudinal muscle (MPLM) preparation of the rat isolated ileum.

Strips of MPLM were dissected from the ileum of male Wistar rats (350 – 550 g) and suspended in organ baths for recording of isometric contractile responses to two different frequencies of electrical field stimulation of 0.5 ms width and 110 % supramaximal voltage. Low frequency (0.05 Hz) evoked twitch contractions, whereas high frequency (30 Hz for 2 seconds every min) evoked rebound contractions. Contractile responses evoked at either frequency were abolished by treatment with either tetrodotoxin (1 μM) or atropine (1 μM). Changes in the amplitude of the electrically evoked contractions to cannabinoids were expressed as a percentage ratio of the amplitude of the contractile response after each addition of the drug, to the amplitude immediately before the first addition of the drug.

At low frequency stimulation, all four agonists, CP 55,940, WIN 55,212-2, anandamide and Δ^9-THC inhibited the twitch response in a concentration related manner. Agonist potencies (IC_{50}) were 3.73 nM, 5.62 nM, 10.81 nM and 28.7 nM respectively (n=6). Rimonabant (10 nM, 100 nM and 1 μM) administered 30 mins prior the addition of the agonist produced concentration-related dextral shifts in the agonist log concentration response curves without a reduction in the maximal response. Using Schild analysis, the pA_2 values were 8.81, 8.75, 8.61 and 8.65 respectively. The slopes of the Schild plots for the different agonists were not significantly different from unity. By itself, at low frequency stimulation, rimonabant induced small but significant concentration related increases in twitch amplitude. For example, 1 μM produced an increase of 11.35 ± 6.55 % respectively (mean ± s.e.m, n= 24). At high frequency stimulation, the IC_{50} values for CP 55,940, WIN 55,212-2, anandamide and Δ^9-THC were 4.67 μM, 3.36 μM, 4.10 μM and 4.33 μM (n=6-14) respectively. Rimonabant (1 μM) only antagonised the inhibitory effect of anandamide, partly of that by WIN 55,212-2 and Δ^9-THC, but not CP 55,940. In contrast to the twitch enhancing action of rimonabant, the rebound contractions were inhibited by 38 ± 5.60 %, (n=38). The optical isomer WIN 55,212-3 (1 nM- 30 μM) was inactive at either frequencies of stimulation. None of the cannabinoids over the concentrations used depressed contractions evoked by exogenously applied acetylcholine. In the rat myenteric plexus, activation of both presynaptic CB_1 and non-CB_1 receptors can inhibit the motor function of the ileum, presumably by suppressing the release of acetylcholine from cholinergic nerve terminals, and the sensitivity of presynaptic inhibition by cannabinoids is dependent on the frequency of electrical stimulation.

ENDOCANNABINOIDS AFFECT LIVER FUNCTION IN THIOACETAMIDE INDUCED HEPATIC ENCEPHALOPATHY

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Introduction: Endocannabinoids function as neurotransmitters and neuromodulators *via* specific receptors. Activation of the endocannabinoid system (ECs) is involved in the vasodilated state associated with liver cirrohosis. We have shown that ECs may play an important role in the pathogenesis of hepatic encephalopathy (HE) in thioacetamide induced fulminant hepatic failure. Modulation of ECs either by specific antagonists to the CB_1 receptors or by exogenous agonists for the CB_2 receptors may have therapeutic potential. We aimed to investigate the possible direct role of the proposed treatment on the cirrhotic liver.

Methods: Sabra mice were assigned to different groups at random. Thioacetamide (TAA) was injected i.p. as a single dose of 200mg kg^{-1}. 24 hours after injection all animals were treated (s. c) with 0.5 ml solution of 0.45% NaCl, 5% dextrose and 0.2% KCl in order to prevent hypovolemia, hypokalemia and hypoglycemia and exposed to infrared light in order to prevent hypothermia(day 2). Endocannabinoids and their antagonists were injected at a dose of 5 mg kg^{-1} i.p one day after TAA administration. Mice were sacrificed by decapitation (day 3) one day after endocannabinoids administration. Livers were assessed for histopathology, T cells, cytokines and 2AG levels. Blood was assayed for liver function: liver enzymes, ammonia, albumin and glucose levels.

Results: TAA administration caused necrosis and inflammation. 2AG , SR141716A , 2AG+SR141716A and HU308 showed less bridiging, necrosis and inflammation and more regeneration. 2AG liver levels were not affected by TAA administration as opposed to elevated brain 2AG levels. TAA administration increased significantly AST, ALT , GGT and ammonia levels. Treatment with SR141716A or HU308 reversed AST, ALT , GGT and ammonia almost to control levels while treatment with 2AG reversed GGT and ammonia to control level. 2AG +SR141716A decreased AST and reversed GGT and ammonia to control levels. TAA administration caused significant decrease in CD3, almost significant in CD4 and no change in CD8 cells in the liver. The treatments involved did not reverse cell counts to control levels. IL6 level significantly decreased after TAA administration. 2AG and HU308 reversed IL6 to control levels.

Conclusions: SR141716A or HU308 have immediate therapeutic effects on liver function while 2AG or 2AG+SR141716A showed partial improvement. The difference in the therapeutic effects on brain and liver suggest that ECs effect is mediated also directly on the brain. Modulation of ECs either by specific antagonists to the CB_1 receptors or by exogenous agonists for the CB_2 receptors may have therapeutic potential in liver disease.

LPS TREATMENT UPREGULATES CB_2 RECEPTORS IN THE ENTERIC NERVOUS SYSTEM OF THE RAT ILEUM

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The distribution and function of the CB_1 receptor has been widely characterized in the gastrointestinal tract. A recent study by Mathison *et al.*, (BJP;142(8):1247-54), reported that in LPS-inflamed rats, the inhibitory actions of CB_1 receptor agonists were abolished wherreas CB_2 receptor agonists were found to exert a novel inhibitory action on motility. The aim of the present study is to investigate the whether CB_2 receptors are upregulated in the rat ileum by LPS treatment.

Whole mounts of rat ileal tissue were used for immunofluorescence staining of CB_2 receptors in the enteric nervous system and on macrophages. Rats were either treated with vehicle or LPS (65 μgkg^{-1}, i.p.) and ileal samples collected. RNA was isolated for RT-PCR and quantitative real time RT-PCR studies and proteins were isolated for western blotting. Tissues from either vehicle or LPS treated rats were mounted in organ baths and electrical field stimulation was applied in the presence or absence of CB_2 receptor agonists.

Immunohistochemical studies demonstrate CB_2 receptors throughout the enteric nervous system of the rat ileum; the distribution is unaffected by LPS treatment. Real Time RT-PCR demonstrates an increase in CB_2 receptor mRNA in the rat ileum. Western blotting reveals enhanced CB_2 receptor protein levels, particularly in the muscle layer, containing the myenteric plexus. The enhanced CB_2 receptor levels do not appear to be due to infiltrating macrophages or upregulation by enteric glia in the myenteric plexus. CB_1 receptor distribution and receptor level is unaffected by LPS pretreatment. Preliminary functional studies indicate that the CB_2 receptor agonist JWH133 inhibits electrically-evoked rebound contractions in LPS-pretreated ileum by acting at a prejunctional neuronal site.

These data indicate that in a LPS model of inflammation, CB_2 receptors are upregulated in the rat myenteric plexus and inhibit motility. These actions appear to be mediated by an inhibition of excitatory enteric neurons and are not due to a neuro-immune interaction. The functional consequence of CB_2 receptor upregulation may be to act as a brake on enhanced motility in pathophysiological conditions.

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CANNABINOID LIGANDS AS ACTIVATORS OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ)

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Peroxisome proliferator-activated receptors (PPARs) are a family of relatively promiscuous nuclear receptors. We have recently demonstrated that Δ^9-tetrahydrocannabinol (THC) is a PPARγ ligand (O'Sullivan et al., 2005), activation of which leads to time-dependent vasorelaxation through increased bioavailability of nitric oxide and hydrogen peroxide production. Our present study has investigated the potential of various other cannabinoid ligands to activate PPARγ with cardiovascular effects. These included the endocannabinoids anandamide, N-arachidonoyldopamine (NADA), and palmitoylethanolamide (PEA), the cannabis plant extracts cannabidiol (CBD) and tetrahydrocannabivarin (THCV), the synthetic ligands WIN55, 212-2 and CP55,940, and the cannabinoid CB_1 receptor antagonists SR141716A and AM251.

Transactivation assays were performed in homologous cultured HEK293 cells transiently overexpressing PPARγ and retinoid X receptor alpha (RXRα) in combination with a luciferase reporter gene (3×PPRE-TK-Luc). Upon incubation with cannabinoid ligands (24 h, 10 μM), it was found that AEA, NADA, THC, CBD, CP55,940, AM251 and SR141716A all significantly increased luciferase activity in cells, indicating PPARγ activation. PEA, THCV and WIN did not significantly activate PPARγ.

In vitro, with the exception of PEA, all the cannabinoid compounds tested caused significant time-dependent vasorelaxation of the rat aorta compared with vehicle controls. However, only the vasorelaxant effects of anandamide and NADA were antagonised by the PPARγ antagonist GW9662 (1 μM). Further investigation of the underlying mechanisms showed that anandamide causes relaxation of isolated rat aortae by similar mechanisms as those revealed for THC (O'Sullivan et al., 2005) and the PPARγ agonist rosiglitizone (Cunnane et al., 2004).

The present results provide evidence that many cannabinoid compounds are capable of activating PPARγ, however, only anandamide and NADA also appeared to cause PPARγ-mediated vascular effects.

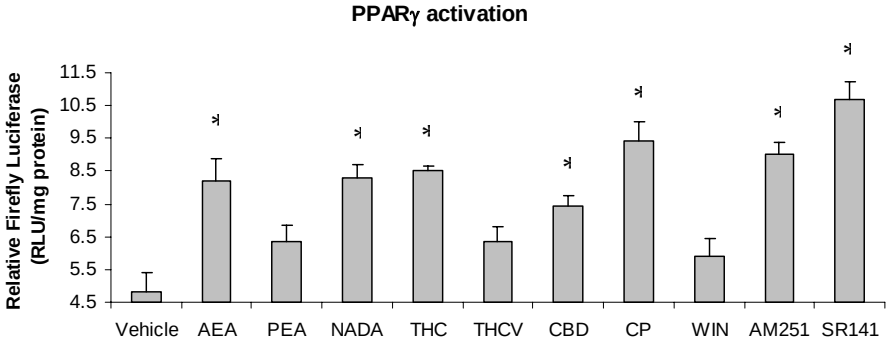

Figure 1. Evidence of PPARγ activation by cannabinoids. Data are given as means with error bars representing SEM. **P* < 0.05 compared to vehicle-treated control cells.

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CB1 CANNABINOID RECEPTOR ANTAGONISM: A NOVEL STRATEGY FOR THE TREATMENT OF LIVER FIBROSIS

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Liver fibrosis is the common response to chronic liver injury, ultimately leading to cirrhosis and its complications (portal hypertension, liver failure, and hepatocellular carcinoma). The fibrogenic process results from activation of non parenchymal cells known as hepatic myofibroblasts that proliferate, synthetize fibrogenic cytokines (TGF-β1), matrix components and inhibitors of matrix degradation. We recently demonstrated that CB2 receptors are overexpressed in hepatic myofibroblasts and elicit potent antifibrogenic properties (Julien et al, 2005, Gastroenterology). In contrast, we also found that daily cannabis smoking is an independent predictor of fibrosis progression during chronic hepatitis C (Hezode et al, 2005, Hepatology). These results suggested that CB1 receptors may promote liver fibrosis. Therefore, the present study examined expression and regulation of CB1 receptors during chronic liver diseases, and evaluated their effects on liver fibrogenesis.

CB1 receptors were markedly expressed in cirrhotic surgical human samples, contrasting with a faint expression in normal liver. CB1 expression predominated in non parenchymal cells within and at the edge of fibrous septa. Double immunohistochemistry identified myofibroblasts as a source of CB1 receptors, and accordingly, CB1 receptors were also expressed in cultured human and murine fibrogenic cells. The function of CB1 receptors during liver fibrogenesis was evaluated in three models, chronic carbon tetrachloride or thioacetamide intoxications, and bile duct ligation, owing to the use of CB1-/- mice or of SR 141716A. SR 141716A markedly prevented progression of liver fibrosis in all three models. Similar results were observed in CB1-/- mice. Antifibrogenic effects of genetic or pharmacological inactivation of CB1 receptors were related to proapoptotic and growth inhibitory effects on hepatic myofibroblasts, resulting in decreased accumulation of these cells during chronic liver injury.

In conclusion, we show here that hepatic myofibroblasts are a target of CB1 receptors during chronic liver diseases. Inactivation of CB1 receptors reduces progression of liver fibrosis, demonstrating their profibrogenic role. These data therefore identify CB1 receptors as a potential novel target for antifibrogenic therapy.

ANANDAMIDE-MEDIATED ANGIOGENESIS: INTERPLAY BETWEEN CB1 RECEPTOR AND NON-CB_1/CB_2 ANANDAMIDE RECEPTOR

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Previous reports from our laboratory showed that endogenous cannabinoid anandamide and its metabolically stable analog methanandamide produced nitric oxide acting on CB_1 cannabinoid receptor as well as non-CB_1/CB_2 "anandamide receptor via the activation of endothelial nitric oxide synthase (eNOS) in endothelial cells (McCollum et al., FASEB J 18(4) pA232, 2004). In the present study, we have showed that methanandamide produced *in vitro* angiogenic responses in CB_1 receptor knockdown (SiRNA) human umbilical vein endothelial cells (HUVEC). We further showed that methanandamide produced an increase in angiogenic sprouting in aortic ring preparations from CB_1 receptor knockout mice. We have also shown in methanandamide produced an increase in matrix metalloprotease (MMP-2 and MMP-9) activity in the conditioned media from CB_1 receptor blocked endothelial and tumor cells. We initially hypothesized that anandamide-mediated nitric oxide production acts as a regulatory switch for MMP-2 and MMP-9 activity in LNCaP cells. However in the present study we found that methanandamide mediated increase in matrix metalloprotease activity is only partially dependent of NO production and negatively regulated by Gi proteins. Thus results from this study suggest that a) CB_1 receptor induces an inhibitory tone on methanandamide-mediated angiogenesis while activation of non-CB_1/CB_2 anandamide receptor stimulates angiogenesis; b) anandamide-mediated increase in MMP-2 and MMP-9 activity is partially regulated by nitric oxide and regulated by heterotrimeric Gi protein mediated signaling in endothelial and tumor cells.

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SATIVEX® CANNABIS BASED MEDICINE IMPROVES SLEEP QUALITY IN PATIENTS WITH MULTIPLE SCLEROSIS, NEUROPATHIC AND RHEUMATIC PAIN

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This presentation reports effects of Sativex® cannabis based medicine (CBM) on sleep in treatment of neuropathic pain (NP), rheumatoid arthritis, cancer pain, spasticity, lower urinary tract symptoms (LUTS), and other sequelae of multiple sclerosis (MS), in a series of placebo-controlled clinical trials. Sativex is a highly characterized pharmaceutical product made from liquid CO_2 extracts from cloned *Cannabis sativa* strains which yield Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in constant proportion. Sativex is formulated for oromucosal administration with each 100 µL pump-action spray containing 2.7 mg of THC and 2.5 mg of CBD plus ethanol: propylene glycol excipients and peppermint oil. In a Phase I study of eight normal subjects Sativex produced less sedation than a THC-predominant oromucosal preparation (Tetranabinex®), demonstrated some alerting properties during sleep, and reduced residual sedative effects of THC the following day (Nicholson et al. 2004). Patients also received Sativex in self-titrated doses in randomized placebo controlled double-blind clinical trials where it was added on to existing medication regimens in patients with intractable symptoms. In Phase II clinical trials, Sativex significantly improved sleep quality in 20 patients with intractable neurogenic symptoms ($p<.05$) (Wade et al. 2003), while 34 patients with intractable NP demonstrated a surprising conversion of subjective sleep from poor or fair to good quality ($p<0.0001$), and longer sleep duration ($p<.0001$) (Notcutt et al. 2004). Patients with intractable LUTS (N=21) also showed significant improvement in sleep self-assessment ($p<.05$) (Brady et al. 2004). In a Phase III RCT in central NP due to MS over 5 weeks in 66 patients, significant benefit of Sativex over placebo was observed in sleep disturbance ($p=.003$)(Rog et al. 2003). A Phase III RCT in intractable pain in 79 subjects also showed benefit on sleep ($p=0.045$). In a Phase III RCT of 125 subjects with peripheral neuropathic pain characterized by allodynia, Sativex produced more marked reductions in sleep disturbance ($p=0.001$) (Nurmikko et al. 2005). In the largest RCT of brachial plexus avulsion and neuropathic pain to date (Berman et al. 2004) in 48 subjects in a double-blind crossover RCT of placebo vs. Tetranabinex vs. Sativex, sleep quality improved from a baseline of 4.8 to 5.2 with placebo (NS), and to 5.9 with Sativex ($p<.01$). In an RCT of 160 MS patients with mixed symptoms, Sativex produced improvement in subjective sleep quality ($p=.047$) (Wade et al. 2004). In a Phase II RCT of nocturnal Sativex in 58 rheumatoid arthritis patients (Blake et al. 2005), significant improvement in sleep quality was noted over placebo ($p=.027$). In a Phase III RCT in intractable LUTS (N=135), Sativex markedly reduced nocturia over placebo ($p=.01$). In a short duration Phase II RCT in intractable cancer pain (N=177), and Phase II RCT in spinal cord injury (N=117), Sativex trended toward improvement in sleep (NSD). Long-term open label studies confirm that the short-term improvement in sleep seen in Sativex RCTs is maintained over prolonged periods of administration.

REPORT ON A CASE SERIES OF PATIENTS USING A BUCCAL CANNABIS EXTRACT CONTAINING THC AND CBD FOR TREATMENT OF NEUROPATHIC PAIN AND SPASTICITY

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A buccal spray extract containing pure extracts of Cannabis sativa with THC and cannabidiol (Sativex®) has received conditional notice of compliance with conditions from Health Canada for use as adjunctive treatment for symptomatic relief of neuropathic pain in multiple sclerosis, making Canada the first country in the world where physicians can prescribe it. The current paper presents a case series of 14 patients followed at a tertiary care pain and neuro-rehabilitation clinic, who have used the buccal cannabis spray for treatment of neuropathic pain and/or spasticity. **Methods:** Consecutive subjects, in whom a trial of buccal cannabis extract has been tried, completed a structured follow-up questionnaire containing demographic and dosing information, a series of 11 point numerical symptom relief rating scales, 7 point global impression of change (GIC) and satisfaction scales (GS), a side effect checklist, and a 7 point subjective measure of improvement in function. **Results:** To date 14 patients have received prescriptions for the buccal extract of cannabis. Questionnaires were obtained on all patients. The mean age was 52 (range 39-78), with 6 men and 8 women. Diagnoses included: spinal cord injury (5), multiple sclerosis (5), neuropathic oro-facial pain (2), post traumatic coccygeal neuropathic pain (1) and spinal stenosis (1). 9 patients used the agent for pain, 4 for pain and spasticity and 1 for spasticity alone. Patients had used the buccal extract for between 1 and 6 months at the time of follow-up. The dose ranged from less than 1 to 8 sprays per day, (average dose between 3 and 4 sprays per day). Of the 13 patients using it for pain 3 patients reported complete relief, 4 reported moderate relief (5-7), 5 reported mild relief (3-4) and 1 reported no relief. Of the 5 targeting spasticity 1 reported complete relief, 1 relief at a level of 9 and 3 mild relief (3-4). Twelve of the 13 patients were satisfied with the agent, 13 reported improvements according to the GIC and 11 reported subjective improvements in function. Side effects were reported by 8 patients the most common of which were; dizziness or feeling faint (3), and nausea, constipation and mild-moderate confusion (2 patients each). **Discussion:** These results are consistent with recent randomized controlled trials demonstrating therapeutic effects of cannabis extracts in neuropathic pain and spasticity. A multi-center randomized controlled trial examining an oral cannabis extract demonstrated an improvement in objective mobility and pain in persons with multiple sclerosis. The effect on the Ashworth scale for spasticity was not significant after 15 weeks of treatment [1], however was significant after 12 months of therapy in the 80% of patients followed for that time [2]. In a RCT of 48 patients using the buccal extract, a statistically significant decrease in pain of brachial plexus avulsion was demonstrated, but not by the full 2 point reduction on the 11 point numeric rating scale required for clinical significance [3]. A further RCT of the buccal extract has demonstrated a significant reduction in pain and improved sleep in 64 patients with central pain due to multiple sclerosis [4]. **Conclusion:** A buccal spray preparation of THC/cannabidiol extract may be emerging as an effective and well tolerated treatment for neuropathic pain and spasticity in a variety of neurological disorders.

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FOLLOW UP STUDY OF PATIENTS WITH NEUROPATHIC PAIN, SPASTICITY SECONDARY TO MULTIPLE SCLEROSIS AND ANOREXIA CAQUEXIA SYNDROME TREATED WITH A WHOLE PLANT CANNABIS EXTRACT

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Introduction: Cannabis based medicines have been evaluated for the treatment of pain and spasticity associated with multiple sclerosis, chemotherapy-induced nausea and vomiting, appetite stimulation and analgesia. Recently, a cannabis extract (CE) has been approved in Canada as an adjunctive treatment for neuropatic pain in adults with multiple sclerosis.

Objective: The first aim of the study is to describe the clinical and epidemiological characteristics of patients receiving a self-administered oromucosal spray CE. As a second aims effectivity, toxicity, well being, most frequent dose used and patient and health care professional satisfaction will be assessed.

Methods: An observational follow up study of 600 patients receiving CE under the administrative status of foreign medication or as compassionate use during one year is on-going. The study is conducted in six University hospitals in Barcelona. A network of neurologists, anaesthetists, oncologists, infectious disease specialists, hospital pharmacists and community pharmacists has been set-up. A specific training programme has been conducted for each group of health care professionals. Patients with the diagnoses previously described and not responding to the reference treatment are included by their hospital doctors who are in charge of the data collection regarding clinical follow-up and quality of life. The CE is dispensed by hospital pharmacists. Community pharmacists train the patients how to self-administer the CE. Data collection regarding dosage administration and side effects is supervised by community pharmacists based on the information regarding dose titration and side effects collected by patients in a diary questionnaire. The duration of the study is planned be one year.

Results and conclusions. Since January 2006 24 patients have been enrolled (8 MS, 14 neurophatic pain and 2 syndrome anorexia caquexia. The main characteristics of patients included during the first six months of the study will be presented. The results of this study will also allow to quantify the need of future research regarding the therapeutic use of cannabis in our setting. Additionally, the study design will be an opportunity to test the feasibility of such a multidisciplinary network.

Agreements: *Departament de Salut de la Generalitat de Catalunya*

VAPORIZATION AS A SAFE AND EFFICIENT SMOKELESS CANNABIS DELIVERY SYSTEM

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INTRODUCTION: The U.S. Institute of Medicine report Marijuana and Medicine published in 1999 suggested that despite cannabis' potential therapeutic value, smoking was not a desirable delivery system. Preclinical studies have determined that vaporization of cannabis delivers active cannabinoids into the collecting receptacle. We conducted a 6-day “proof of concept” study to investigate vaporization using the Volcano® device as an alternative means of delivery of inhaled *Cannabis sativa,* to characterize pharmacokinetic and pharmacodynamic effects and to assess whether it may be an appropriate system for use in future clinical effectiveness studies.

METHODS: Eighteen healthy subjects were recruited and admitted to the inpatient ward of the General Clinical Research Center (GCRC) at San Francisco General Hospital to investigate the delivery of cannabinoids by vaporization of marijuana compared to marijuana smoked in a standard National Institute on Drug Abuse cigarette. One dose (1.7, 3.4 or 6.8% tetrahydrocannabinol) and delivery system (smoked cannabis cigarette or vaporization system) was randomly assigned for each of the six study days. The primary endpoint was the comparison of plasma concentrations of delta-9-tetrahydrocannabinol (THC), cannabidiol, cannabinol, and metabolites, including 11-OH-THC resulting from inhalation of cannabis after vaporization *vs* smoking. Maximum concentrations, concentration at 6 hours and areas under the curve were calculated. Expired carbon monoxide was measured to evaluate whether the vaporizer reduces exposure to gaseous toxins as a secondary endpoint. We also evaluated physiologic and neuropsychologic effects and queried patients for their preference of blinded dose day and delivery method. All adverse events were reported.

RESULTS: 21 participants were enrolled to obtain the 18 who completed the 6-day inpatient study. 15 men and 3 women, mean age 30 years, were included in the final analysis. There was no significant difference in THC concentrations delivered into the bloodstream between smoking and vaporization at any of the three initial strengths inhaled. Expired carbon monoxide concentrations were significantly elevated with smoked compared to vaporized cannabis. 14 participants preferred vaporization, 2 smoking and 2 reported no preference. No adverse events were observed.

CONCLUSION: Vaporization of cannabis is a safe and effective mode of drug delivery. Participants had a clear preference for vaporization over smoking as a delivery system for the cannabis used in this trial. Future studies of medicinal cannabis could consider utilizing vaporization as a bioequivalent mode of delivery to smoking combusted material.

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RISK OF CANNABIS DEPENDENCE IN CANNABIS USERS AMONG TRAUMA INPATIENTS

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Cannabis can be considered to have less addictive risk than some other substances, such as nicotine, cocaine, and heroin, based on the proportion of users who develop dependence. We evaluated this issue in a sample of 1,118 adult trauma inpatients (mean age 37 years [range 18-96 years], 72.1% male, 56.3% white) hospitalized for at least 2 days and interviewed with the substance use disorders section of the Structured Clinical Interview for DSM-IIIR (Soderstrom et al., J Amer Med Assoc 1997;277:1769-1774). Logistic regression models were used to test whether subjects who met criteria for lifetime or current cannabis dependence differed in demographic characteristics from those who were lifetime users but not dependent on cannabis. Results from the logistic regression models are presented as odds ratios [95% confidence intervals] adjusted for other demographic characteristics. Four hundred and fourteen patients (37%) had used cannabis at least 20 times in their lifetime. Of these cannabis users, 40.1% met criteria for lifetime cannabis dependence and 24.6% for current (past 6 months) dependence. By comparison, 80.7% and 57.8% of lifetime (at least 6 times) opiate users, 70.9% and 48.8% of lifetime cocaine users, and 60.6% and 33.5% of lifetime alcohol users developed lifetime or current dependence, respectively. As compared to lifetime cannabis users without dependence, those with lifetime cannabis dependence were less likely to be white (36.1% vs. 51.6%, adjusted odds ratio: 0.59 [0.37-0.95]). Those with current cannabis dependence were also less likely to be white (24.5% vs. 52.2%, adjusted odds ratio: 0.37 [0.20-0.67]) and three times more likely to be younger ($\leq$ 33 years old) (83.3% vs. 59.9%, adjusted odds ratio: 3.02 [1.61-5.67]). These findings suggest that users of cannabis have less risk of developing dependence than do users of opiates, cocaine, or alcohol; and that the cannabis users most at risk are younger non-whites.

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DYNAMIC REGULATION OF SERUM ENDOCANNABINOID CONTENT IN AFFECTIVE DISEASE AND FOLLOWING SOCIAL STRESS IN HUMANS

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Preclinical data has suggested that the endocannabinoid system may be involved in stress regulation and affective diseases, however data from human populations in this regard is lacking. The first aim in this research examined was to examine serum endocannabinoid content in women suffering from minor or major depression and healthy matched controls. The second aim examined serum endocannabinoid content in women with major depression and healthy, matched controls under basal conditions, immediately following social stress (public speaking and mental arithmetic) and during a recovery phase. In the first experiment, it was determined that serum content of 2-arachidonylglycerol (2-AG) was significantly reduced in individuals with major depression and that this reduction significantly and negatively correlated to episode duration, such that the longer the depressive episode the lower the 2-AG content. Serum anandamide (AEA) content was not significantly lower in this sample, however, serum AEA content exhibited a robust, negative correlation with anxiety symptoms in depression, such that individuals with high scores on anxiety scales exhibited lower AEA content. Interestingly, individuals with minor depression exhibited an opposite profile, such that serum AEA was significantly elevated in this population. In the second experiment, both basal 2-AG and AEA were significantly reduced in women with major depression, replicating our initial findings. Furthermore, immediately following stress both serum AEA and 2-AG content significantly increased and subsequently decreased during the recovery period. This stress-induced increase in serum endocannabinoid content was present in both subjects with major depression and matched controls. Collectively, these data extend preclinical data by suggesting that the endocannabinoid system is activated during stress, possibly acting as a buffer system to counteract the adverse effects of stress. Furthermore, the deficit in serum endocannabinoid content in major depression may represent a compromised buffer system that has resulted in the manifestation of major depression, where as the elevation seen in minor depression may represent an actively engaged system which is acting to prevent the development of this disease.

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PREVIOUS EXPOSURE TO THC ALTERS THE REINFORCING EFFICACY AND ANXIETY-RELATED EFFECTS OF COCAINE IN RATS

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To evaluate the hypothesis that prior cannabis exposure increases the likelihood of abusing or becoming addicted to other drugs, we studied the effects of THC exposure on subsequent self-administration of cocaine in Experiment 1. Rats were given six THC or vehicle injections, ending one week before the start of cocaine self-administration training. THC pre-exposure did not alter the acquisition of cocaine self-administration or the amount of cocaine taken under a fixed-ratio 1 schedule (FR1), with one response required for each injection. Under a progressive-ratio schedule, with the response requirement increasing exponentially with each injection, cocaine-seeking was significantly reduced in THC-exposed rats, suggesting that THC exposure causes cocaine to be devalued as a reinforcer. In contrast, THC exposure did not alter the value of heroin as a reinforcer under the progressive-ratio schedule in an earlier study, but it increased heroin self-administration under the FR1 schedule. In Experiment 2, locomotor effects of cocaine and heroin were measured in rats pre-exposed to THC or vehicle. THC pre-exposure produced cross-tolerance to the motor-depressant effects of heroin; this may explain the shortened post-injection pauses exhibited by THC-exposed rats when self-administering heroin under FR1. When given cocaine, THC-exposed rats showed normal elevations in locomotion, but they avoided the center of the open field, suggesting that THC exposure enhances the anxiogenic effects of cocaine. This enhanced anxiogenic effect — which was confirmed in Experiment 3 using another model of anxiety, the light-dark test — may explain the reduced reinforcing value of cocaine observed in THC-exposed rats in Experiment 1.

ENDOCANNABINOIDS MEDIATE ETHANOL-INDUCED MODULATION OF EXCITATORY POSTSYNAPTIC CURRENTS IN CULTURED HIPPOCAMPAL NEURONS

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Alcohol increases extracellular endocannabinoids levels and inhibits their transport in neuronal cells, although the molecular mechanisms by which this occurs and physiological significance are unknown. The participation of endocannabinoids and their receptors in the effect of acute alcohol on spontaneous synaptic transmission was studied using whole-cell voltage clamp. We examined the synaptic mechanisms underlying the reduced excitatory activity; alcohol actions on properties of action-potential-independent miniature excitatory postsynaptic currents (mEPSCs) were studied in cultured hippocampal neurons. Immunocytochemical analysis suggested that these neurons have the highest levels of cannabinoid type-1 receptor (CB1) and are distributed on the axons and dendrites. Properties of mEPSCs generated by activation of N-methyl-D-aspartate receptors (NMDARs) and non-NMDA receptors were examined during acute exposure to 50mM alcohol. In the presence of 50mM alcohol, the amplitude (23 ± 0.99 %, n=12 neurons) and the frequency (30 ± 2.3 %, n=12 neurons) of mEPSCs increased ($p<0.0001$) in the first 5 min exposure. In contrast, at 20 min exposure both amplitude and frequency decreased to 6 ± 0.82% and 78 ± 6.35 % (n= 12 neurons) of control ($p<0.0001$), respectively. Based on the quantal theory of neurotransmitter release, changes in the frequency of miniature currents are correlated with changes in transmitter release, suggesting that alcohol decreased presynaptic glutamate release. Since alcohol changed the amplitude of mEPSCs, indicating that the presynaptic changes were associated with changes in the properties of postsynaptic neurons. To understand the contribution of the endocannabinoid system in alcohol action, we used the CB1 receptor antagonist rimonabant (SR141716A). Rimonabant antagonized alcohol effects on amplitude (100 ± 0.89 %, n= 12 neurons) and frequency (32 ± 5.6 %, n=12 neurons) ($p<0.0001$) of mEPSCs. These results suggest that alcohol-induced endocannabinoids are partially responsible for the alcohol-induced inhibition of excitatory neurotransmission. This suggests a retrograde messenger role of endocannabinoids in alcohol action on excitatory neurotransmission and highlights the endocannabinoid system as a valuable target in the therapy for alcoholism. (BSB Supported by NIH grant, AA14411)

INVOLVEMENT OF THE CB_1 CANNABINOID RECEPTOR ON SALVINORIN-A-INDUCED REWARD

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Salvinorin A is the major active ingredient of *Salvia divinorum*, a potent psychoactive and hallucinogenic herb, usually smoked or buccally absorbed by chewing, that is currently available in the United States and in the Europe, and widely available on the internet (Drug Enforcement Administration, 2002). It is a kappa-opioid receptor agonist (Roth et al. 2002; Sheffler and Roth 2003; Chavkin et al. 2004). Salvinorin A (0.001-0-032 mg/kg) has discriminative stimulus effects similar to those of the centrally penetrating k-agonist U69,593, following s.c. administration in rhesus monkeys (Butelman et al., 2004). A dose-dependent decrease of dopamine levels in the caudate putamen but not in the nucleus accumbens accompanied by a conditioned place aversion has been shown after treatment with salvinorin A (1.0 mg/kg and 3.2 mg/kg, i.p.) in C57BL/6J mice (Zhang et al., 2005). Since conditioned place aversion was obtained with doses (1-3.2 mg/kg) higher than those effective in the smoked *Salvia divinorum* in humans (Valdes, 1983; Siebert, 1994), the aim of the present study was to investigate lower doses on the Conditioned Place Preference (CPP) test and i.c.v. self-administration paradigm in Wistar rats. Because an interaction between k-opioid and cannabinoid system on self-administration has been recently reported (Mendizabal et al., 2005), we also investigated the role of cannabinoid system on salvinorin-A effects. In the CPP test, Salvinorin-A produced a significant increase in the time spent in the drug-paired compartment on the post-conditioning day in a range between 0.1 and 40 μg/kg, when compared with that obtained in the pre-conditioning period. Pretreatment with rimonabant completely reduced the salvinorin-A-induced increase of the time spent in the drug-paired compartment during postconditioning. Using the i.c.v. self-administration paradigm, there was a progressive increase in the number of pressings of the drug-associated lever starting from 0.1 to 0.5 μg/infusion. The highest concentration (1 μg/infusion) produced a gradual decrease in the number of drug-associated lever pressings. When combined with salvinorin A, peripheral pretreatment with rimonabant significantly decreased the number of drug-associated lever pressings in comparison with salvinorin-A alone. Binding studies revealed that salvinorin A binds the CB1 receptor in a competitive manner, causing the displacement of the CB_1 receptor agonist CP55,940 in brain tissue.

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MAPPING CANNABIDIOL-INDUCED ALTERATIONS IN REGIONAL BRAIN ACTIVITY BY 2-DEOXYGLUCOSE IMAGING

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Cannabidiol (CBD) is a 'non-psychoactive' component of *Cannabis sativa* which has been known since the 1970s to moderate the actions of the principal active constituent of cannabis, Δ^9-tetrahydrocannabinol (THC). There are also reports of CBD having actions of its own, including anxiolytic, anticonvulsant and neuroprotective effects (reviewed in Pertwee, in Di Marzo. Cannabinoids. Kluwer, 2004). There is much current interest in the concomitant use of CBD and THC on the basis that some of the adverse effects of THC can be abrogated by CBD. It is, however, as yet unclear how CBD exerts its effects, which appear not to be by a CB_1 receptor mechanism, nor is it yet established in which brain areas CBD has its actions. The aim of the present study was to determine the effects of CBD on regional cerebral activity in the rat.

Local cerebral glucose use (LCGU) was imaged in male Long-Evans hooded rats by quantitative 2-deoxyglucose autoradiography (Sokoloff et al, J Neurochem, 1977, 28, 897-916) in freely moving animals (Crane and Porrino, Brain Res, 1989, 499, 87-92). Rats were administered CBD (5, 15 or 50 mg/kg ip) or vehicle (1% Tween 20 in 0.9% saline) 30 mins prior to initiating the LCGU measurement.

CBD elicited changes in LCGU in a restricted number of brain regions, including the ventral pallidum, the CA3 region of the hippocampus and regions of the prefrontal cortex. There was a general pattern of increases in LCGU at 5 and/or 15 mg/kg, but no change from control at 50 mg/kg. This is in contrast to the decreases in LCGU in limbic structures and some other brain areas involved in sensory-motor processing seen in response to 5 mg/kg THC (Brett et al, Neuroreport, 2001, 12, 3573-3577). These results contribute to the understanding of mechanisms underlying the differential behavioural effects of THC and CBD, and the ability of CBD to modulate some aspects of the effects of THC. Further studies are under way investigating the effects of THC-rich and CBD-rich cannabis extracts on cerebral activation. A clearer knowledge of the effects these compounds exert on brain function *in vivo* will increase understanding of their therapeutic versus side effect profile and facilitate decisions on the introduction of these agents for clinical use.

ERK-DEPENDENT MODULATION OF CEREBELLAR SYNAPTIC PLASTICITY FOLLOWING CHRONIC Δ9-TETRAHYDROCANNABINOL EXPOSURE

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Cerebellar circuits have been designated as a major neurobiological substrate for the chronic effects of THC and for the expression of behavioural signs of THC withdrawal *in vivo*. There is substantial evidence for cerebellar CB1R molecular adaptations and modifications in receptor signalling after prolonged THC exposure. Recent reports also suggest a role for the Ras/ERK cascade in CB1R-mediated plasticity in motor control regions, including the cerebellum (Rubino et al., 2005; Rubino et al, 2006). However, very little is known about the effects of chronic THC exposure on cerebellar synaptic plasticity, which may contribute to the development of behavioural tolerance.

Our study investigated the neurophysiological adaptive responses occurring at cerebellar Parallel Fiber (PF)-Purkinje Cell (PC) synapses in slices obtained 24 hours following 4.5 consecutive daily s.c. injections of THC (10 mg/Kg) or its vehicle. The role of the Ras/ERK pathway in chronic THC-induced synaptic plasticity has been addressed by means of the genetically engineered mouse model lacking RasGRF1, an activator of the Ras/ERK cascade, and by *in vivo* pharmacological inhibition of ERK1,2 activation.

Chronic THC administration induced a long-lasting potentiation of the synaptic transmission between PF and PC, together with a facilitated induction of slow metabotropic glutamate receptor type 1-mediated excitatory postsynaptic currents. In addition, cerebellar PKA-dependent LTP (Salin et al., 1991) was affected by repeated THC administration. In THC tolerant mice, activation of the cAMP/PKA pathway by forskolin application or by brief tetanic stimulation of PF (8Hz, 15s), instead of triggering LTP, induced an adenosine A1 receptor (A1R)-mediated LTD. These results suggest a functional interaction between cerebellar A1R- and CB1R-dependent signalling on those neurophysiological mechanisms underlying the development of tolerance to the THC-induced locomotor effects.

The pharmacological inhibition of ERK activation during chronic THC exposure prevented the alterations in PF-PC synaptic activity and plasticity described in THC tolerant mice. Similar results were obtained in RasGRF1 null mice.

In summary, the present work reveals for the first time that, beside CBR1 molecular adaptations and modifications in receptor signalling, prolonged THC exposure induces ERK-dependent changes in PF-PC synaptic plasticity. This contributes to generate forms of pathological plasticity which might play a role in cannabinoid dependence.

THE ENDOCANNABINOID SYSTEM IN THE EFFECTS OF ALCOHOL ON LIMBIC NEURONS: ELECTROPHYSIOLOGICAL EVIDENCE *IN VIVO*

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The endogenous cannabinoid system has been implicated in the modulation of addictive behaviours and in the mechanism of action of different drugs of abuse. Specifically, several lines of evidence indicate that the endocannabinoid system is involved in the pharmacological and behavioural effects of alcohol. For example, chronic exposure to alcohol induces a selective increase of anandamide and 2-arachidonoyl-glycerol in cultured cells, and downregulates CB1 receptor function and number in rodents. Additionally, evidence suggests that endocannabinoid signalling may be involved in the modulation of alcohol reinforcing effects and alcohol drinking behaviour. Reciprocally interconnected regions of the limbic circuits, the mesolimbic dopaminergic (DA) system, the nucleus accumbens (NAc) and the basolateral amygdala (BLA), process many responses evoked by drugs of abuse, such as their rewarding properties, affective motor behaviour and emotional responses. Both alcohol and cannabinoids, among other drugs of abuse, modulate the activity of neurons in this regions, whereas endocannabinoids have been shown to regulate synaptic functions and mediate short- and long-term forms of synaptic plasticity.

Thus, we studied the contribution of the endocannabinoid system in the electrophysiological effects of alcohol in the limbic reward circuit. We utilized extracellular single cell recordings from ventral tegmental area (VTA) DA, NAc and BLA neurons in anesthetized rats. DA and BLA neurons were antidromically identified as projecting to the shell of NAc, whereas NAc putative medium spiny neurons were identified by their evoked responses to BLA stimulation.

Alcohol (0.25-1 g/kg, i.v.) stimulated firing rate of VTA DA neurons and inhibited BLA-evoked NAc neuron spiking responses and spontaneous firing of BLA projection neurons. The CB1 receptor antagonist rimonabant (SR141716A, 1 mg/kg, i.v.) fully antagonized alcohol effects in all regions. Blockade of FAAH, the major catabolising enzyme for anandamide, or acute and chronic treatments with the CB1 agonist WIN55212-2 modulated the response to alcohol of recorded neurons.

Our results demonstrate that electrophysiological effects of alcohol in the mesolimbic reward pathway rely upon stimulation of CB1 receptors by endogenous cannabinoids. Thus, the reduced motivational properties of alcohol following SR141716A administration or CB1 deletion found in behavioural studies suggest that the endocannabinoid system is necessary for full development of alcohol-induced appetitive behaviours and addiction. Our results further corroborate the notion that the endogenous cannabinoid system is a potential therapeutic target in alcoholism, and more generally in drug addiction.

CB1 RECEPTOR ANTAGONIST AM251 INHIBITS COCAINE'S REWARDING EFFECTS AND COCAINE-PRIMED RELAPSE TO DRUG-SEEKING BEHAVIOR IN RATS BY A DA-INDEPENDENT MECHANISM

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Previous studies demonstrate that blockade of CB1 receptors by SR141716A inhibits the rewarding effects of Δ^9-THC, heroin, ethanol or nicotine, but not cocaine-conditioned place preference or cocaine self-administration. In the present study, we examined whether the novel highly potent and selective CB1 receptor antagonist AM251 inhibits cocaine self-administration, cocaine-induced enhancement of brain stimulation reward, cocaine-induced reinstatement (relapse) of drug-seeking behavior and cocaine-induced changes in extracellular dopamine (DA), glutamate, and γ-aminobutyric acid (GABA) in the reward-related nucleus accumbens (NAc). Systemic administration of AM251 (1, 3, 10 mg/kg i.p., 30 min prior to testing) dose-dependently lowered (37%, 43%, 60%, respectively) the break-point for cocaine self-administration under a progressive-ratio reinforcement schedule, and dose-dependently inhibited (maximally 60%) cocaine-triggered reinstatement of drug-seeking behavior. AM251 (0.3, 1, 3 mg/kg) also dose-dependently attenuated (74%, 65%, 96%, respectively) the enhancement of electrical brain-stimulation reward produced by 2 mg/kg cocaine. The same doses of SR141716A as used for AM251 maximally lowered the break-point for cocaine self-administration by 30% and cocaine-enhanced brain stimulation reward by 40%. *In vivo* brain microdialysis showed that cocaine priming significantly elevated extracellular NAc DA (+400% over baseline) and NAc glutamate (+180%), but had no effect on NAc GABA. AM251 alone (1, 3, 10 mg/kg) dose-dependently elevated extracellular NAc glutamate levels, but not DA or GABA. AM251 (same doses) selectively attenuated cocaine-induced increases in NAc glutamate, but not in DA. Taken together, these data (although correlational in nature) suggest that CB1 receptors tonically inhibit NAc glutamate release, whereas blockade of CB1 receptors inhibits cocaine's rewarding effects and cocaine-primed relapse by a glutamatergic NAc mechanism, rather than by reducing cocaine-induced increases in NAc DA. The present findings are therefore conceptually congruent with recent suggestions in the literature that glutamatergic mechanisms may be a crucial neural substrate for cannabinoid action in the NAc (e.g., Robbe et al., ***Proc Natl Acad Sci USA*** 99:8384-8388, 2002; Lupica et al., ***Br J Pharmacol*** 143:227-234, 2004; Gardner, ***Pharmacol Biochem Behav*** 81:263-2284, 2005) and other brain regions (e.g., Gerdeman and Lovinger, ***J Neurophysiol*** 85:468-471, 2001; Marsicano et al., ***Nature*** 418:530-534, 2002; Huang et al., ***J Neurosci*** 23:10311-10320, 2003).

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CANNABINOID CB_1 RECEPTOR DELETION AND CB_1 RECEPTOR ANTAGONISM BOTH *ENHANCE* MORPHINE CONDITIONED PLACE PREFERENCE

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Introduction: A growing body of evidence suggests that the cannabinoid CB_1 receptor system is involved in the conditioned effects of morphine. For example, Martin et al (2000) reported that a morphine conditioned place preference (CPP) was attenuated in CB_1 knockout (CB_1 KO) mice as compared to wild type littermate (WT) controls. Likewise, the CB_1 receptor antagonist SR141716 blocks the *acquisition* of morphine CPP (Chaperon et al 1998) in rats when it is administered prior to morphine conditioning sessions. Preliminary data from our laboratory revealed that the *expression* of morphine CPP was significantly *enhanced* in mice when SR141716 was administered only on the test day (in the absence of morphine), suggesting an alternative role of CB_1 receptors in morphine's conditioned effects. *Methods:* The role of CB_1 receptors in the conditioned effects of 5.0 mg/kg morphine were assessed with a standard CPP procedure in which mice received 1, 2, 3, or 4 pairings of morphine and saline in alternating compartments within the CPP testing chamber. In Experiment I, the role of the CB_1 receptor in the *expression* of morphine CPP after 1, 2, 3 or 4 conditioning pairings was investigated in male C57Bl/6 mice pre-treated with either vehicle or 3.0 mg/kg SR141716 prior to the test session. In Experiment II, the effect of CB_1 receptor deletion on morphine CPP after 1, 2, 3, or 4 conditioning pairings was assessed in CB_1 KO mice and WT controls. *Results:* SR141716 significantly enhanced the expression of morphine CPP after both 2 and 4 pairings of morphine and saline. Similarly, morphine CPP was enhanced after 2 conditioning pairings in CB1 KO mice as compared to WT controls. *Conclusions:* Although there are reports that the acquisition of a morphine CPP is attenuated by SR141716 in rats (following 4 conditioning pairings), the data reported here indicate that SR141716 enhances the expression of an established morphine CPP in mice under specific conditions. SR141716 has memory-enhancing effects (Wolff and Leander 2003; Lichtman 2000), which may explain the increased expression of morphine's conditioned effects by SR141716 in mice that have already acquired a morphine CPP. Morphine's conditioned effects were also enhanced in CB_1 KO mice after 2 pairings, and CB_1 KO has also been demonstrated to facilitate learning and memory (Reibaud et al 1999; Martin et al 2002). It is somewhat surprising, however, that these CB_1 KO mice acquired morphine CPP given that Martin et al (2000) reported a lack of morphine CPP in CB1 KO mice following 3 conditioning pairings. These findings point to the overall importance of assessing the behavioral effects of pharmacological and genetic manipulation across a wide range of experimental conditions.

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THE NOVEL ANTICANCER CANNABINOID QUINONE HU-331 IS MORE POTENT AND LESS CARDIOTOXIC THAN DOXORUBICIN – A COMPARATIVE *IN-VIVO* STUDY

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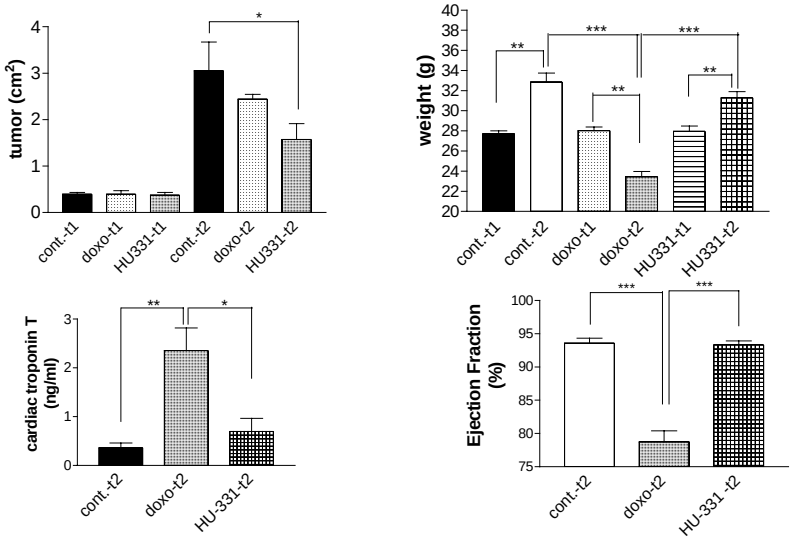
Quinones of various chemical families serve as biological modulators, and both natural and synthetic quinones are widely used as drugs. Anthracyclines, a large group of quinonoid compounds produced by different strains of streptomyces, exert antibiotic and antineoplastic effects and are used to treat several forms of cancer. The best-known members of this family are daunorubicin and doxorubicin, the first identified anthracyclins. Although these and other quinonoid compounds are effective in the treatment of many different forms of cancer, their side effects, the most severe being cumulative heart toxicity, limit their use. The development of quinonoid compounds that display antineoplastic activity, but are less toxic, is a major therapeutic goal.

We have reported the synthesis of a new anticancer quinone, HU-331, from cannabidiol, one of the most abundant cannabinoids of *Cannabis sativa.* HU-331 was found to be highly effective against tumor xenografts in *nude* mice. It is strongly anti-angiogenic both *in-vitro* and *in-vivo*, making this compound a promising scaffold for new anti-angiogenic drug, and it specifically inhibits topoisomerase II. Here we report that HU-331, while being more active than doxorubicin on HT-29 colon carcinoma model in *nude* mice is much less cardiotoxic (assayed by echocardiography and cardiac troponin T plasma levels).

In summary, *in-vivo* HU-331 was more active and less toxic than doxorubicin and thus it has a high potential to develop into a new anticancer drug.



t1- at the beginning of the study, t2- at the end of the study (after 2.5 months)

ANTI-CANCER EFFECTS OF CANNABIDIOL IN HUMAN BREAST CARCINOMA: CELLULAR AND MOLECULAR MECHANISMS OF ACTION AND INHIBITION OF METASTATIC SPREADING

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Δ^9-tetrahydrocannabinol (THC) and activation of cannabinoid receptor type 1 (CB_1) have been shown to inhibit growth and metastatic spreading of human breast cancer cells (De Petrocellis et al., 1998; Grimaldi et al., 2006). However the main limitation of CB_1 receptor agonists remains their potential psychotropic action. In this study, the anti-tumor activities of five non-psychotropic plant cannabinoids, i.e. cannabidiol (CBD) and CBD-acid, cannabigerol (CBG), cannabichromene (CBC) and THC-acid, and of a CBD-rich cannabis extract, have been investigated with the aim to determine whether there is any advantage in using CB_1-receptor-inactive phytocannabinoids or *Cannabis* extracts in cancer therapy.

The highly malignant, non-estrogen sensitive MDA-MB-231 cell line, and the estrogen-sensitive MCF-7 cell line, were used as models. Of all natural drugs tested, we found that CBD was the most potent at inhibiting tumour cell growth, followed by CBC, THC-acid and CBG, whereas CBD-acid was inactive. CBD-rich extract was as potent as pure CBD. CBD was significantly less active at inhibiting the growth of non-cancer mammary cells. When administrated subcutaneously to athymic mice, both CBD and CBD-rich extract inhibited the growth of xenograft tumors obtained by s.c. injection of MDA-MB-231 cells in athymic mice, and reduced the lung metastatic nodules originated from the intra-paw injection of these cells. While the growth inhibitory effect of CBD in MCF-7 cells was due to cell cycle arrest at the G1-S transition, the effect in MDA-MB-231 cells was due to induction of caspase-3 release from pro-caspase and subsequent apoptosis, and was attenuated by ~50% by a mixture of cannabinoid CB_2 and vanilloid TRPV1 receptor antagonists, and by ~ 50% by anti-oxidant agents. Accordingly, MDA-MB-231 cells were found to express both CB_2 and TRPV1 receptors, and CBD, CBC and CBG were capable of inhibiting endocannabinoid reuptake (and hence of potentially elevating endocannabinoid levels) and/or to activate to some extent the human TRPV1 receptor. Furthermore, CBD also caused a dose-dependent elevation of reactive oxygen species (ROS) in MDA-MB-231 cells.

In conclusion, we report that CBD inhibits MDA-MB-231 cell growth in vitro and in vivo by inducing apoptosis, in part via "direct" or "indirect" activation of cannabinoid CB_2 and vanilloid TRPV1 receptors, and in part by causing direct elevation of ROS. Different mechanisms underlie CBD effects on the estrogen-sensitive MCF-7 cell line. Our data support the testing of cannabis-based medicines, and particularly of CBD and CBD-rich cannabis extracts, in clinical trials for the potential treatment of mammary carcinoma.

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ANANDAMIDE INHIBITS ENDOTHELIAL CELL SPROUTING ANGIOGENESIS IN VITRO AND IN VIVO

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Endocannabinoids are now emerging as suppressors of key cell-signalling pathways involved in cancer cell growth, invasion and metastasis. We reasoned that anti-tumor effect could be at least in part ascribed to inhibition of neo-angiogenesis. In support of this, we previously reported that anandamide inhibited both VEGF and Flt-1 expression in thyroid tumors.

In this study we demonstrated the anti-angiogenic activity of the metabolically stable anandamide analogue, 2-methyl-2'-F-anandamide (Met-F-AEA) using *in vitro*, two- and three-dimensional, and *in vivo* assay systems. *In vitro*, Met-F-AEA inhibited endothelial cell proliferation and capillary-like tube formation, in a cannabinoid CB1 receptor dependent manner. The Met-F-AEA anti-proliferative effect was accompanied by apoptosis. Analyzing the signalling pathways implicated in the process of angiogenesis we observed a decreased phosphorylation of pERK, and we found that p38 MAPK was involved in Met-F-AEA induced apoptosis, while activation of NFkB, *via* stimulation of the CB1 receptor, was not necessary for apoptotic signalling in this system. Met-F-AEA was also able to inhibit *in vivo* angiogenesis in the chick chorioallantoic membrane assay. Moreover, in a 3-D model using different polymeric matrices, that reproduce the in vivo microenvironment and architecture, Met-F-AEA suppressed tumor-induced angiogenesis of endothelial (PAE) and tumor cells (KiMol) spheroids co-coltures.

Obtained results and the methodological approaches used could provide the rationale for further studies *in vitro* and *in vivo*, aimed to investigate deregulated angiogenesis related diseases.

INHIBITION OF COX-2 PROTECTS HUMAN CERVICAL CARCINOMA CELLS FROM R(+)-METHANANDAMIDE-INDUCED APOPTOTIC DEATH

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The role of cyclooxygenase-2 (COX-2) in apoptosis remains controversial. Recently, we have shown that the endocannabinoid analogue, R(+)-methanandamide (R(+)-MA) elicits apoptotic death of human neuroglioma cells via a COX-2-dependent pathway.[1,2] To assign this hitherto unknown mechanism to peripheral tumor cells, the present study investigated the role of COX-2 in R(+)-MA-induced apoptotic death of human cervix carcinoma cells (HeLa). R(+)-MA caused a profound and sustained up-regulation of COX-2 expression and prostaglandin E_2 (PGE_2) synthesis accompanied by decreased cellular viability and enhanced accumulation of cytoplasmic DNA fragments. R(+)-MA-mediated DNA fragmentation and cell death were significantly inhibited when COX-2 expression was prevented by COX-2-silencing small-interfering RNA (COX-2 siRNA). A similar protection was observed in the presence of the selective COX-2 inhibitor NS-398. Moreover, inhibition of COX-2 expression by the cholesterol-depletor ß-methyl-cyclodextrin and the ceramide synthase inhibitor fumonisin B_1 significantly interfered with R(+)-MA-induced cell death, suggesting a contribution of membrane lipid rafts and de novo synthesized ceramide to this response. In contrast, COX-2 expression and cell death by R(+)-MA were not affected by antagonists of CB_1-, CB_2- and VR_1 receptors. A substantial up-regulation of COX-2 expression and PGE_2 synthesis was also observed in the presence of anandamide and the well-established chemotherapeutic paclitaxel. Again, transfection of cells with COX-2 siRNA significantly inhibited apoptotic cell death elicited by these agents. Collectively, this study defines COX-2 as an important target by which endogenous cannabinoids and paclitaxel induce apoptosis of cervix carcinoma cells, suggesting that cotreatment with COX-2 inhibitors during chemotherapy could diminish its efficiency.

[1] Ramer et al., Mol Pharmacol 2003; 64:1189-98
[2] Hinz et al., Mol Pharmacol 2004; 66:1643-51

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NEUROPROTECTIVE EFFECTS OF CANNABINOIDS INDUCE THERAPEUTIC PROPERTIES IN A VIRAL MODEL OF MULTIPLE SCLEROSIS

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Cannabinoids (CB) have demonstrated beneficial effects in different animal models of multiple sclerosis (MS), as well as neuroprotective properties against excitotoxicity. In the present study, we postulate that the potential therapeutic effects of CB in MS could be explained, at least in part, by their anti-excitotoxic activity and a reduction of damage to axons.

First, we observed the amelioration of motor deficits by the chronic administration of the synthetic cannabinoid HU210 in mice suffering demyelination after infection with the Theiler's virus (TMEV). This beneficial effect was correlated with a reduction in axonal damage.

Second, NBQX, an antagonist of AMPA receptors, was sub-chronically administrated to TMEV infected mice. This treatment reduced both motor deficits and axonal damage, in the same manner as HU210 did. This observation suggests that excitotoxic processes participate in the pathology of this model of multiple sclerosis and may be responsible for axonal disorders.

Using primary cultures of astrocytes and neurons, we observed that the non-selective CB agonist HU-210 exerted a neuroprotective effect against AMPA-induced neuronal death. Interestingly, this effect was reversed by antagonists of both types of CB receptors, namely CB1 and CB2. While neither CB1 nor CB2 agonists demonstrated any neuroprotective effect when applied alone, the co-application of both agonists (ACEA and JWH133) induced a neuroprotection comparable to that observed with the non-selective agonist HU-210.

Over-activation of AMPA/Kainate receptors has been implicated in acute lesions to the central nervous system, and is thought to be involved in axonal injury in the context of chronic disorders. We induced excitotoxic lesions by microinjection of AMPA in the spinal cord of rats and observed a protective effect of HU210 in this model of acute lesion to the spinal cord.

In animal models of chronic pathologies, such as the TMEV model used in this study, in which axonal lesions have been observed in the spinal cord, blockade of AMPA/Kainate receptors could exert beneficial effects by reducing axonal injury. In this context, the neuroprotective effect of CB agonists against AMPA-mediated damage could represent one of the mechanisms responsible for their therapeutic action in animal models of chronic disorders of the central nervous system, such as MS.

CB1, BUT NOT CB2, MEDIATES STIMULATION OF BONE FORMATION INDUCED BY TRAUMATIC BRAIN INJURY

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Enhanced osteogenesis induced by traumatic brain injury (TBI) is a common clinical complication of which the underlying mechanisms are not fully understood. Using closed head injury (CHI), an established model to mimic TBI in mice, we show here CHI-induced rapid and sustained increase in peripheral bone formation. Because TBI also stimulates central endocannabinoid production, we investigated the role of CB1 and CB2 in mediating the CHI-induced stimulation of bone formation. We used sexually mature mice, normal C3H or C57BL/6J and *cb1*$^{-/-}$ and *cb2*$^{-/-}$ mice established in a C57BL/6J background. CHI was induced using a weight drop device. The severity of the brain damage was assessed by the Neurological Severity Score and only moderately-severely injured mice were included in the study. Newly formed bone was vitally double labelled by the fluorochrome calcein and femoral bone formation and resorption parameters were determined by histomorphometric analysis. Determination of bone 2-AG levels was performed by LC-MS. Bone norepinephrine (NE) was determined using HPLC-EC analysis. CHI resulted in increased trabecular bone formation by 65% and 35% after 4 and 8 days, respectively. This was accompanied by 3-fold increase in periosteal bone formation. Bone resorption was unaffected. A substantial stimulation of osteoblast activity was evident already one day post-CHI. It occurred equally in C3H and C57BL/6J male and female mice. CB1 but not CB2 deficient mice were entirely resistant to the CHI-induced stimulation of bone formation. The stimulation of osteoblast activity was preceded by increased bone 2-AG and decreased NE levels. In an attempt to mimic the CHI-induced stimulation of bone formation 2-AG was administered i.p. once and bone formation measured 24 h later. Indeed, the 2-AG administration closely simulated the effect of CHI. By contrast, a peripherally selective cannabinoid agonist that does not cross the blood brain barrier had no such effect. The TBI-induced stimulation of bone formation is perhaps the best documented clinical phenomenon connecting the CNS and the skeleton. The present findings demonstrate that this phenomenon depends on the presence of functional CB1 receptors. Our data further suggests that the main CB1 activation occurs in the CNS, rather than peripherally in bone, leading to reduced bone NE levels and alleviation of sympathetic inhibition of bone formation. These findings will potentially lead to the development of therapeutic measures to combat the TBI stimulation of ectopic osteogenesis by CB1 antagonists.

SR141716A IMPROVES COGNITIVE AND NEUROLOGICAL FUNCTION IN A MODEL OF SECONDARY BILIARY CIRRHOSIS IN THE RAT

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Introduction: Hepatic encephalopathy (HE) is a major neuropsychiatric complication of both acute and chronic liver failure. However, its pathogenesis is still unknown. It has been suggested that the cognitive deficits characterizing this state result from changes in some neurotransmitter systems in the brain including the glutamatergic, cholinergic and monoaminergic systems. Endocannabinoids (EC) function as neuromodulators via specific receptors. Recently the endocannabinoid system was found to be involved in the vasodilated state associated with liver cirrhosis. We hypothesize that it might be involved also in hepatic encephalopathy.

Methods: Male, Sprague-Dawley rats were subjected to ligation of the bile duct (BDL). Sham operated animals were used as controls. 2 weeks post-surgery, animals receiving either vehicle or SR141716A, a CB_1 receptor antagonist, were evaluated for cognitive and neurological function in the Object Recognition and in the Neurological Severity Score (NSS) tests, respectively. Neurological score was evaluated after 4 weeks as well. The animals were sacrificed and their hippocampi were taken to determine 2-AG levels by GC-MS analysis, and density of CB_1 receptors using radiolabeled ligand.

Results: Brain 2-AG levels were elevated and cognitive and neurological functions were significantly impaired in BDL rats. SR141716A improved these deficits. CB_1 receptor density was significantly lower in the hippocampi of BDL rats after 4 weeks.

Conclusion: These results indicate an involvement of the endocannabinoid system in the pathogenesis of HE. Modulation of the CB_1 cannabinoid receptor might have therapeutic potential.

IMMUNOHISTOCHEMICAL STUDY OF THE ENDOGENOUS CANNABINOID SYSTEM IN MULTIPLE SCLEROSIS POSTMORTEM HUMAN SAMPLES

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Increasing evidence suggest that cannabinoid administration may relieve the progression of certain clinical symptoms in different multiple sclerosis animal models. In clinical trials performed with multiple sclerosis (MS) patients, cannabis and individual cannabinoids may be effective in ameliorating symptoms such as tremor, spasticity and pain. Despite the possible beneficial effects of cannabinoids in MS, there are few studies about the changes in cannabinoid CB_1 receptors in animal models of this disease and no data exist in postmortem brains of human patients with MS. Previous data indicate that the endocannabinoid system may participate in neuroinflammatory responses. We have recently shown dramatic changes in the pattern of expression of cannabinoid CB_2 receptor and the enzyme fatty acid amido hidrolase (FAAH) as a consequence of beta-amyloid deposition in Alzheimer's disease and of viral infection of the brain in an animal model of encephalitis (SIVE model) and HIV-associated dementia (HIVE) patients.

We seek to study the pattern of distribution of specific components of the endocannabinoid system in the characteristic areas of demyelination of MS pathology. To that end, we used tissue samples from cortex brain regions of healthy individuals and of MS donors to first identify and sort these lesions. Afterwards, we performed an immunohistochemical study of cannabinoid CB_1 and CB_2 receptors and of the FAAH enzyme. Our preliminary results show an overexpression of CB_2 receptor in microglial and macrophage cells in demyelinated areas while FAAH expression was increased only in astrocytes.

The changes of expression in CB_2 receptor and the enzyme FAAH in glial cells agree with previous results in Alzheimer's disease, SIVE and HIVE tissue samples. This observation suggests a common pattern of response of the endocannabinoid system in the neuroinflammatory process triggered in these neuropathologies.

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WIN 55,212-2 IMPROVES CEREBRAL TISSUE RECOVERY AFTER EXPERIMENTAL NEWBORN HYPOXIA-ISCHEMIA IN A CB1 AND CB2 RECEPTOR DEPENDENT MANNER

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Introduction.

Experimental newborn hypoxia-ischemia causes an infarcted area in brain that includes a "core" region, affected by irreversible necrotic damage, and a "penumbra" region, which can be potentially recovered from the injury. The aim of this study was to test whether the exogenous activation of both CB1 and CB2 receptors by the agonist WIN 55,212-2 could promote the recovery of the "penumbra" and improve the functionality of the brain parenchyma after newborn hypoxia-ischemia.

Methods.

Left common carotid artery was ligated and sectioned under anesthesia in 7-day-old Wistar rats. After two hours or recovery from surgery, rats were placed in airtight glass containers in hypoxic conditions (8% O_2/92% N_2) during 120 minutes. Then, single doses of vehicle (0.5% DMSO in PBS/BSA; n=10), WIN 55,212-2 (0.1 mg/kg; n=13), WIN+SR141716A (3mg/kg; n=6) or WIN+SR144528 (2 mg/kg; n=6) were administered subcutaneously. Control group had sham surgery with no hypoxia or treatment.

Measurement of brain damage was performed by magnetic resonance imaging (T2WI, DWI, ADC maps) at 24h, 72h and 7 days after hypoxia-ischemia (Biospec 47/40, 7 Tesla). Then, rats were perfused intracardiacally with 4% paraformaldehyde (in 0.1M PBS, pH 7.4) and brains were removed, postfixed and crioprotected for Nissl staining and GFAP inmmunofluorescence.

Results.

No significant differences in the infarct volume were found between the experimental groups at 24h. Interestingly, at 72h rats treated with WIN showed a reduction of the infarct volume, an effect being stronger at 7 days (fig. 1). Moreover, the number of viable neural cells observed in the "penumbra" by Nissl staining was increased and reactive gliosis was reduced in animals treated with WIN. Finally, apparent diffusion coefficient in the temporal cortex of WIN-treated rats indicates a normalization of water diffusion in the "penumbra" (fig. 2). The antagonism of both CB1 and CB2 receptors reversed the effects of WIN administration.

Fig. 1

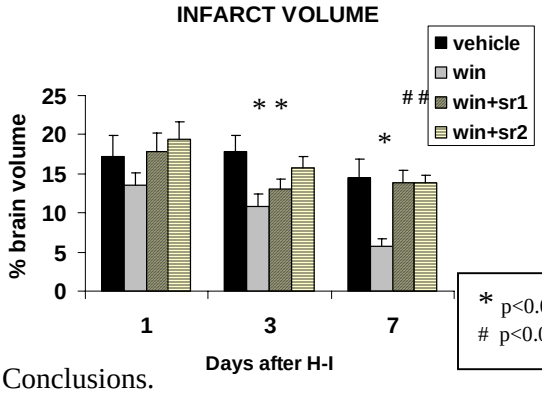
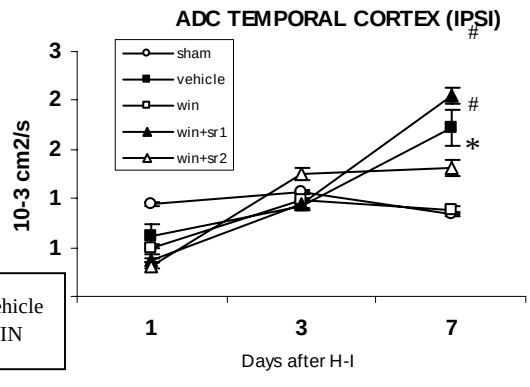


Fig. 2



Conclusions.

WIN protects newborn rats from damage caused by hypoxia-ischemia, reducing the magnitude of the infarction, and promoting the histological and functional recovery of the "penumbra" region.

A SINGLE ULTRA-LOW DOSE OF THC INDUCES LONG-LASTING COGNITIVE DEFICITS IN MICE: IMPLICATIONS FOR CHRONIC EXPOSURE TO CANNABINOID DRUGS

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Acute application of cannabinoid drugs has been shown to protect laboratory animals from various brain insults, including physical trauma, excitotoxicity and ischemia. On the other hand, chronic administration of the same drugs has been reported to induce cognitive deficits and morphological changes in the brain of the same species of animals. Based on our previous in vitro studies on the concentration-dependent dual activity of cannabinoids on intracellular calcium, we hypothesized that, while high doses of cannabinoids may protect from neurological insults, low doses are expected to induce brain damage *(Sarne and Keren, Med. Hypoth. 2004;63:187-192).* In the present study we tested this hypothesis in regard to the harmful effect of an ultra-low dose of delta-9 tetrahydrocannabinol (THC). We first determined the doses of THC that exerted either the "conventional" inhibitory effects, or a "paradoxical" stimulatory activity, in a battery of acute tests in mice. We found that regular (high) doses of THC (1-10 mg/kg) suppressed nociception, reduced body temperature and decreased motor activity in adult ICR mice, as expected. On the other hand, much lower doses (0.0003-0.003 mg/kg) potentiated nociception, elevated body temperature and increased motor activity of the mice. We then studied the long term effects of a single low dose of THC (0.001 mg/kg), using two different cognitive tests: Morris water maze and step-through passive avoidance. The experimental results indicated a significant impairment of cognitive functions up to 4 months following a single injection of this low dose of THC. The same low dose of THC induced a significant activation of caspase-3, a key enzyme in apoptotic and necrotic neuronal cell death. This activation of caspase was detected in the cortex as early as 6 hours following the i.p injection of THC. These experimental findings point to the neurotoxic profile of THC and to possible deleterious consequences of its repeated application that exposes the organism to low concentrations of the drug for long periods of time (due to the pharmacokinetics of this lipophilic substance) and should be taken into account while evaluating the therapeutic potential of cannabinoid drugs.

CHRONIC EXPOSURE TO Δ^9-THC INDUCES INCREASED DOPAMINE TRANSPORTER AVAILABILITY

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Introduction: A tight association exists between the cannabinoid system and brain dopaminergic circuits involved in addiction, schizophrenia and movement disorders. Ex vivo measurements have shown that delta-9-tetrahydrocannabinol (Δ^9-THC), a cannabinoid-1 receptor agonist and the major active constituent of marijuana, may alleviate a reduced striatal dopamine transporter (DAT) density in schizophrenia (Dean et al., Biol Psychiatry 2003). Moreover, cannabinoids have been reported to provide neuroprotection in acute and chronic neurodegeneration, e.g. against in vivo and in vitro toxicity of the 6-hydroxydopamine model for PD (Lastres-Becker et al, Neurobiol Dis 2005). The aim of this study was to investigate and quantify the in vivo effect of Δ^9-THC on striatal and midbrain DAT binding by microPET.

Method: Six adult healthy Wistar rats (female, body weight 200-250g, age range 15-18 weeks) were investigated in baseline condition, after acute (single injection of 0.5mg/kg IV) and chronic (during 2 weeks, 3mg/kg IP daily) administration of Δ^9-THC. For scanning, animals were anesthetized using 60 mg/kg pentobarbital IP. After IV bolus injection of 17 MBq of ^{18}F-FECNT, static acquisitions (40 min, start 3h p.i.) were conducted on a FOCUS microPET system (CTI-Concorde, USA). FPB reconstructed data were anatomically standardized and analyzed using a predefined VOI approach. Regional DAT binding ((specific uptake/cerebellar non-specific uptake)-1) was calculated and statistical analysis was done using paired t-testing. The effect on motor behavior was measured using the cylinder test at baseline, day 2, 7 and 14. Results were expressed in percentages of right paw use and also analyzed by a paired t-test.

Results: ^{18}F-FECNT binding in the striatum and substantia nigra was not affected by a single injection of Δ^9-THC (all $p>0.05$). However, chronic Δ^9-THC administration resulted in augmented DAT binding in the striatum, compared to baseline level (+13 %, $p=0.04$), with a large intersubject variability. In the substantia nigra, DAT binding was not affected by chronic Δ^9-THC exposure. Cylinder tests showed no significant group effect in right/left paw use during chronic Δ9-THC exposure (all $p>0.05$).

Conclusion: Chronic Δ^9-THC exposure induces DAT expression and/or affinity changes. These first-time in vivo findings contradict a possible role of Δ^9-THC as dopamine reuptake inhibitor to explain increases in extracellular dopamine levels (Sakurai-Yamashita et al, Pharmacol Biochem Behav 1989), but support alternative mechanisms by which Δ^9-THC can augment dopamine neurotransmission e.g. by changes in cell firing (Pistis et al, Brain Res 2002; French et al, Neuroreport 1997). This quantitative pharmacological effect needs to be considered when investigating the potential neuroprotective effect of cannabinoid agonists in animal models of PD using DAT imaging as biomarker, in the evaluation of cannabinoid-dopamine interactions in drug abuse and schizophrenia, or in evaluating patients for neurodegenerative movement disorders with previous chronic exposure to cannabis.

LYSOSOMAL DYNAMICS IN Δ^9-TETRAHYDROCANNABINOL-INDUCED APOPTOSIS IN RAT CORTICAL NEURONS

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The plant-derived cannabinoid Δ^9-Tetrahydrocannabinol (THC) is the predominant psychoactive moeity of cannabis. We have previously shown that THC causes apoptosis via the CB_1 receptor and activation of the stress activated kinase c-jun N-terminal Kinase (JNK) in the rat cortex[1]. The tumour supressor protein, p53, has been shown to induce apoptosis through a lysosomal-mitochondrial pathway that is initiated by lysosomal destabilisation[2]. The impact of cannabinoids on the lysosomal system and lysosomal cathepsin proteases has not been elucidated. Spleen Tyrosine Kinase (SyK) has been reported to have a role in the lysosomal branch of apoptosis[3] and its role in cannabinoid signaling was examined in this study.

Cultured cortical neurons were prepared from Wistar rats. THC (5μM, 5min) significantly increased the mean phospho-$p53^{SER15}$ expression from 300±44 (Mean±S.E.M, arbitrary units) to 800±71 ($p<0.001$, ANOVA, n=6). The THC-induced increase in phospho-p53 was abrogated by the JNK_1 inhibitor DJNK1 (10μM).

THC (5μM, 5mins) also increased the mean phospho-SyK expression from 105±15 (Mean±S.E.M, arbitrary units) to 357±133 ($p<0.05$, ANOVA, n=6) and this activation was attenuated by the CB_1 antagonist, AM251 (10μM) . The THC-induced increase in SyK phosphorylation was initiated within 5 minutes and sustained up until 90 minutes. THC treatment significantly increased the % apoptotic cells from 20±6% to 55±8% and this was blocked following SyK inhibition with Sulfonamide (50nM; $p<0.01$, ANOVA, n=7). The role of p53 in coupling THC to activation of SyK will be discussed.

To determine the impact of THC on lysosomal integrity, acridine orange (AO; 5μg/ml) was used to label inact lysosomes and the number of lysosomes per cell was determined as an index of lysosomal integrity. THC (5μM, 2hrs) significantly reduced lysosomal membrane integrity ($p<0.001$, ANOVA, for 65-149 cells) and the p53 inhibitor, Pifithrin-α blocked the THC-induced reduction in lysosomal membrane integrity. Loss of lysosomal integrity causes leakage of the lysosomal contents (*e.g.* Cathepsins) into the cytosol and the THC-induced apoptosis was significantly blocked by the Cathepsin-L inhibitor (Cathepsin-L inhibitor I , Z-FF-FMK, 10μM)

The results from this study indicate that THC impacts on the lysosomal system, possibly via SyK and p53, and a lysosomal branch of apoptsis is pertinent in THC-induced neurotoxicity.

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NEUROPROTECTION WITH CANNABINOIDS IN A RAT MODEL OF PARKINSON'S DISEASE

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We have recently demonstrated that two plant-derived cannabinoids, Δ^9-tetrahydrocannabinol and cannabidiol (CBD), are neuroprotective in Parkinson's disease (PD), presumably because of their antioxidant and cannabinoid receptor-independent properties (Lastres-Becker et al., Neurobiol. Dis. 19, 96-107, 2005). Now, we wanted to further explore this issue by examining the neuroprotective effects of a series of cannabinoid-based compounds, with more selectivity for different elements of the cannabinoid signalling system, in rats with unilateral lesions of nigrostriatal dopaminergic neurons caused by 6-hydroxydopamine. We used the CB_1 receptor agonist arachidonyl-2-chloroethylamide (ACEA), the CB_2 receptor agonist HU-308, the non-selective agonist WIN55,212-2, and the inhibitors of the endocannabinoid inactivation AM404 and UCM707. As expected, the application of 6-hydroxydopamine caused a significant depletion of dopamine (DA) contents and a reduction of tyrosine hydroxylase (TH) activity in the striatum, that were accompanied by a reduction in TH-mRNA levels in the substantia nigra. None of these events occurred in the contralateral structures. Daily administration of ACEA or WIN55,212-2 did not reverse 6-hydroxydopamine-induced DA depletion in the lesioned side, whereas HU-308 produced a small recovery that supports a possible involvement of CB_2 but not CB_1 receptors. In addition, AM404 produced a marked recovery of 6-hydroxydopamine-induced DA depletion and TH deficit in the lesioned side. Possibly, this is caused by the antioxidant properties of AM404, which are derived from the presence of a phenolic group in its structure, but not by the capability of AM404 to block the endocannabinoid transporter, because UCM707, another transporter inhibitor but devoid of antioxidant properties, did not produce the same effect. None of these effects were observed in non-lesioned contralateral structures. We also examined here the timing for the effect of those cannabinoids, such as CBD, that provided neuroprotection in this rat model of PD due to its antioxidant properties. We found that CBD, as expected, was able to recover 6-hydroxydopamine-induced DA depletion when it was administered immediately after the lesion, but it failed to do that when the treatment started 1 week later. In addition, the effect of CBD implied an upregulation of mRNA levels for Cu,Zn-superoxide dismutase, a key enzyme in endogenous defenses against oxidative stress. In summary, our results indicate that those cannabinoids having antioxidant cannabinoid receptor-independent properties provide neuroprotection against the progressive degeneration of nigrostriatal dopaminergic neurons occurring in PD. However, the activation of CB_2 (but not CB_1) receptors or other additional mechanisms might also modestly contribute to the potential of cannabinoids in this disease.

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SPECIES TREES AND GENE TREES AS METHODS FOR INFERRING COEVOLUTION

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Periodically our group has presented data regarding *Cannabis* plant parasites (ICRS'94, '00, '02). We used parasites as "plant taxonomists" to address questions regarding taxonomy of the *Cannabis* host plant. This method is based upon the observation that obligate parasites coevolve with their hosts. Parasite phylogeny mirrors host phylogeny. At ICRS'02 we demonstrated host-parasite coevolution as "parallel cladogenesis," where a parasite species tree mirrored a host species tree. The trees used in that investigation were cladograms, which are trees that show branching patterns; the branch lengths in cladograms have no significance. Branch lengths become significant in phylograms, whose lengths are drawn proportional to amounts of evolutionary change.

A species tree contains smaller trees descending within its branches – an assemblage of gene trees. Gene tree topology mostly mirrors species tree topology, but may differ due to gene duplication events, differential lineage sorting, or horizontal gene transfer. Pellegrini et al. (1999; *PNAS* 96:4285) proposed that pairs of genes may coevolve within a genome; Pellegrini quantified these relationships using a "phylogenetic profile." The genes for receptors and ligands must coevolve to maintain their structural relationships and binding affinities. Fraser et al. (2004; *PNAS* 101:9033) noted that coevolution may be a property of genes whose gene products do not interact physically, "…genes that work together in a single metabolic pathway may show coordinated changes in evolutionary rates because of increased or decreased utilization of those genes over evolutionary time." This property is à propos to cannabinoid receptors and cannabinoid ligand enzymes. We examined cannabinoid receptors (CB1, CB2, TRPV1, GPR55) and endocannabinoid enzymes (FAAH, MAGL, COX2, NAPE-PLD, DAGLα and DAGLβ) for evidence of coevolution. Gene trees of receptors and enzymes were constructed from orthologs BLASTed from ten phylogenetically diverse organisms. Multiple sequence alignments were used to make cladograms of gene trees within species trees. Evidence for coevolution was assessed with mirrored trees (adapted from host-parasite studies) and Pellegrini's phylogenetic profile method. Lastly, receptor-ligand relationships were reexamined with phylograms whose branch lengths were proportional to three sets of maximum likelihood metrics: nucleotide substitutions, NS/SS ratios, or Ka/Ks ratios. We employed a linear regression analysis to correlate branch lengths from equivalent branches in paired trees of receptors and ligand enzymes.

All three methods (mirrored cladograms, phylogenetic profiles, and regression analysis of phylograms) suggested close associations between cannabinoid receptors and DAGL enzymes. Researchers have debated whether AEA or 2-AG is the "natural" or "intrinsic" ligand of CB1 and CB2. Our coevolutionary analysis may weigh upon this debate.

THE EVOLUTIONARY ORIGIN OF CANNABINOID RECEPTORS: EVIDENCE OF AN ANCIENT ROLE IN PRESYNAPTIC REGULATION OF NEURONAL SIGNALLING IN INVERTEBRATE CHORDATES

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We have previously reported the identification of CiCBR, an ortholog of vertebrate CB_1/CB_2 cannabinoid receptors in the urochordate Ciona intestinalis and the first cannabinoid receptor to be discovered in an invertebrate (Elphick et al., 2003; Gene 302, 95-101). Further investigation of the phylogenetic distribution of cannabinoid receptor orthologs in deuterostomian invertebrates has recently been facilitated by sequencing of the genome of the sea urchin Strongylocentrotus purpuratus (phylum Echinodermata). Importantly, we have found that an ortholog of CiCBR and the vertebrate CB_1/CB_2 receptors is not present in this species, indicating that cannabinoid receptors of this type originated in an invertebrate chordate ancestor of urochordates and vertebrates. Therefore, our discovery of CiCBR provides a unique opportunity to investigate cannabinoid receptor function in an invertebrate species and to obtain evidence of the ancestral roles of cannabinoid receptors in invertebrate chordates before the emergence of CB_1 and CB_2 receptors in vertebrates.

We have used Western blotting and immunocytochemistry to examine the expression of CiCBR in Ciona, employing novel antibodies to the C-terminal tail of CiCBR. Consistent with the expected mass for CiCBR, a ~ 47 kDa band was detected in membrane fractions of homogenates of adult Ciona and immunocytochemical analysis of serial sections of adult Ciona revealed intense immunoreactivity in a dense meshwork of fibres throughout the central neuropile region of the cerebral ganglion. CiCBR-immunoreactivity was also observed in fibres exiting the ganglion via the anterior and posterior nerves and analysis of whole-mount preparations revealed that these CiCBR-immunoreactive axons project over the interior surface of the inhalent and exhalent siphons. Isolated CiCBR-immunoreactive axons not associated with the anterior and posterior nerves were also observed projecting through the cortical neuronal cell body layer of the cerebral ganglion. Central and peripheral CiCBR-immunoreactive fibres were studded with intensely stained varicosities, indicative of a role for CiCBR in regulation of axonal release of neurotransmitters, neuromodulators or neurohormones. Collectively, these data suggest that the well-established role that the CB_1 receptor has as a presynaptic regulator of neurotransmitter release in mammalian nervous systems may have originated in ancestral-type invertebrate cannabinoid receptors before the emergence of CB_1- and CB_2-type receptors in vertebrates. Furthermore, the neuroanatomical data presented here provide a framework for experimental analysis of the physiological roles of CiCBR, exploiting Ciona intestinalis as an invertebrate model system for research on endocannabinoid signalling.

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MAGL (MONOACYLGLYCEROLIPASE) ACTIVITY IN *TETRAHYMENA*

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We have earlier shown that major components of the endocannabinoid system such as FAAH [*V. Karava et al. Biochimie 87 967-974 (2005)*], CB receptors and NAEs are present in the unicellular eukaryote *Tetrahymena pyriformis.* Here we present evidence showing that *Tetrahymena pyriformis* cells are able to take up and metabolize the endocannabinoid 2-arachidonoylglycerol by the action of a MAGL-like activity. The enzymatic activity was found in the cell homogenate as well as in subcellular fractions and it was partially characterized. The activity was not inhibited by the irreversible and rather selective FAAH inhibitor URB-597. The possibility of the secretion of MAGL activity by *Tetrahymena* cells was also investigated. The presence of MAGL activity in a unicellular eukaryote is shown for the first time and suggests the importance of this enzyme throughout the evolution. The results along with results from previous studies further support the notion that both enzymes MAGL and FAAH could inactivate endogenous signaling lipids which might have manifold roles in protists as it has been suggested for mammals and invertebrates. These roles remain to be elucidated.

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PLASMON WAVEGUIDE RESONANCE (PWR) SPECTROSCOPY: A NOVEL TOOL TO EXAMINE CANNABINOID RECEPTOR SIGNALING

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Plasmon waveguide resonance (PWR) spectra can provide information about ligand-mediated conformational changes in optically anisotropic systems, such as lipid-embedded receptor proteins. PWR detects resonant electron oscillations (plasmons) generated by polarized laser light in a dielectric-coated silver film on the surface of a glass prism (resonator). When a proteolipid bilayer is deposited on the surface of the resonator, plasmon resonance will occur at an incident light angle that is dependent on refractive index anisotropy and thickness of the bilayer. In the present work we employed this novel method to detect ligand-mediated conformational changes in the human cannabinoid receptor (hCB1). A recombinant epitope-tagged hCB1 construct was solubilized and partially purified using using WGA- and metal affinity chromatography. The partially purified hCB1 receptor protein was incorporated into a lipid (egg PC) bilayer on the surface of the resonator. PWR measurements indicate that cannabinoid ligands exhibit high affinity to the reconstituted, purified hCB1. In addition, we found that two structurally different cannabinoid agonists (WIN 55,212-2 and CP 55,940) shift the PWR spectra in opposite directions, indicating that these ligands may interact with different hCB1 receptor conformations. Further investigations are in progress to study the effect of other cannabinoid ligands on the conformation of the hCB1.

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LIGAND-DEPENDENT ALTERATIONS IN CB_1 RECEPTOR FUNCTION AND LOCALIZATION IN CB_1 RECEPTOR-EXPRESSING CHO CELLS

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Previous research from our laboratory has shown that chronic Δ^9-tetrahydrocannabinol (THC) or WIN 55,212-2 (WIN) administration produces brain region-dependent decreases in CB_1 receptor-mediated G-protein activation and downregulation of CB_1 receptor binding sites. Recently, these adaptations have been shown to persist for more than one week following cessation of THC in some brain regions. The current study utilized a Chinese Hamster Ovary (CHO) cell line stably expressing mouse CB_1 receptors to further characterize potential mechanisms of CB_1 receptor adaptation. Using cannabinoid-stimulated [^{35}S]GTPγS binding, we determined that cannabinoid agonists stimulated G-protein activation in cell membranes, with an order of efficacy of CP55,940 (CP) = WIN $\geq$ methanandamide > THC. In a separate series of experiments, membranes were prepared from CB_1 receptor-expressing CHO cells that were treated for 21 hours with WIN, THC, CP, methanandamide or vehicle (containing 0.05% ethanol). Treatment with WIN, THC or CP attenuated agonist-stimulated [^{35}S]GTPγS binding independent of the agonist used to activate G-proteins. Interestingly treatment with methanandamide did not desensitize CB_1-mediated G-protein activation. Furthermore, treatment with WIN, THC, CP or methanandamide did not result in significant downregulation of CB_1 receptor binding sites. To determine whether the desensitization observed following cannabinoid treatment was accompanied by CB_1 receptor internalization, CHO cells expressing GFP-tagged CB_1 receptors were treated with WIN, THC, CP or vehicle and analyzed using confocal microscopy. WIN, THC and CP treatment resulted in internalization of CB_1 receptors. These results demonstrate that treatment with cannabinoids, with the exception of methanandamide, results in desensitization of CB_1 receptors. Desensitization occurs without altering the total number of CB_1 receptors, although internalization of the CB_1 receptor was observed. Thus, CB_1 receptors expressed in CHO cells exhibit similarities and differences in their responses to acute and chronic agonist exposure compared to CB_1 receptors in mouse brain. Comparison of the current cell culture model with brain CB_1 receptors will aid in elucidation of the mechanisms involved in CB_1 receptor adaptation to chronic agonist treatment.

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DUAL MODULATION OF ADENYLYL CYCLASE BY CANNABINOIDS IN HUMAN BRONCHIAL EPITHELIAL CELLS

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Introduction

Cannabinoids (CB) have been shown to possess immunomodulatory properties and may be involved in airway pathophysiology. Cannabinoid receptors couple to $G_{i/o}$ proteins inhibiting adenylyl cyclase activity. We investigated CB receptor subtype expression and the effects of the endogenous cannabinoid virodhamine and the synthetic agonist CP55,940 on cAMP accumulation in human bronchial epithelial cells.

Methods

All measurements were carried out using 16HBE14o- cells. CBR mRNA was isolated and detected by RT-PCR. cAMP accumulation in cell suspension was measured, after cells were lysed, by competitive radioligand binding assay.

Results

mRNA of both CB receptor subtypes was detected in 16HBE14o- cells.

Virodhamine and CP55,940 decreased concentration dependently forskolin induced cAMP accumulation which was mediated by CB_2 receptors, as shown by using the CB_2 receptor antagonist SR1445228 and was sensitive to pertussis toxin. However, pretreatment with pertussis toxin also unmasked a stimulating component by both virodhamine and CP55,940, which could be blocked by the CB_1 receptor antagonist SR141716.

Our results demonstrate the presence of both CB_1 and CB_2 receptors in human bronchial epithelial cells which are coupled to distinct pathways, mediating stimulation and inhibition of adenylyl cyclase activity, respectively.

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THE NITRIC OXIDE SENSITIVE GUANYLYL CYCLASE ALPHA1 SUBUNIT IS ABUNDANT IN DIFFERENT TYPES OF INTERNEURONS BUT NOT IN PYRAMIDAL CELLS IN RAT HIPPOCAMPUS

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Nitric oxide (NO) plays an important role in synaptic plasticity as a retrograde messenger and it also modulates CB_1 receptor signaling in interneuron terminals in the hippocampus. The NO receptor, soluble guanylyl cyclase (sGC) is present in the brain in two functional subunit composition: α1β1 and α2β1. The β1 subunit is expressed both in pyramidal cells and in interneurons in the rat hippocampus, however the exact localization of the α subunits was unknown. Here we describe the expression pattern of sGC α1 subunit in the CA1 region.

We found that α1 subunit positive interneurons were always positive for β1 and vice versa, however pyramidal cells stained only for β1, but not for α1 subunit. With double immunofluorescent staining, we found that two thirds of cholecystokinin positive, three fourth of parvalbumin positive, one third of somatostatin positive and one fifth of neuronal nitric oxide synthase (nNOS) positive neurons are positive for the α1 subunit. From the α1 positive neurons about 20% was positive for cholecystokinin, 40% for parvalbumin, 7% for somatostatin and about 11% for nNOS. Therefore, in a subpopulation of interneurons sGC may serve as an autoreceptor for intracellularly released NO. We found no co-localization between nNOS and type 1 cannabinoid receptor. There was about 50% overlap between the substance P receptor positive and nNOS containing cells from both directions.
Using preembedding immunogold and immonoperoxidase double labeling technique, we also investigated whether sGC is transported to the axon terminals. We found that the α1 subunit is present in parvalbumin and cholecystokinin positive basket cell terminals and in cholecystokinin positive terminals on pyramidal cell dendrites.

Our results suggest that sGC composed of α1β1 subunits is selectively expressed in different types of interneurons, and is transported to the terminals as well, where it potentially detects NO released from pyramidal cells or interneurons and can play a role in CB_1 receptor signaling.

ALLOSTERIC MODULATION OF THE CANNABINOID CB_1 RECEPTOR: AGONIST DEPENDENCE

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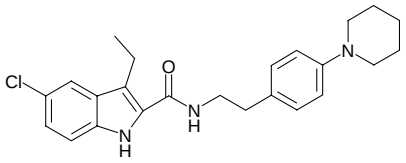
In a recent publication (Price et al, 2005) we identified three novel allosteric modulators of the cannabinoid CB_1 receptor. Interestingly, these compounds are allosteric *enhancers* of agonist binding *affinity*, but allosteric *inhibitors* of agonist signalling *efficacy*. The nature of the effect of allosteric modulators is known to be ligand-dependent (Christopoulos & Kenakin, 2002). By definition, investigation of the functional effect of an allosteric modulator on the endogenous ligand for the orthosteric site is crucial to the development of clinically useful compounds. While both anandamide and 2-arachidonoyl glycerol (2-AG) have nanomolar affinity for the CB_1 receptor they may well have different physiological and pathophysiological roles, being released in different tissues and initiating diverse signalling events. In this study we investigated whether one of these compounds (Org 27569) differentially affects anandamide and 2AG, thereby potentially affording ligand-dependant modulation. We have extended the study to investigate the effect of this compound on several other CB_1 receptor agonists.



Org 27569

We investigated the effect of Org 27569 on responses to CB_1 receptor agonists using two functional assays: stimulation of [^{35}S]GTPγS binding to mouse brain membranes and inhibition of electrically-evoked contractions of the mouse vas deferens. The E_{max} of CP55940 was significantly reduced by Org 27569 at 100nM and 1μM in the [^{35}S]GTPγS binding assay and at 1, 10 and 100nM in the vas deferens. CP55940 was more susceptible to antagonism by Org 27569 than WIN55212-2. The potency of Org 27569 against anandamide matched its potency against CP55940 in the [^{35}S]GTPγS binding assay but not in the vas deferens in which it significantly reduced the E_{max} of anandamide at 1μM but not at 100 nM. Org 27569 produced little antagonism of 2-AG in the [^{35}S]GTPγS binding assay even at 1 μM. Org 27569/2-AG experiments were not performed in the vas deferens. However, it was found in additional experiments with the vas deferens that the E_{max} of Δ^9-THC was significantly reduced by Org 27569 at 10, 100 and 1000nM but not at 1 nM.

These data demonstrate that the potency exhibited by Org 27569 as an allosteric modulator of the CB_1 receptor is both agonist- and assay-dependent.

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DIFFERENTIAL REGULATION OF TYROSINE HYDROXYLASE EXPRESSION IN NEUROBLASTOMA CELLS BY DISTINCT CANNABINOID CB_1 RECEPTOR AGONISTS

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Functional interactions between catecholamine and cannabinoid transmission systems could explain the influence of Δ^9-THC on several central activities including modulation of motor and emotional behaviours. Many studies have reported the lack of co-localisation of CB_1 cannabinoid receptors with tyrosine hydroxylase (TH), the rate-limiting enzyme implicated in catecholamines biosynthesis. Hence, it is generally suggested that cannabinoids could mediate regulation of neurotransmission by modulating presynaptic influences on catecholaminergic neurons. In contrast, other immunohistochemical investigations have demonstrated expression of CB_1 cannabinoid receptors in TH containing cells of the mesocorticolimbic system, suggesting a possible direct control of cannabinoid system on catecholamines synthesis. We recently identified the expression of functional CB_1 cannabinoid receptors on the murine neuroblastoma cell line N1E-115, a widely used model for the study of catecholamines synthesis. In the present, study we examined the influence of structurally different cannabinoid ligands on TH expression and gene transcription in this model.

The expression of TH was examined by Western blot analysis of protein extracts from N1E-115 cells previously exposed to cannabinoid agonists. The regulation of TH gene transcription was examined using a rodent TH promoter luciferase reporter assay in transiently transfected cells.

Analysis of Western blot studies indicated that exposure of the cells for 5 to 24h to HU210 (100 nM-10 µM) resulted in a significant reduction of TH expression. In contrast, such treatments with CP55940 elicited no change, suggesting an agonist dependent effect. Besides, the use of the luciferase-based assay revealed that HU210 mediates an inhibition of TH gene transcription while CP55940 induces an increase in the transcriptional activity. Both responses were inhibited by SR141716A, confirming the involvement of CB_1 cannabinoid receptors. This system was used for evaluating the properties of a variety of cannabinoid ligands from different chemical families. Contrasting with the inhibitory response mediated by the majority of tested compounds that behaved like HU210, the members of the family of non-classical cannabinoid ligands (CP55940-like compounds) were found to markedly increase the activity of the reporter gene.

Taken together, the present data indicate that cannabinoid receptors present in this neuroblastoma cell line operate a direct modulation of TH expression through the regulation of its gene transcription. In addition, the existence of different responses to chemically distinct agonists suggests the independent coupling of the CB_1 cannabinoid receptor to multiple signalling pathways.

ANANDAMIDE INHIBITS CALCIUM CHANNELS IN DORSAL ROOT GANGLION NEURONS INDEPENDENT OF CANNABINOID-1 RECEPTORS

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The endocannabinoid system may serve an important function in the peripheral regulation of nociceptive transmission. One mechanism by which anandamide (AEA) modulates activity of primary afferent neurons is through inhibition of the increase in free intracellular calcium concentration ($[Ca^{2+}]_i$) evoked by depolarization with KCl (50 mM). Changes in $[Ca^{2+}]_i$ were measured in isolated adult dorsal root ganglion (DRG) neurons by microfluorimetry 40-48 h after dissociation. In capsaicin-sensitive DRG neurons (cell area < 500 μm^2) AEA (10 nM) and its stable analog (R)-(+)-methanandamide (MAEA, 10-1000 nM) attenuated the KCl-evoked increase in $([Ca^{2+}])_i$ through inhibition of L-type calcium channels but the effect was independent of the cannabinoid-1 receptor (CB1R)- and pertussis toxin (PTX). The inhibitory effect of AEA was attenuated by inhibition of the membrane transporter and regulated by activity of fatty acid amide hydrolase. Neither AEA nor MAEA modulated capsaicin-insensitive DRG neurons (cell area > 500 μm^2). Although no independent effects occurred in response to CB1R antagonists SR141716A or AM251 alone, co-application of antagonists with AEA resulted in inhibition of the KCl-evoked increase in $[Ca^{2+}]_i$ in capsaicin-insensitive DRG neurons. Pre-treatment with PTX also unmasked the inhibitory effect of AEA. The effect of AEA on DRG neurons was terminated by brief disruption of membrane integrity with methyl-β-cyclodextrin (1mM).

Together these findings provide evidence for direct inhibitory effects of AEA on primary afferent neurons and suggest complexity and dissimilarity in endocannabinoid regulation of populations of sensory neurons.

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THE ENDOCANNABINOID SYSTEM IN RABBIT BRAIN. EVIDENCE FOR THE PRESENCE OF CB2 RECEPTORS.

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The endocannabinoid system is a lipid signaling system consisting of the cannabinoid receptors (CB1 and CB2), endogenous ligands such as anandamide and 2-arachidonoylglycerol and mechanisms for the termination of the signaling process. This system has been characterized in different tissues of vertebrates as well as in some invertebrates. The presence of basic elements of the endocannabinoid system has been shown even in a protist by our group. Rabbit is a widely used experimental animal and its platelets have extensively been used in studies concerning the function of endocannabinoids. To our knowledge, the endocannabinoid system has never been studied in rabbit brain. In this study we provide evidence for the existence of basic elements that constitute the endocannabinoid system in rabbit brain, as expected. Namely: a) FAAH activity was detected and the enzyme was identified and characterized in brain homogenate as well as in subcellular fractions, with highest activity in membrane and microsomal fractions, b) MAGL activity was also detected and characterized, c) CB1 receptors were detected ,as expected and finally most interesting was the finding that CB2 receptors were present. Rabbit brain might provide an excellent model for further studies on the action and the role of CB1 and CB2 receptors in the brain.

ROLE OF CB1 IN DIFFERENT NEURONAL SUBPOPULATION IN THE BRAIN

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The endocannabinoid system is involved in the control of synaptic transmission via a retrograde mechanism, as well as in the regulation of different forms of short- and long-term plasticity. The expression of cannabinoid receptor CB1 in distinct neuronal sub-populations of the central nervous system poses the question which neurons mediate the physiological effects of cannabinoids in the brain. We generated a number of CB1 conditional knockout mouse lines using the Cre/loxP system to dissect the involvement of different neuronal subpopulations in the physiological functions of the endocannabinoid system and the pharmacological effects of drugs acting at the CB1 receptor. Using an electrophysiological approach, the results show that the endocannabinoid system is functionally present in different neuronal subpopulation in several brain subregions.

EXPRESSION ANALYSES OF TWO HUMAN CB2 PROTEIN VARIANTS

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Very recently we discovered that the CB2 receptor is involved in bone mass regulation in mice (1). Additionally, in a human genetic study we found an association of single nucleotide polymorphisms (SNP) in the CB2 receptor gene (*CNR2*) with human osteoporosis (2). In order to identify potential CB2 variants that may be causally involved in the pathogenesis of osteoporosis, we sequenced the CB2 coding exon in nearly 400 osteoporotic patients and healthy controls. We identified disease-associated SNPs in the coding and the untranslated regions of the *CNR2* gene, which are in complete linkage disequilibrium. Interestingly, two SNPs were on adjacent nucleotides resulting in the amino acid exchange glutamine to arginine at protein position 63, which is localised in the first intracellular loop. The arginine CB2 variant was more frequent in osteoporotic patients.

Because CB2 knockout mice develop symptoms reminiscent of postmenopausal osteoporosis, we hypothesized that the disease associated CB2 variant might result in lower CB2 mRNA expression levels, or in a functional deficit of the CB2 receptor. To test these possibilities, we isolated genomic DNA and RNA from blood cells of 59 volunteer healthy blood donors. Genomic DNAs were analyzed for the genotype of the SNPs rs2502992 and rs2501432, which define the glutamine/argenine polymorphism, using TaqMan SNP Assays from ABI. We thus identified homozygous “glutamine”, homozygous “arginine” and heterozygous “glutamine/arginine” subgroups. For expression studies, we used real-time PCR, with primers from the non-coding exon 1 and the coding exon 2 of the *CNR2* gene. Our results showed that both CB2 variants were expressed at equal levels in human blood samples.

We next analyzed the sub-cellular localization of the two CB2 protein variants. For this purpose, we fused a V5-tag with the CB2 coding region of both protein variants. MC3T3 E1 cells transfected with these tagged CB2 expression vectors were stained with an antibody against the V5-tag. Both protein variants showed a similar staining pattern with a prominent membrane localization.

In conclusion, our results did not provide any evidence for differential expression or cellular localization of the osteoporosis-associated CB2 protein variant. Functional studies concerning the signalling properties of the variant receptors are ongoing.

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CELL SURFACE CB1 AND GPR55 ARE TARGETED TO DISTINCT CELLULAR DOMAINS WHEN EXPRESSED RECOMBINANTLY IN CULTURED HIPPOCAMPAL NEURONS

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The CNS actions of cannabis and related compounds (cannabinoids) are thought to be primarily mediated by the CB_1 subtype of cannabinoid receptor. However, an increasing number of functional studies have identified novel cannabinoid-like actions in the brain and two recent patent applications have suggested that cannabinoid ligands can also bind to the orphan G-protein coupled receptor GPR55 (Baker et al., 2006; TIPS. 27:1-4.). As GPR55 mRNA is expressed within human brain tissue, this receptor may therefore play a role in the CNS actions of cannabinoids. In this study we have compared the cellular expression of recombinant, N-terminally tagged, GFP-fusions of CB_1 and GPR55. The constructs were transfected into neurons (5-14 days in vitro) using calcium phosphate procedures. Live confocal imaging of GFP revealed expression of both constructs throughout individual neurons. However, GPR55-GFP was often associated with the somatodendritic plasma membrane, whereas CB_1-GFP had a more punctuate distribution in the soma and prominent axonal labeling. Using an antibody to GFP we demonstrated that both chimeras were expressed at the cell surface, but with markedly different cellular distributions. Surface GPR55 was detected on both axons (L1 or GAP-43-positive) and soma/dendrites (MAP2 positive), whereas CB_1 was found predominantly on axons with little or no expression within somatodendritic regions. In conclusion, the differing cellular distributions of CB_1 and GPR55 suggests that they might sub-serve distinct functional roles in the CNS, with the potential for both receptors to act presynaptically, but with GPR55 mediating postsynaptic actions of cannabinoids.

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ANANDAMIDE MODULATION OF CALCIUM SIGNALLING IN CULTURED HIPPOCAMPAL NEURONS AND GLIA

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The endocannabinoid anandamide plays an important role in modulating neuronal function, where in addition to classical cannabinoid receptors, it acts on a wide variety of molecular targets, including $TRPV_1$ channels, K^+ channels, Na^+ channels and NMDA receptors. In this study we have used digital epifluorescence microscopy combined with single cell flura-2 microfluorimetry to investigate the effects of anandamide on intracellular Ca^{2+} signalling in cultured hippocampal cells. Exposure of cultures to anandamide (1-10 µM) resulted in a marked increase in intracellular calcium levels in both neurons (0.30 ratio units; n=7) and glia (0.33 ratio units; n=7). The response was delayed by up to 5 minutes after the initial anandamide exposure and was often oscillatory in nature. In some cells the response did not fully recover on washout (up to 45 minutes). The source of the signal primarily reflected activation of a calcium influx pathway as it was blocked in the presence of calcium-free medium (99.5%; n=10) and depletion of intracellular calcium stores with thapsigargin (2 µM) had no significant effect ($P>0.05$; n=10). In order to investigate the pharmacology of the anandamide response we tested the actions of pharmacological blockers of CB_1 (SR141716A) and $TRPV_1$ receptors (SB366791). However, neither SR141716A (1 µM) nor SB366791 (25 µm) had any significant effect on responses to anandamide ($P>0.05$; n=10). Moreover, the well established cannabinoid (Win55212-2; 10 µM) and $TRPV_1$ (capsaicin; 10 µM) receptor agonists did not elevate intracellular calcium levels ($P>0.05$; n=10). These experiments suggest that a novel, non-CB1, non-$TRPV_1$ molecular target is responsible for anandamide-mediated calcium influx in hippocampal neurons and glia.

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THE IMPORTANCE OF RESIDUES, S7.39 AND S2.60 IN MODULATING THE FUNCTION OF THE HUMAN CB_1 CANNABINOID RECEPTOR

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The CB_1 cannabinoid receptor is a member of the GPCR Family 1A typified by rhodopsin. However, unlike rhodopsin, the crystal structure of CB_1 remains elusive. Furthermore, a series of structurally diverse ligands are able to recognize and bind at the CB_1 receptor, suggesting the existence of multiple ligand binding sites on the receptor. The rhodopsin X-ray crystallographic study is a valuable template on which to model the CB_1 binding sites. In this study, we have used *in silico* computational ligand docking models (based on previously identified residues) complemented with *in vitro* site-directed mutagenesis and heterologous receptor expression to map the ligand binding sites of the human CB_1 (hCB_1) receptor. Previous modeling studies have suggested that S2.60(173) and S7.39(383) as possible site(s) of hydrogen bonding with AM841, a high affinity CB_1 agonist (*Picone, R.P., et al. Mol Pharmcol.,68:1623-1635, 2005*). We hypothesized that the mutation of S2.60 and S7.39 would disrupt hydrogen bonding sites for cannabinoid compounds with dimethylheptyl sidechains (at C-3) and hydroxylation (at C-9 or C-11), such as AM4056 (an AM841 analogue) and HU-210.

Recombinant hCB_1 receptors stably expressed in HEK 293 cells, were used to investigate the consequences of mutating S2.60 (to S2.60A) or S7.39 (to S7.39A) in radioligand binding studies and GTPγS functional assays. The S2.60A mutant receptor bound to [^{3}H]CP-55,940 with K_d and Bmax values that were comparable to wild-type (WT) hCB_1 receptors. In competition binding studies and the GTPγS functional assay, the S2.60A mutant displayed a modest but significant (~6-fold) reduction in affinity and potency for HU-210. In contrast, the affinity and potency of AM 4056 was unaltered at this mutant.

Surprisingly, the S7.39A mutant receptor did not bind [^{3}H]CP-55,940. However, the [^{3}H]WIN-55,212 binding properties at this mutant were similar to the WT receptor. Therefore, displacement studies on this mutant were characterized using [^{3}H]WIN-55,212. HU-210 displayed a drastic reduction in the binding affinity (K_i, >500-fold) and potency (>200-fold) at the S7.39A mutant as compared to the WT hCB_1, suggesting that S7.39 plays a crucial role in the recognition and binding of the agonist, HU-210. Similarly, the EC_{50} for AM 4056-mediated activation of the S7.39A receptor was increased by ~500-fold. The binding properties of other ligands tested (WIN-55,212, SR 141716A, Anandamide) were unaltered at the S7.39A mutant. These results suggest that, S2.60 and S7.39 residues play a crucial role in mediating ligand specific interaction at the hCB_1 receptor.

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CB1-RECEPTOR DEPENDENT INHIBITION OF STRIATAL DOPAMINE RELEASE IS INDIRECT VIA GABA RELEASE INHIBITION

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The main psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC), acts in the CNS via cannabinoid 1 receptor (CB1R) activation. These receptors are highly localized in the striatum, where they may mediate suppression of motor activity following administration of THC or synthetic CB1 agonists. One explanation for this behavioral consequence of CB1R activation is that CB1 agonists might suppress striatal dopamine (DA) release; however, evidence from previous studies has been inconclusive. Here we addressed this question in striatal slices, with detection of locally evoked extracellular DA concentration ($[DA]_o$) using carbon-fiber microelectrodes and fast-scan cyclic voltammetry. Consistent with previous reports, DA release evoked by a single-stimulus pulse was unaffected by a CB1R agonist, WIN55,212-2. However, when DA release was evoked by a train of stimuli, WIN55,212-2 caused a significant decrease in evoked $[DA]_o$, implicating the involvement of local striatal circuitry. Evoked $[DA]_o$ was not altered by either an inverse agonist, AM251, or by a neutral antagonist, VCHSR1, arguing against regulation by endogenously released cannabinoids with the stimulation protocol used. However, both antagonists could prevent and reverse suppression of evoked $[DA]_o$ by WIN55,212-2. The effect of WIN55,212-2 was also prevented by picrotoxin, a $GABA_A$ receptor antagonist, and by catalase, a metabolizing enzyme for hydrogen peroxide (H_2O_2) scavenging enzyme. These data indicate that suppression of striatal DA release by CB1R activation occurs via a novel indirect mechanism that involves GABA release inhibition and subsequent generation of modulatory H_2O_2. Local circuitry-mediated inhibition of striatal DA release may contribute to motor consequences of cannabinoid agonists.

PKC EPSILON NULL MICE SHOW ENHANCED BEHAVIORAL RESPONSES TO CANNABINOIDS

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Endocannabinoids and Δ9-tetrahydrocannabinol, the major psychoactive compound in marijuana, bind to and activate the cannabinoid CB1 receptor. The CB1 receptor is the most abundant G-protein coupled receptor in brain, with high levels of expression in the hippocampus, striatum, cortex and reward centers of the basal ganglia. Activation of CB1 leads to altered neurotransmitter release in several brain regions. Despite the receptor's abundance, little is known about mechanisms that modulate CB1 receptor signaling.

Here we show that protein kinase C epsilon (PKCε), a member of the novel subclass of PKC isozymes, mediates acute behavioral sensitivity to both endogenous and exogenous cannabinoids. In these studies, PKCε null mice showed enhanced analgesia, hypothermia and catalepsy and reduced spontaneous activity compared to wild-type littermates following CB1 agonist WIN55,212-2 administration. Additionally, PKCε null mice displayed significant hypothermia following administration of the endogenous cannabinoid anandamide, while their wild-type littermates did not. Furthermore, although PKCε null mice show enhanced sensitivity to acutely administered cannabinoids, tolerance development following chronic administration of cannabinoids was, depending on the measure, normal or increased.

Our findings suggest that PKCε is a novel regulator of CB1 receptor signaling and may provide a therapeutic target for the modulation of the endogenous cannabinoid system.

IN SILICO MODELING OF GPR55: CREATION OF AN INITIAL MODEL AND REFINEMENT BASED UPON SEQUENCE DIVERGENCES IN TMH6 AND TMH7

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Recently, the orphan receptor, GPR55,[1-3] has been reported to be a third cannabinoid receptor subtype.[4] GPR55 exhibits low sequence identity (10–15%) to cloned CB1 or CB2 receptors and has been reported to signal via G_{13}.[4] The amino acid sequence of GPR55 contains many of the highly conserved sequence motifs that are commonly used as alignment guides across Class A G protein-coupled receptors (GPCRs): N1.50 in TMH1, D2.50 in TMH2, W4.50 in TMH4, P5.50 in TMH5 and F7.60 in the intracellular extension of TMH7, Hx8. These later two motifs are not found in CB1/CB2. In addition, the E-2 loop of GPR55 is likely similar to that of rhodopsin, as GPR55 has a Cys at 3.25 and a Cys near the middle of the E-2 loop. In rhodopsin, corresponding Cys residues are linked by a disulfide bridge, a bridge that is not possible in CB1/CB2. However, GPR55 diverges from rhodopsin and CB1/CB2 in TMH3, TMH6 and TMH7. A minor divergence in TMH3 is the presence of DRF in GPR55 that aligns with the Class A (D/E)RY motif. More significant divergences are found in TMH6 and TMH7. The TMH6 CWXP motif found in rhodospin, CB1 and CB2 aligns with SFLP in GPR55 and the TMH7 NPXXY motif aligns with DVFCY in GPR55. Overall, the GPR55 sequence has sufficient homology with rhodopsin that the rhodopsin crystal structure[5] has been used by us as a template for the creation of an initial model of GPR55. However, because of the important sequence divergences in TMH6 and TMH7 noted here, it is very important to understand the conformational requirements dictated by the GPR55 sequence in these regions. For this reason, we have undertaken Monte Carlo/simulated annealing studies of TMH6 and TMH7 in GPR55 in order to identify appropriate helix conformations for incorporation in our rhodopsin-based model of GPR55. The results of these simulations will be presented. **Support:** DA03934 and DA00489 (PHR).

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CANNABINOID CB2 RECEPTOR CONSTITUTIVE ACTIVITY MAY BE DUE TO LACK OF AROMATICITY AT RESIDUE 6.44

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It has been proposed that G protein-coupled receptor (GPCR) activation is characterized by Trp 6.48 χ1 g+ $\rightarrow$ trans rotamer shift. Trp 6.48 in the Class A GPCR, rhodopsin, is flanked by aromatic residues, F6.44 and Y6.51 that restrict the mobility of the helix favoring Trp 6.48 χ1 g+. Residue 6.44 is a locus at which mutation to certain non-aromatic residues in several receptors has led to constitutive activity. Han and coworkers(Han et al., 1996) reported that a F6.44(261)V mutation in Rho led to measurable constitutive activity. Chen and coworkers reported that a F6.44(303)L mutation in the alpha-1B-adrenergic receptor(Chen et al., 1999) and a F6.44(282)L mutation in the beta-2-adrenergic receptor (Chen et al., 2002) led to constitutively activated receptors, as well. In the CB2 receptor, residue 6.44 is a Leu. In work to be presented, we test the hypothesis that the high constitutive activity of the CB2 receptor is in part due to the lack of an aromatic residue at position 6.44. Monte Carlo/simulated annealing (Conformational Memories) calculations of WT CB2 TMH6 and L6.44F TMH6 revealed that a Phe at 6.44 tends to promote the W6.48 g+ (inactive state) rotamer. To test this result experimentally, a L6.44F mutant CB2 receptor was stably transfected into the HEK293 cells and the effects of this mutation were examined with Western blot, ligand binding, and cAMP accumulation assays. Consistent with our working hypothesis, the CB2 L6.44F mutant was found to lose constitutive activity. **Acknowledgements:** This study was supported by NIH grants DA03934 and DA00489 (PHR); DA11551 and EY13632 (ZHS).

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IP-ONE: A NEW HTRF® ASSAY TO MONITOR Gq-COUPLED GPCR RESPONSE

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GPCRs (G- Protein Coupled Receptors) are currently the most important target class to be investigated in the drug discovery process. Upon activation, GPCRs are carrying the information within the cell via two major signalling pathways: one results in variations of the cyclic AMP (cAMP) level whereas the other one results in a transient increase of intracellular Ca2+ triggered by inositol (1,4,5) triphos-phate (IP3). Around 40% of the GPCRs, coupled to the Gq subtype of G proteins, are linked to the IP3/Ca^{2+} pathway.

Even if Ca^{2+} response is quite distal from the initial event occurring at the GPCR level, it is universally used as a tool to probe GPCR activation in a HTS environment. In the last years, many efforts have been dedicated to identify new ways to monitor Gq coupled GPCR by detecting upstream signalling events. Particularly, IP3 production from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by PLC seems to be the best indicator of GPCR activation. However, IP3 is rapidly degraded in the cell (less than 1 min) leading to the accumulation of various inositides in the cytosol. For decades, a well established, low throughput radioactive method was used to quantify the total amount of inositol phosphates produced. In the last years, efforts to directly monitor IP3 levels in a high throughput, non-radioactive format successfully lead to the development of new assay kits. Nevertheless, the transient accumulation of IP3 remain the main hurdle to quantify with both high accuracy and robustness the production of this second messenger.

The aim of the study was to set-up a new assay kit to monitor Gq coupled GPCR by detecting the major IP3 degradation product, inositol (1) phosphate (IP1), that can be accumulated in the cytosol. This assay based on HTRF technology is homogenous, non-radioactive, well adapted to HTS and to miniaturisation. Comparison with reference functional assays such as the radioactive method or Ca^{2+} monitoring showed a close correlation. Therefore, this assay appears to be a major alternative to these methods. Data will be presented on the assay format as well as its validation on various GPCR models, including either stable or transient cell lines, and chimeric GPCR constructs.

PURIFICATION AND CHARACTERIZATION OF RECOMBINANT NAPE-PLD EXPRESSED IN ESCHERICHIA COLI

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In animal tissues, *N*-acylethanolamines (NAEs) including the endocannabinoid anandamide are principally formed from their corresponding *N*-acylphosphatidylethanolamines (NAPEs) by a membrane-associated phospholipase D (NAPE-PLD). Our cDNA cloning revealed that the enzyme was a novel member of the metallo-ß-lactamase family and was structurally and functionally distinguishable from the known PLDs (Okamoto et al., *J. Biol. Chem.* 279, 5298, 2004). In an attempt to highly purify recombinant NAPE-PLD, we constructed cDNA of a fusion protein of rat NAPE-PLD and glutathione *S*-transferase (GST), and expressed it in *E. coli* together with a chaperone protein. This fusion protein was solubilized with CHAPS, and purified by glutathione affinity chromatography. The GST tag was cleaved by PreScission protease, and removed by the second cycle of glutathione chromatography. The NAPE-PLD protein was finally purified with hydroxyapatite chromatography. The purified enzyme preparation gave a single protein band around 46 kDa on SDS-PAGE, and showed a specific enzyme activity of about 2.0 μmol/min/mg protein with *N*-palmitoyl PE as substrate. The purified enzyme was markedly stimulated in a dose-dependent manner by millimolar concentrations of Ca^{2+}. However, this effect was fully replaced with Mg^{2+}. The enzyme showed high reactivity for various NAPEs with C4 or longer *N*-acyl chains, while the enzyme was much less active with *N*-acetyl PE and *N*-formyl PE. In addition, the purified enzyme was almost inactive with major glycerophospholipids existing in biomembranes (PE, phosphatidylcholine, phosphatidylinositol, and phosphatidylserine) in contrast with *Streptomyces chromofuscus* PLD showing wide substrate specificity. These results suggested that NAPE-PLD is constitutively active *in vivo* and degrades different NAPEs at similar rates without damaging other membrane phospholipids. We also performed site-directed mutagenesis studies on several histidine and aspartic acid residues of NAPE-PLD that are highly conserved within the metallo-β-lactamase family and presumed to be catalytically important. Single mutations of D147, H185, H187, D189, H190, H253, D284, and H321 caused abolishment or remarkable reduction of the catalytic activity, suggesting that NAPE-PLD functions through a mechanism similar to those of other members of this family. Further mutagenesis studies also suggested catalytic importance of L207, C224 and H380.

Ref. Wang, J., Okamoto, Y., Morishita, J., Tsuboi, K., Miyatake, A., and Ueda, N. *J. Biol. Chem.*, doi:10.1074/jbc.M512359200, 2006.

INHIBITION OF FATTY ACID AMIDE HYDROLASE BY ANALOGUES OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

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There is good evidence to show that non-steroidal anti-inflammatory drugs (NSAIDs) can interact with the endocannabinoid system. For example, the beneficial effect of indomethacin in the carrageenan model of inflammation in the mouse can be blocked by SR144528 (Holt et al., Br J Pharmacol 146 [2005] 467-76), and the antinociceptive effect of spinally administered indomethacin in the formalin test of inflammatory pain is blocked by AM251 and is not seen in $CB1^{-/-}$ mice (Gühring et al., Eur J Pharmacol 454 [2002] 153-163). Several NSAIDs (including ibuprofen and indomethacin) inhibit FAAH in a pH-dependent manner (see e.g. Holt et al., Br J Pharmacol 133 [2001] 513-20). As yet, it is not possible to say whether FAAH inhibition contributes to the in vivo effects of ibuprofen and indomethacin, but the findings themselves motivate a study of the structural requirements for inhibition of FAAH by NSAIDs.

Two series of compounds were investigated. In the first series, the ability of five indomethacin analogues to inhibit the hydrolysis of 2 µM AEA by rat brain FAAH was investigated. Indomethacin completely inhibited FAAH with IC_{50} values of 19 µM (pH 6.2) and 96 µM (pH 8.4). Indomethacin ester 4-methoxyphenyl, indomethacin ester, n-heptyl, indomethacin N-octyl amide, N-(2-phenylethyl)-indomethacin amide and N-(4-acetamidophenyl)-indomethacin amide showed little or no pH dependency, implicating the carboxyl hydroxy group of indomethacin in the generation of the pH sensitivity. The only compound showing a greater degree of inhibition of anandamide metabolism than indomethacin at a concentration of 10 µM was the 4-methoxyphenyl ester. This compound inhibited FAAH with IC_{50} values (with maximum inhibition attained in brackets) of 1.8 µM (48 %) and 3.3 µM (59 %) at pH 6.2 and 8.4, respectively. A similar result was seen for this compound using mouse brain homogenates and a pH of 7.0.

In the second series, eight ibuprofen heterocyclic amides were investigated using mouse brain homogenates and an assay pH of 7.0. Ibuprofen itself was rather weak, producing ~40 % FAAH inhibition at a concentration of 100 µM. Six of the ibuprofen heterocyclic amides were more potent than ibuprofen, and the most potent, the 6-methyl-pyridin-2-yl analogue, completely inhibited FAAH activity with an IC_{50} value of 8.3 µM. Using rat cerebellar homogenates and an assay pH of 7.2, the compound inhibited FAAH with an IC_{50} value of 2.7 µM. The corresponding value for ibuprofen was 133 µM. Interestingly, the 6-methyl-pyridin-2-yl analogue has been reported to be the most effective of these compounds in a test for analgesia, but shows a lower ulcerogenic activity than ibuprofen (Cocco et al., Eur J Med Chem 38 [2003] 513-8).

In conclusion, the present data would suggest that the NSAID molecules can be modified to increase their potency towards FAAH. The combination of cyclooxygenase and FAAH inhibition may be a useful therapeutic approach for the treatment of inflammatory pain.

3-NITRO-*N*-PENTADECYLBENZAMIDE IS A TEMPLATE FOR SELECTIVE INHIBITORS OF 2-ARACHIDONOYLGLYCEROL METABOLISM

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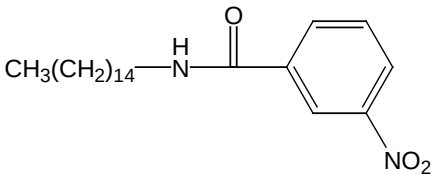
Introduction: Three enzymes responsible of the hydrolysis of endocannabinoids have been identified. Fatty acid amide hydrolase (FAAH), a monoacylglycerol lipase (MAGL) activity, and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) have all been cloned. In contrast to the situation for FAAH, only a few MAGL inhibitors are available, and it is unclear as to how many different enzymes capable of the hydrolysis of 2-AG and its homologue 2-mono-oleoylglycerol (2-OG) are present in the brain. There is a need of selective inhibitors of this enzyme, that should be useful as pharmacological tools as well as promising leads with therapeutic potential. Here we report that 3-nitro-*N*-pentadecylamide inhibits 2-OG metabolism by cytosolic fractions from rat brain.

Chemical structure of 3-Nitro-*N*-pentadecylbenzamide



Methods : MAGLcy and FAAH activities were assessed using, respectively, cytosolic and membrane rat cerebellar homogenates incubated with [^{3}H]-2-OG or [^{3}H]-anandamide. Membrane-bound MAGL activity (MAGLm) was also investigated using the membrane fractions co-incubated with URB597 in order to rule out hydrolysis of [^{3}H]-2-OG by FAAH. Rat lung homogenates were used as source of NAAA enzyme incubated with [^{14}C]-palmitoylethanolamide. Affinity for the cannabinoid receptors was determined using CHO-hCB_1 or CHO-hCB_2 transfected cell membranes and [^{3}H]-CP55,940 or [^{3}H]-WIN55,212-2, respectively as ligands. Metabolism of anandamide and 2-OG was also investigated in intact C6 glioma cells.

Results: 3-nitro-*N*-pentadecylamide (**1**) is devoid of affinity for the cannabinoid receptor but inhibits the hydrolysis of 2-OG by cytosolic fractions with with an IC_{50} value of 1.04 µM. This compound does not interfere with FAAH nor NAAA (41 and 14% inhibition at 10 µM, respectively) but inhibits 2-OG hydrolysis by membrane fractions (IC_{50} 4.3 µM). MAGLcy inhibition by **1** is time-dependent, showing maximal effect when the compound is pre-incubated 1 hour before the substrate. However, the inhibitory activity of **1** against FAAH remains very low even after 1 hour pre-incubation. In intact C6 glioma cells, **1** moderately inhibits anandamide and 2-OG metabolism, producing 50% inhibition at 100µM.

Conclusion: 3-nitro-*N*-pentadecylamide effectively inhibits 2-OG hydrolysis in vitro and in spite of its weaker activity on intact cells, it might be considered as a lead for the design of potent and selective MAGL inhibitors

PHARMACOLOGICAL CHARACTERIZATION OF THE INITIAL AND TIME-DEPENDENT UPTAKE OF ANANDAMIDE

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Evidence is emerging to suggest that the cellular uptake of anandamide (AEA) has both a rapid initial and a time-depenent phase, and that the pharmacological properties of these phases may be different (see e.g. Glaser *et al., Proc Natl Acad Sci* USA 100 [2003], 4269-74). In addition, there is data to suggest that the uptake of AEA may show different properties in different cells (see e.g. Hillard & Jarrahian, *Neuropharmacology* 48 [2005] 1072-8. In order to investigate this further, we investigated the initial and the time dependent uptake of [^{3}H]AEA in four different cell types: C6 glioma, ND7/23 neuroblastoma x dorsal root ganglion cells, P19 embryonic carcinoma and RBL2H3 basophilic leukaemia cells.

Experiments were conducted in the presence of 15 µM fatty acid-free BSA and a low (100 nM) added concentration of [^{3}H]AEA. Initial rates of uptake were defined as those seen using a short (~1 min) [^{3}H]AEA incubation time. Time dependent rates were calculated from slope replots of incubations at ~1, ~4, ~7 and ~10 min. Inhibitors (1 µM URB597 and 30 µM AM404) were dissolved in ethanol and preincubated for 15 min prior to addition of [^{3}H]AEA.

AM404 appeared to reduce the initial rates of [^{3}H]AEA uptake in all cell lines, but a similar effect was seen for the wells alone. URB597, on the other hand, did not affect the binding to the wells, but significantly reduced the initial uptake of [^{3}H]AEA into wells seeded with C6 or RBL2H3 cells, but not with ND7/23 or P19 cells. Inhibition of uptake into ND7/23 or P19 cells was seen at longer [^{3}H]AEA incubation times. The initial uptake into C6 and P19 cells was reduced by pretreatment with methyl-ß-cyclodextrin, which depletes cholesterol. The combination of methyl-ß-cyclodextrin and URB597 treatments did not produce a greater inhibition of uptake into the C6 cells than seen with methyl-ß-cyclodextrin alone.

The time-dependent uptake of [^{3}H]AEA was very low in the absence of cells, thus allowing measurement of rates to be made without confounding data due to binding to plasticware. Although as a caveat it should be pointed out that the time-dependent (but not initial) uptake was sensitive to the vehicle used, the time-dependent uptake into C6 cells was reduced by AM404 relative to vehicle-treated controls, but not by URB597. The time-dependent uptake into P19 cells was found to be sensitive to both URB597 and AM404.

The current data are consistent with a model whereby AEA rapidly translocates the plasma membrane by a process driven by FAAH and dependent upon the presence of membrane cholesterol. The intracellular AEA is thereafter redistributed in a time-dependent manner that in C6 cells is sensitive to AM404 but not URB597.

ENDOVANILLOID DEGRADATION: COMPARATIVE ANALYSIS OF FAAH, COMT AND TRPV1 EXPRESSION IN THE MOUSE BRAIN

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Anandamide (AEA) and *N*-arachidonoyl-dopamine (NADA), have been proposed to activate both cannabinoid CB_1 and vanilloid TRPV1 receptors (Di Marzo et al., 2002) and hence to act as both endocannabinoids and endovanilloids. NADA occurs in nervous tissues, with the highest concentrations being found in the hippocampus and cerebellum (Huang et al., 2002). Whereas the main AEA degrading enzyme is fatty acid amide hydrolase (FAAH), one of the suggested inactivation pathways for NADA is the methylation of its cathechol moiety by cathechol-*O*-methyl-transferase (COMT) (Huang et al., 2002). The potential role for AEA and NADA as TRPV1 ligands has been investigated here by comparing the distribution of FAAH and TRPV1, and of COMT and TRPV1, in the mouse brain by means of immunohistochemistry.

We used commercially available specific anti-FAAH, anti-COMT and anti-TRPV1 antibodies and exploited the availability of the TRPV1 null mice (-/-), of a blocking peptide for the FAAH antibody and omission of the primary antibodies, as controls of staining specificity. Four anesthetized male mice were transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were removed, cryoprotected in sucrose and cut on a cryostat into 20 μm-thick frozen transversal sections. Double immunofluorescence was carried out on free floating sections incubated for 2 days in a mixture of goat anti-TRPV1 and either rabbit anti-FAAH or rabbit anti-COMT. Subsequently, the sections were incubated for 2 hours at room temperature in a mixture of secondary IgG antibodies including rabbit anti-goat Alexa 546 and goat anti-rabbit Alexa 488. For single ABC immunohistochemistry the same primary antibodies as for immunofluorescence were used followed by biotin-conjugated IgG secondary antibodies and avidin-biotin-peroxidase (ABC, Vector).

Both methods used point to the same results. FAAH and TRPV1 co-expression was detected in the hippocampus and cerebellum of the mouse brain. Consistent with previous analyses (Egertova et al., 2003), FAAH-immunoreactivity was found in the somata and dendrites of hyppocampal pyramidal cells and in the somata of Purkinje cells and granular cells. In the hippocampus, high FAAH and TRPV1 co-expression was detected in the principal neurons of the Ammon's horn, whereas in the cerebellum high co-expression of FAAH and TRPV1 was found in the Purkinje cell bodies. Among other areas, COMT and TRPV1 co-expression was also detected in the hippocampus and cerebellum. COMT-ir was found in the somata and dendrites of hippocampal pyramidal cells, in the somata and initial axon segments of Purkinje cells and in basket cells of the cerebellar molecular layer. In the hippocampus, COMT and TRPV1 co-expression was detected in the principal neurons of the Ammon's horn, whereas in the cerebellum co-expression of COMT and TRPV1 was found in Purkinje cell bodies and initial axon segments. The finding, in the mouse hippocampus and cerebellum, of a similar expression pattern of FAAH or COMT and TRPV1 supports the concept that AEA and other FAAH-substrates, such as oleoylethanolamide and linoleoyl-ethanolamide, act as endogenous ligands of TRPV1 receptors (Di Marzo et al., 2002; Ross, 2003; Movahed et al., 2005), and that COMT plays a role in the inactivation of NADA in rodents (Huang et al., 2002).

PHARMACOLOGICAL CHARACTERIZATION OF ANANDAMIDE UPTAKE AND FAAH INHIBITORS

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Although the exact mechanism of endocannabinoid uptake and disposal remains unknown, several compounds have been reported to selectively inhibit anandamide uptake into the cell, anandamide degradation by fatty acid amide hydrolase (FAAH), or both processes simultaneously. It is critical that the extent of selectivity is fully understood to make conclusions about the specific mechanisms involved in endocannabinoid uptake and degradation. A previous report comparing several anandamide uptake inhibitors concluded that none of the reported inhibitors were entirely selective and results were dependent upon cell type and assay conditions (Fowler *et al.*, 2004). Since this published comparison, there have been reports of more potent compounds, as well as a binding site that is independent of FAAH and involved in endocannabinoid transport. Here, we present a full comparison of a subset of compounds (AM404, AM1172, LY2077855, LY2183240, OMDM-1, OMDM-2, a Sepracor compound, UCM707, URB597, and VDM11) in assays measuring 1) functional [^{14}C]-anandamide uptake in cells containing (RBL-2H3) and cells lacking (HeLa) FAAH, 2) hydrolytic activity of purified rat ΔTM-FAAH, and 3) displacement of [^{3}H]-LY2183240 binding in RBL-2H3 and HeLa cell membranes. With the exception of OMDM-1, OMDM-2 and AM1172, all compounds inhibited 1) anandamide uptake in both RBL-2H3 and HeLa cells, 2) hydrolytic activity of rat ΔTM-FAAH, and 3) binding of [^{3}H]-LY2183240 to RBL-2H3 and HeLa cell membranes. General rank order potency of the compounds was maintained across the three assays. Only three compounds were found to have inhibition constants in low- or sub-nanomolar concentrations: LY2077855, LY2183240, and URB597. However, concentration response curves for these compounds were significantly right-shifted for anandamide uptake in HeLa cells compared to RBL-2H3. In addition, total levels of anandamide uptake were significantly lower in HeLa cells compared to RBL-2H3 cells. In conclusion, the present study allows for a more direct comparison of these compounds and the binding assay provides a better understanding of the mechanisms involved in anandamide uptake and degradation. Although it is apparent that at least two proteins are involved in the uptake of anandamide, FAAH is required to maintain a concentration gradient and may have a direct interaction with the putative transport protein to maintain a functional high affinity binding state.

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THE HYDROLYSIS OF 2-AG IN RAT CEREBELLAR MEMBRANES IS NOT INHIBITED BY A POTENT MONOGLYCERIDE LIPASE INHIBITOR URB754

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2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid that binds to CB1 and CB2 cannabinoid receptors, inducing cannabimimetic effects. However, *in vivo* effects of 2-AG are weak due to its rapid enzymatic hydrolysis. The main enzyme responsible for 2-AG hydrolysis has been reported to be a monoglyceride lipase (MGL), which is inhibited by serine hydrolase inhibitors such as MAFP (methyl arachidonylfluorophosphonate) and HDSF (hexadecylsulphonyl fluoride). Additionally, previous studies have demonstrated that MGL is sensitive to inhibition by sulfhydryl-specific reagents like *p*CMB (*p*-chloromercuribenzoic acid) and NEM (*N*-ethylmaleimide). We have earlier showed that MGL-like activity in rat cerebellar membranes is inhibited by MAFP and HDSF (Saario et al., 2004). Additionally, *N*-arachidonylmaleimide (NAM), which is a maleimide compound and 2-AG analog, inhibits MGL-like activity with an IC_{50} value of 140 nM (Saario et al., 2005). Recently it was reported that recombinant rat brain MGL is inhibited by URB754 with an IC_{50} value of 200 nM (Makara et al., 2005).

In this study, inhibition of 2-AG hydrolysis by URB754 was investigated in rat cerebellar membranes and brain homogenates. Inhibition activity of URB754 was determined by the production of arachidonic acid, the hydrolysis product of 2-AG, as measured by HPLC.

As a result, the hydrolysis of exogenously added 2-AG ($5x10^{-5}$ M) by rat cerebellar membranes was not inhibited by URB754 (100 µM) at all. Moreover, only approximately 10 % of 2-AG hydrolysing enzyme activity in brain homogenates was inhibited by URB754 (100 µM).

In conclusion, as URB754 did not inhibit enzymatic degradation of 2-AG in rat cerebellar membranes, we suggest that the previously characterized MGL-like activity in rat cerebellar membranes is different from the URB754-sensitive recombinant rat brain MGL activity (Makara et al., 2005). Further, since URB754 inhibited 2-AG hydrolysis only by 10 % in native rat brain preparations, our data suggest that the majority of brain's 2-AG degrading enzymatic capacity is due to MGL-like enzyme(s) distinct from the molecularly identified MGL.

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ALL TRANS ANADAMIDE SYNTHESIS AND ITS EFFECTS ON PLATELET FUNCTIONS. INTERFERENCE WITH CIS-ANANDAMIDE HYDROLYSIS BY FAAH

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It has been shown that mammalian cells, including platelets, may contain some polyunsaturated fatty acid geometrical isomers. In recent years, the role of the lipid naturally occurring cis-geometry has attracted attention, in particular for the cis-trans geometry isomerization catalyzed by free radical species. Previous studies showed that these isomers can compete in vitro with arachidonic acid metabolism and alter aggregation. In a recent study from our laboratory [*Anagnostopoulos D., Chatgilialoglu C., Ferreri C., Samadi A. and Siafaka-Kapadai A. Bioorg.Med.Chem.Lett. 15 2766-70 (2005)*] we synthesized all-trans arachidonic acid and studied its effects on rabbit platelet aggregation in comparison with the respective cis-isomer. All-trans arachidonic acid was able to induce platelet aggregation and alter the aggregation induced by strong platelet agonists. In the present study we used all-trans arachidonic acid methylester, formed by thiyl radical-based isomerization, as intermediate for the synthesis of all-trans anandamide, in a straightforward procedure. The structure of the synthesized all-trans anandamide was verified by NMR. We then investigated its effects on platelet aggregation and found that all-trans anandamide was able to induce platelet aggregation in a concentration dependent manner and interfere with aggregation induced by strong platelet agonists, such as PAF and thrombin. Its effects on platelet serotonin release was also investigated. Furthermore, we found that all-trans anandamide can compete with cis anandamide metabolism hydrolysis by FAAH. In conclusion all-trans anandamide is a very interesting molecule and further studies such as binding to CB receptors, uptake and transfer through the cell membrane are required and are currently under investigation. These studies might provide further insights on the role and mechanism of action of its cis counterpart as well.

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CELLULAR ACTIVITY AND LOCALIZATION OF FAAH-GFP FUSION PROTEINS

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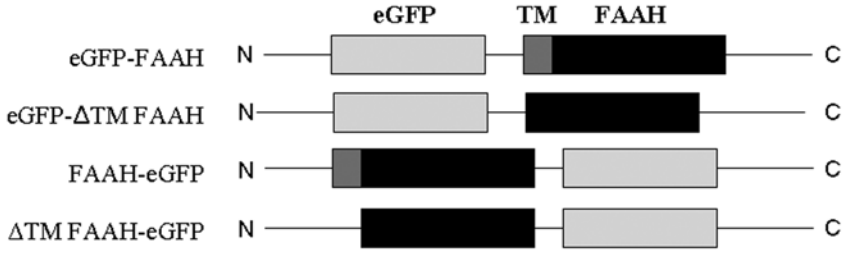
Fatty acid amide hydrolase (FAAH) is a membrane-associated enzyme involved in endocannabinoid inactivation. The signaling capacity of the endocannabinoid anandamide (AEA) is regulated by its rapid uptake, and metabolism by FAAH. The mechanism(s) of AEA membrane transport is currently debated (1) and it is not known how AEA is intracellularly transported to FAAH for hydrolysis. Through its hydrolysis of AEA, FAAH maintains an inward concentration gradient that promotes AEA uptake in the steady-state. FAAH is localized primarily on the endoplasmic reticulum (ER) in cultured cells, with minor localization reported in the mitochondria and the plasma membrane. FAAH contains an N-terminal transmembrane helix (residues 1-29) which aids its dimerization. However, removal of this region does not appreciably affect FAAH activity (2).

To examine FAAH activity and dynamics, several constructs of FAAH fused to green fluorescent protein (GFP) were prepared and analyzed following transfection into COS-7 cells. Both WT FAAH and transmembrane-deleted (ΔTM) FAAH were fused to GFP through either their N- or C-termini (see Figure).



All of the fusion proteins were successfully expressed in COS-7 cells. The presence of the various FAAH-GFP proteins augmented AEA uptake to levels indistinguishable from untagged FAAH, suggesting that GFP does not adversely affect FAAH activity nor its ability to recruit AEA for hydrolysis. The localization of the FAAH-GFP fusion proteins was also examined. To investigate whether the N-terminal transmembrane helix may play a role in restricting the cellular localization of FAAH, the ΔTM-FAAH fusion proteins were compared to WT-FAAH and other constructs. The possible localization and activity of FAAH in the mitochondria will also be discussed.

Acknowledgements: NIH grants DA9374 and DA16419

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FATTY ACYL AMIDES OF ENDOGENOUS TETRAHYDROISOQUINOLINES ARE TRPV1 ANATAGONISTS

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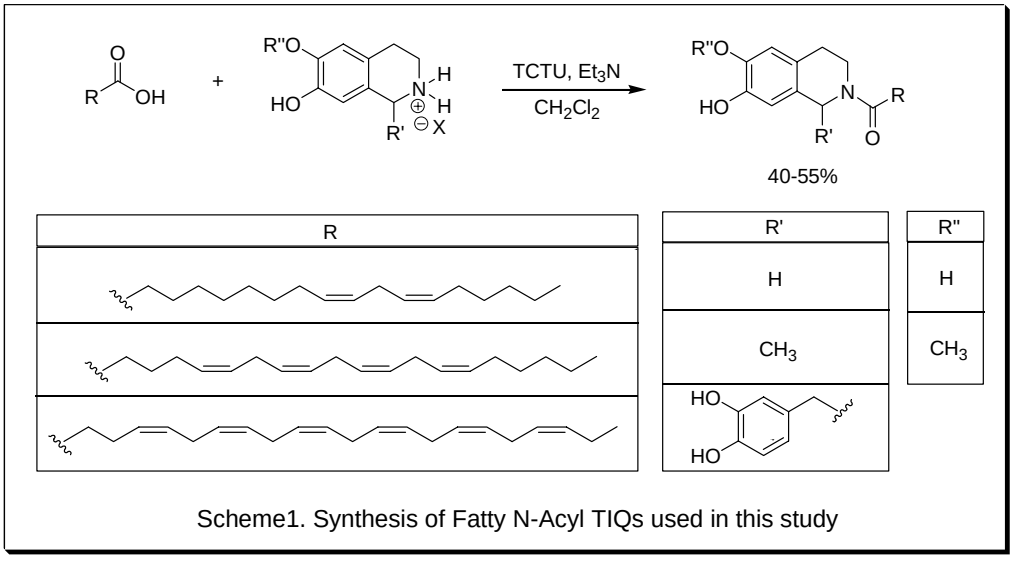
As part of the ongoing effort in our laboratory to identify new bioactive lipids, we have been synthesizing putatively endogenous amides and subsequently looking for ESI-MS evidence for their existence in tissue homogenates. Previous research on the structure activity relationship of capsazepine at TRPV1 channels reveals that the tetrahydroisoquinoline (TIQ) moiety is a core pharmacophore of TRPV1 receptor antagonism. Tetrahydroisoquinolines derived from Pictet-Spengler reactions of dopamine with aldehydes are well known endogenous alkaloids, and include salsolinol, norsalsolinol, isosalsoline, and tetrahydropapaveroline. We hypothesized that conjugation of TIQs with fatty acids would produce antagonists of TRPV1 receptors. Utilizing standard peptide coupling conditions (TCTU, Et_3N, CH_2Cl_2), salsolinol, norsalsolinol, isosalsoline, and tetrahydropapaveroline were conjugated to fatty acids to give the amides in moderate yield (Scheme 1). These compounds, which exist as mixtures of interconvertable rotamers, were tested for calcium mobilization in HEK293 cells stably transfected with TRPV1 receptors and for antagonism of the canonical TRPV1 agonist capsaicin. The IC_{50} values for these compounds were in the high nM to low μM range. Dose-response studies on N-arachidonoyl salsolinol indicate that capsaicin antagonism occurs in a non-competitive manner.



Scheme1. Synthesis of Fatty N-Acyl TIQs used in this study

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FURTHER CHARACTERISATION OF A FLUORESCENCE-BASED ASSAY FOR FAAH ACTIVITY

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Fatty acid amide hydrolase (FAAH) is the principal endocannabinoid hydrolysing enzyme. It provides an alternative therapeutic route for manipulation of the cannabinoid system. In order to exploit this pathway, however, it is necessary to establish assays which allow rapid screening of potential modulators. Here, we describe the further characterisation of a fluorescence-based assay of FAAH, assessing the impact of alternative substrates, solvents, detergents and bovine serum albumen.

Rat liver microsomes were prepared as previously described (De Bank *et al.* 2005) and stored at -80°C until required. FAAH activity was assessed by determination of the ammonia evolved from primary amide hydrolysis using OPA derivatization (Alexander *et al.* this meeting).

Comparing alternative primary amides as substrates for FAAH activity, oleamide appeared a better substrate than it's trans isomer elaidamide, the saturated analogue stearamide or the longer chain monounsaturated analogue erucamide. Thus, hydrolysis rates at 100 µM were 3.37 ± 0.54; 1.42 ± 0.73; 1.36 ± 0.79 and 0.37 ± 0.26 nmol/min/mg protein, respectively.

The solvent dimethylsulfoxide was well tolerated in the assay, with no change in FAAH oleamide hydrolysis activity at concentrations up to 5 % (v/v). In contrast, the inclusion of detergents in the assay evoked concentration-dependent inhibitions of activity: sodium deoxycholate (0.05 % w/v, 79 ± 15; 0.1 % 21 ± 4 % control); Triton X-100 (0.05 %, 74 ± 10; 0.1 % 60 ± 7 % control) and SDS (0.05 %, 2.2 ± 0.2; 0.1 % 1.2 ± 0 % control). Thus, the non-ionic example was better tolerated than the charged detergents.

Addition of fatty acid-free BSA to the incubation evoked a concentration-dependent enhancement of FAAH activity (0.01 % w/v 114 ± 2; 0.05 % 125 ± 3; 0.1 % 132 ± 2 % control). It is possible that BSA acts simply as a protein stabiliser or, potentially, as a 'carrier' for the substrate/product.

Taken together, these results suggest a combination of conditions (oleamide as substrate, DMSO as solvent, low concentrations of Triton X-100 and BSA up to 0.1 %) which should facilitate the screening of novel chemical entities as modulators of FAAH.

De Bank *et al.* (2005) Biochem Pharmacol 69: 1187-1193

A NOVEL FLUORESCENCE-BASED ASSAY FOR FAAH ACTIVITY

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Fatty acid amide hydrolase (FAAH) is a major catabolic enzyme for regulation of endocannabinoid levels, which shows promise as a potential novel therapeutic target. In this report, we describe a simple, inexpensive assay for FAAH activity which makes use of fluorescence derivatization of the ammonia evolved from oleamide hydrolysis.

Microsomes were prepared as previously described (De Bank *et al.* 2005) and stored at -80°C until required. FAAH activity was assessed by determination of the ammonia evolved from oleamide hydrolysis using o-phthaldehyde derivatization in the presence of hydrosulfite ions, monitoring the fluorescent isoindole-1-sulfonate product after 30 min (Mana and Spohn, 2001).

The assay was used to monitor FAAH activity in preparations from rat liver and brain where V_{max} values of 15 and 1 nmol/min/mg protein, respectively, were observed. K_m values were similar (129 and 179 μM) in the two tissues. Oleamide hydrolysis was sensitive to the selective FAAH inhibitor URB597, but not CCP (*N*-cyclohexanecarbonylpentadecylamine), an inhibitor of *N*-acylethanolamine acid amidase activity. Analysis of increasing concentrations of URB597 and CCP allowed calculation of pIC_{50} values of 6.6 ± 0.1 and <5.0, respectively. URB597 inhibition of FAAH activity appeared to be non-competitive and time-dependent. Investigation of the effects of a series of organophosphates revealed a rank order of potency of chlorpyrifos > azamethiphos = malaoxon > diazinon > chlorfenvinphos > DFP, with pIC_{50} values ranging from 6.5 to 4.0.

In summary, therefore, we describe here a fluorescence-based FAAH assay with potential application for high throughput screening.

De Bank, P.A., Kendall, D.A., & Alexander, S.P.H. (2005). *Biochem. Pharmacol.*, 69, 1187 - 1193.
Mana, H. & Spohn, U. (2001). *Anal. Chem.*, 73, 3187 - 3192.

DEOXY BICYCLIC COMPOUNDS 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 and 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 cyclohexenone. The 1-deoxy-CP-47,497 analogs were obtained by stereoselective reduction of the carbonyl group to form a series of compounds for both epimeric alcohols. 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 (Thompson, A.L.S.; *et. al. Synthesis* 2005, *4*, 547).

3-[4-(1,1-Dimethylalkyl)phenyl]-4-(3-hydroxypropyl)-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). The structure and conformation of *cis*-3-[4-(1,1-dimethylethyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol has been confirmed by X-ray crystallography.

The affinities at both receptors increased with increased chain length (up to 1,1-dimethylheptyl or 1,1-dimethyloctyl) with the CB_2 receptor affinities being higher than those at CB_1, but still very weak. The syntheses 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.

EFFECTS OF HALOGEN SUBSTITUENTS UPON CB_1 AND CB_2 RECEPTOR AFFINITIES IN THE 1-ALKYL-3-(1-NAPTHOYL)INDOLE SERIES

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The structure of THC, as determined in 1964 by Gaoni and Mechoulam, has a dibenzopyran nucleus. This led to the development of structure-activity relationships (SAR) based upon this skeleton. Pfizer extended these SAR to their non-traditional cannabinoids, which do not contain the dibenzopyran nucleus. In the late 1980's it was found by a group at Sterling-Winthrop that pravadoline, an indole based non-steroidal anti-inflammatory agent inhibited contractions of the mouse vas deferens. It was subsequently determined that this was caused by interaction of the compound with the CB_1 receptor. This compound and other structurally related aminoalkylindoles have since been shown to exhibit typical cannabinoid pharmacology *in vivo* and *in vitro.*

Many of these indole derivatives have high affinity for both the CB_1 and CB_2 receptors. 1-Pentyl-2-methyl-3-(1-naphthoyl)indole (JWH-007) has $K_i = 9.5 \pm 4.5$ at CB_1 and $K_i = 2.9 \pm 2.6$ at CB_2. The propyl analog (JWH-015) has good affinity for the CB_2 receptor with $K_i = 13.8 \pm 4.6$ while it has weak affinity for CB_1 with $K_i = 164 \pm 22$. (Huffman, J.W.; *et.al. Bioorg. Med. Chem.* 2005**,** *13,* 89).. Substituents on the naphthyl ring of these indoles causes variation in the affinities for both the CB_1 and CB_2 receptors. To investigate the effect of halogen substituents upon receptor affinity the synthesis of a series of indole derivatives with halogens as substituents in the C-4 and C-8 positions of the naphthyl ring was carried out

The CB_1 and CB_2 receptor affinities for the *N*-alkyl-3-(4-halo-1-napthoyl)indoles, *N*-alkyl-3-(8-halo-1-napthoyl)indoles and their 2-methyl analogs will be discussed (*N*= C_3H_7, C_5H_{11}; X= Br, Cl, I, F) . In this series of compounds the CB_1 receptor affinities of the *N*-pentyl-4-halo compounds are greater than those of the *N*-propyl analogs (K_i = 1.2 nM to 14 nM) while the *N*-pentyl-8-halo series has little to no affinity in comparison (K_i = 73 nM to 522 nM). Data for the *N*-pentyl series shows a greater affinity for the CB_2 receptor relative to the *N*-propyl series, with K_i values ranging from 1.1 nM to 13.1 nM. JWH-416 and JWH-417, the 8-iodo-*N*-pentyl compound and its 2-methyl analog have 22-fold and 40-fold selectivity, respectively, for the CB_2 receptor. The *N*-propyl series has much greater affinity for the CB_2 receptor (K_i = 22 nM to 38 nM in the 4-halo series) relative to the CB_1 receptor (K_i = 93 nM to 530 nM).

The synthesis of these compounds as well as further SAR and GTPγS data, for selected compounds, will be discussed.

DIMETHYLHEPTYL DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL (2-AG) AND 2-ARACHIDONYL GLYCERYL ETHER (2-AGE): SYNTHESIS AND CB1 RECEPTOR ACTIVITY

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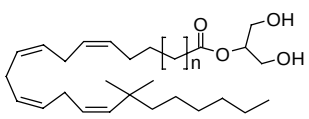
Introduction

Dimethylheptyl (DMH) analogues of two endocannabinoids, 2-arachidonoyl glycerol (2-AG) and 2-arachidonyl glyceryl ether (2-AGE) were synthesized and their ability to activate the CB1 receptors was determined by the [^{35}S]GTP$_\gamma$S binding assay using rat cerebellar membranes. The main goal of the study was to examine how the DMH end tail affects the activity properties of 2-AG (**1**) and its stable ether (**2)** and urea analogues (**5)**. The importance of the chain length was also explored by synthesizing 2-AG and 2-AGE derivatives (**3**, **4**) possessing the chain length C_{21} instead of C_{22}.



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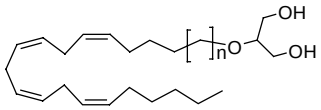
3 n= 0



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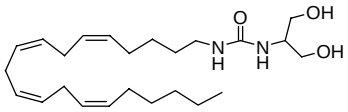
4 n= 0



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Results

Modification of a pentyl tail of 2-AG led to a dramatic potency decrease while the impact on the efficacy was much weaker. Replacement of the pentyl chain of 2-AGE resulted in similar loss of potency, whereas the efficacy remained comparable to 2-AGE. Shortening of the chain length from C_{22} to C_{21} did not improve the potency values but, interestingly, led to agonists with increased efficacy. Introducing a more hydrophilic and stable urea bond produced only weak agonistic activity.

Conclusion

Based on these results, we conclude, that unlike arachidonoyl ethanol amide -type compounds and classical cannabinoids, the activity properties of 2-AG and 2-AGE, cannot be improved by the replacement of the end pentyl chain with the DMH structure.

1-ALKYL-3-KETO-INDOLES: IDENTIFICATION AND IN VITRO CHARACTERIZATION OF A SERIES OF POTENT CANNABINOID LIGANDS

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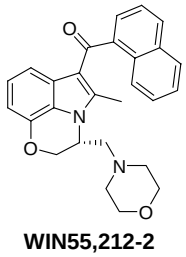
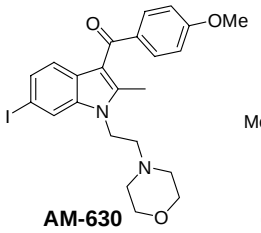
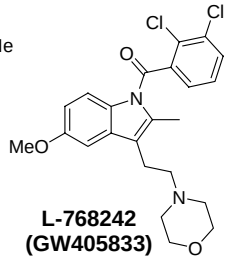
The indole core has been commonly incorporated into the design and synthesis of numerous cannabinoid ligands since Sterling disclosed results from SAR investigations on the anti-inflammatory agent pravadoline 15 years ago (Bell *et al*., J. Med. Chem., 1991, *34*, 1099). The aminoalkylindole (R)-(+)-WIN55212-2 emerged from these efforts (D'Ambra *et al., J. Med. Chem.*, 1992, *35*, 124), and this potent but only marginally selective agonist has been extensively utilized to characterize the pharmacological actions mediated by both CB_1 and CB_2 receptors. Indole analogs with increased levels of selectivity for the CB_2 receptor have since been identified and are exemplified by the antagonist AM-630 (6-iodopravadoline) (Ross *et al., Brit. J. Pharmacol*., 1999, *126*, 665) in addition to the CB_2-selective agonists L768242 (GW405833) (Gallant, *et al*., *Bioorg. Med. Chem. Lett.*, 1996, *6*, 2263), JWH-015 (Huffman, *et al*., *Bioorg. Med. Chem. Lett*., 1994, *4*, 563), and AM-1241 (Malan *et al.*, Pain, 2001, *93*, 239). The CB_2-selective agonists L768242 (Valenzano *et al., Neuropharm*., 2005, *48*, 658) and AM-1241 (Ibrahim *et al*., *PNAS*, 2003, *100*, 10529) exhibit robust efficacy in several preclinical models of inflammatory and neuropathic pain, thus providing support for the concept of utilizing CB_2 agonists as therapeutically effective analgesic agents.



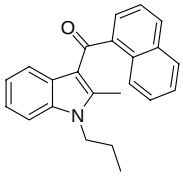
WIN55,212-2



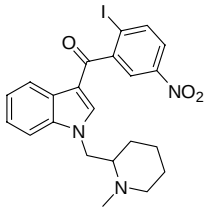
AM-630



L-768242 (GW405833)



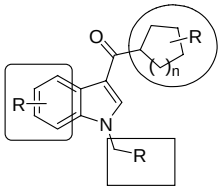
JWH-015



AM-1241

The indole scaffold was exploited in attempts to design novel potent, highly selective CB2 agonists. Indole analogs possessing a variety of pendant nitrogen sidechains and saturated cyclic ketones as the C(3)-acyl substituent were synthesized and evaluated. Potency was assessed by radioligand binding assays performed in cell lines that express recombinant human CB2 or CB1 receptors. Functional efficacy at the CB2 receptor was measured in a calcium flux (FLIPR) assay. Numerous agonists were identified that possess the combination of potent CB2 activity and high selectivity versus the CB1 receptor. The emerging SAR trends from this compound series will be described.

Ring Substitution
hydroxy, methoxy, benzyl-oxy, halo, alkyl



C(3)-Acyl Substituent
saturated cyclic ketones

Indole N(1)-substituent
alcohols, ethers, cyclic ethers, amines, sulfonamides, heterocycles

IN VITRO AND IN VIVO PHARMACOLOGY OF SYNTHETIC OLIVETOL-DERIVED CANNABINOID RECEPTOR LIGANDS

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[1]Endocannabinoid Research Group, Institute of Biomolecular Chemistry C.N.R. Pozzuoli (Naples), Italy; [2]Department of Experimental Medicine – Section of Pharmacology "L. Donatelli", Second University of Naples, Naples, Italy; [3]Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK; [4]Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via A. Moro 2, 53100 Siena (Italy). [*]Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA), Italy.

We have previously reported the development of several olivetol-derived compounds as ligands of cannabinoid receptors with metabolic stability and high affinity in receptor binding studies with human recombinant CB_1 and CB_2 receptors (Brizzi et al., J. Med. Chem., 2005). Of these compounds, CB-25 (K_i=5.2 and 13 nM, for CB_1 and CB_2, respectively) and CB-52 (K_i=210 nM and 30 nM, for CB_1 and CB_2, respectively) were selected here for further pharmacological characterization.

The functional activity of the two compounds at cannabinoid receptors was assessed by determining their capability to inhibit forskolin-induced cAMP formation in intact mouse neuroblastoma N18TG2 cells (which constitutively express CB_1 receptors) and in CHO cells over-expressing either human recombinant CB_1 or CB_2 receptors (denoted as CB_1-CHO and CB_2-CHO cells, respectively). Membranes from mouse brain or CB_2-CHO cells were used to assay CB_1- or CB_2-induced GTP-γ-S binding, respectively. The two compounds were also tested in rats in vivo on: 1) acute nociception, in healthy animals using the plantar test; and 2) formalin-induced nocifensive behaviour.

At mouse CB_1 receptors, CB-25 enhanced forskolin-induced cAMP formation in N18TG2 cells (EC_{50}~20 nM, max. stimulation~48%), thus behaving as an inverse agonist, but it stimulated GTP-γ-S binding to mouse brain membranes, thus behaving as a partial agonist (EC_{50}=100 nM, max. stimulation=48%). At human CB_1 receptors, CB-25 behaved again as a partial agonist as shown by its capability to inhibit cAMP formation in CB_1-CHO cells (EC_{50}=1200 nM, max. inhibition=68%). On the other hand, CB-25 showed little or no activity *per se* in all assays of CB_2-coupled functional activity and antagonized CP55940-induced stimulation of GTP-γ-S binding. Regarding CB-52, it always behaved as a partial agonist at both mouse and human CB_1 receptors, by inhibiting forskolin-induced cAMP formation by N18TG2 cells (EC_{50}~450 nM, max. inhibition~40%) and CB_1-CHO cells (EC_{50}=1400 nM, max. inhibition=62%), and by stimulating with low efficacy GTP-γ-S binding to mouse brain membranes (EC_{50}=11 nM, max. stimulation~16%). At CB_2 receptors, CB-52 exhibited no activity *per se* and antagonized CP55940-induced stimulation of GTP-γ-S binding. In vivo, both compounds, administered i.p., behaved like CB_1 inverse agonists by producing dose-dependent nociception in the plantar test carried out in healthy rats, and by antagonising the anti-nociceptive effect of i.p. WIN55,212-2. However, in the formalin test, the compounds counteracted both phases of formalin-induced nociception in a way antagonized by a CB_1 receptor antagonist, thus behaving as CB_1 partial agonists.

These findings indicate that CB-25 and CB-52 might be useful pharmacological tools as the first neutral CB_2 antagonists ever developed, and as potential analgesics for the treatment of non-acute pain.

STUDIES TOWARDS ENHANCED BINDING AND ACTIVITY OF NEUTRAL ANTAGONISTS FOR THE CB1 RECEPTOR

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RTI International, California Pacific Medical Center Research Institute[*], Medical College of Georgia[≠], College of Judea and Sumaria[§], University of North Carolina-Greensboro[π].

In earlier reports, analogs of SR141716 were designed, synthesized and tested to probe the role of the heteroatoms of the carboxyhydrizide moiety in binding to active and inactive conformations of the wild type and mutant CB1 receptors. These studies computationally and pharmacologically supported a receptor conformation that is stabilized upon binding of the inverse agonist SR141716. This work identified H-bond formation with K3.28 (192) in the stabilization of the inactive receptor conformation by SR141716 and neutral antagonism by analogs absent this H-bond capability. In the absence of H-bonding between receptor and ligand, there is a decrease of stabilization of the inactive state conformation and a decrease of a significant contribution to binding affinity. We have designed new analogs that are aimed at restoring affinity while maintaining neutral antagonism. The work also probes the role of structural modifications in providing additional binding affinity. We will present the syntheses, computational modeling to support the design of the compounds, binding and functional assay results and behavioral studies on these compounds.

DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW 1,8-NAPHTHYRIDIN-4(1H)-ON-3-CARBOXAMIDE AND QUINOLIN-4(1H)-ON-3-CARBOXAMIDE DERIVATIVES AS CB_2 SELECTIVE AGONISTS

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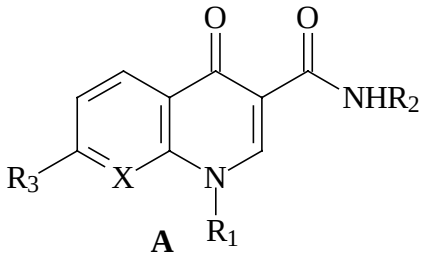
[a]Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 6, 56126 Pisa, Italy, [b]Dipartimento di Neuroscience B.B. Brodie, Università di Cagliari, Cittadella Universitaria, SS 554 Km 4.5, 09042 Monserrato, Italy, [c]Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G. Pieraccini 6, 50139, Firenze, Italy.

Interest in cannabinoid pharmacology has rapidly increased since the discovery of the endocannabinoid system (ECS), which includes cannabinoid receptors, the endocannabinoids, metabolizing enzymes, and a specific cellular uptake system. We have previously reported the binding results of a series of 1,8-naphthyridin-4(1H)-on-3-carboxamide derivatives (Ferrarini et al. 2004). These compounds generally exhibit a higher affinity for the CB_2 than for the CB_1 receptor, and for some of these compounds the K_iCB_1/K_iCB_2 ratio was higher than 20. Furthermore, we recently constructed the three-dimensional models of the CB_1 and CB_2 receptors by means of a molecular modeling procedure, and a series of CB_2 ligands were docked into both receptors, showing that the CB_2 model was reliable and predictive (Tuccinardi et al, 2006). In the present study, basing on the docking results, new 1,8-naphthyridin-4(1H)-on-3-carboxamide and quinolin-4(1H)-on-3-carboxamide derivatives of general structure **A** were designed, synthesized and tested for their affinities towards the cannabinoid CB_1 and CB_2 receptors. The binding results showed that the 1,8-naphthyiridine derivatives, which present arylalkyl and carboxycycloalkylamide substituents in position 1 and 3 respectively, generally possess a high CB_2 affinity, with a K_i value < 20 nM. Furthermore, the substitution of the naphthyridine-4-one nucleus with the quinoline-4-one system determined a general increase in CB_2 affinity, accompanied by a high selectivity for the CB_2 receptor (K_iCB_1/K_iCB_2 ratio > 210). Moreover the [^{35}S]GTPγ binding assay, and functional studies on human basophils indicated that the 1,8-naphthyridin-4(1H)-on-3-carboxamide derivatives behaved as CB_1 and CB_2 receptor agonists.



A

Ferrarini P. L.; Calderone, V.; Cavallini, T.; Manera, C.; Saccomanni, G.; Pani, L.; Ruiu, S.; Gessa, G.L. Bioorg. Med. Chem. 2004, 12, 1921–1933.

Tuccinardi, T.; Ferrarini, P. L.; Manera, C.; Ortore, G.; Saccomanni, G.; Martinelli, A. J. Med. Chem. 2006, 49, 984 -994.

PHARMACOLOGICAL PROFILE OF A NEW LIGAND FOR CB1 AND CB2 CANNABINOID RECEPTORS

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Emerging evidence implicates endocannabinoids in a wide variety of physiological and pathophysiological processes. To date, most cannabinoid drugs used therapeutically are derived from cannabis and produce their effects by activation of cannabinoid receptors. However, the psychoactivity of these compounds has prevented their widespread acceptance in clinical practice. Newly developed cannabinoids may hold the promise of the development of useful and safe drugs. This study aimed to investigate the pharmacological characteristics of a novel derivative of THC, DPG-4. It was evaluated for CB1/CB2 receptor affinity and activity using radioligand binding and functional studies. Specific behavioral and neurochemical indices were examined in order to assess cannabinoid activity. Behavioral paradigms such as open field test, bar test and intracranial self-stimulation were used with the aim to compare the profile of DPG-4 to that of WIN 55,212-2. Dopaminergic activity, in discrete rat brain regions such as the striatum nucleus accumbens and prefrontal cortex, was also examined following acute administration of DPG-4 and WIN 55,212-2. DPG-4, displayed low nanomolar affinities for CB1/CB2 receptors and increased the basal [^{35}S]GTPgS binding with an EC_{50} value similar to that found for WIN 55,212-2. Preliminary data using the aforementioned behavioral paradigms show a specific behavioral profile reminiscent of a CB1 agonist. The neurochemical findings further support the agonist nature of DPG-4 for the CB1 receptor. These data support that DPG-4 has high affinity and acts as an agonist for the CB1 receptor. Studies are in progress in order to elucidate the structure-activity relationship that might be useful in the development of substances with promising therapeutic value

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NOVEL ORALLY ACTIVE CB_2 RECEPTOR AGONISTS WITH POTENTIAL ANTI-HYPERALGESIC EFFECTS

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Introduction

Cannabinoid receptor agonists are being developed as potential analgesic and anti-hyperalgesic therapeutics. Sativex, a CB_1/CB_2 receptor agonist, is already launched in markets for the conditions of chronic and neuropathic pain. Here we present a series of orally available CB_2 receptor agonists with excellent pharmacological profile.

Methods

The CB_1 receptor characterization was done in hCB_1 transfected CHO cells & rat whole brain membrane. The CB_2 receptor binding was studied in hCB_2 transfected CHO cells and rat spleen membrane. The functional CB_1/CB_2 – mediated stimulation of GTPγS binding was carried out using the rat cerebellar membrane and inhibition of forskolin-induced stimulation of cAMP in CHO cells. The pharmacokinetic profiling was done in Sprague-Dawley (SD) rats. Efficacy of the compounds were characterized in FCA induced hyperalgesia and models of neuropathic pain in SD rats.

Results

One of the lead candidates in the series, GRC 10622 had a high affinity for CB_2 receptor with a K_i of 10.56 nM (hCB_2) and 3.36 nM (rat CB_2). GRC 10622 significantly inhibited forskolin-induced stimulation of cAMP in hCB_2 receptors with an EC_{50} of 0.6 nM compared to that of 857 nM on the hCB_1 receptor. In pharmacokinetic studies, GRC 10622 (10 mg/kg, p.o.) was found to be orally available with a C_{max} of 1029 ng/ml and a plasma half-life ($t_{1/2}$) greater than 6h in rats. GRC 10622 at 3 mg/kg p.o. produced >80% reversal of chronic constriction injury induced mechanical hyperalgesia and >50% inhibition of the FCA induced mechanical hyperalgesia.

Another molecule from this series, GRC 10514 had a high affinity for CB_1 receptor with a K_i of 19.2 nM (hCB_1) and 0.45 nM (rat CB_1). GRC 10514 also had a nanomolar affinity towards human and rat-spleen CB_2 receptors with K_i of 12.1 nM and 1.40 nM, respectively. GRC 10514 significantly stimulated [^{35}S]GTPγS binding with EC_{50} of 193 nM and showed full agonism in inhibition of forskolin-induced stimulation of cAMP. In pharmacokinetic studies, GRC 10514 (10 mg/kg, p.o.) was found to be orally available with a C_{max} of 433 ng/ml observed at 30 min with a $t_{1/2}$ of 3.0 h in rats. GRC 10514 (0.1, 0.3 & 1 mg/kg, p.o.) produced a significant dose dependent reversal of mechanical hyperalgesia in the ligation model of neuropathic pain.

Conclusion

The data suggests that these CB_2 agonists may be beneficial in chronic inflammatory as well as neuropathic pain.

BEHAVIORAL EFFECTS OF CB2 CANNABINOID RECEPTOR LIGANDS

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We have identified the presence and functional neuronal expression of the so called "peripheral" cannabinoid CB2 receptors in mammalian brain. We have also shown that direct CB2 antisense oligonucleotide administration into the brain modifies mouse behavior. We now describe the effects of the putative CB2 agonist, JWH015, the mixed CB1/CB2 agonist WIN55212-2 and CB2 antagonist SR144528 on mouse general activity, including, distance traveled, ambulatory activity and stereotype behavior. The performance of mice in the two compartment black and white box was also assessed following acute treatment with the agonist JWH015 and the antagonist SR144528. Acute treatment with CB2 agonist (JWH015) altered mouse spontaneous locomotor activities in a strain and gender dependent fashion. However, there was increase in activity by the males and a reduction in activity by the females of the DBA/2 strain. A general pattern of depression in locomotor activity was induced by JWH 015 in both males and females in the three mouse strains tested as the dose was increased. The effects of acute administration of JWH015 on stereotype behavior were similar to those described for locomotor activity. In the two compartment black and white box the acute effects of the putative CB2 agonist JWH015 at low doses (1-20 mg/kg) did not induce robust anxiolytic response rather this peripheral administration of JWH015 induced an anxiogenic profile of response in the black-white test box and the females of the C57BL/6 strain were more sensitive to the aversions in the white chamber. In contrast chronic treatment of control mice with JWH015 induced an anxiolytic profile of response in comparison to the chronic mild stress animals. The locomotor activities following the acute treatment with JWH015 by the female C57BL/6 mice in the white chamber was significantly reduced compared to the males without significant modification in the black chamber by both male and female C57BL/6 mice in comparison to vehicle treated controls. However, the total zone entries into both chambers were significantly reduced with increasing dose with the female mice more susceptible to the locomotor depressant effects of JWH015. Using the DBA/2 strain the spontaneous locomotor activity and stereotype behavior was enhanced by acute administration of low doses of SR144528, with the males more susceptible to the enhanced locomotor behavior than the female mice except at the 20 mg/kg dose when the male mice were also more sensitive to the locomotor depressant effects of CB2 cannabinoid receptor antagonism. SR144528 did not induce stereotype behavior in female mice at the doses used. In the two compartment black and white box test box treatment with SR144528 had little or no effect on the time mice spent in both chambers by male or female DBA/2 mice except a reduced time spent in the white chamber by the male mice at the highest dose used of 20 mg/kg. The spontaneous locomotor activities in both chambers by both DBA/2 male and female treated with SR144528 were also not significantly different from vehicle treated control mice at 20 mg/kg when the locomotor activity was significantly reduced in the white chamber in comparison to control animals. The transition between the chambers measured as total zone entries were also not significantly different from control mice, except at the lowest dose of 1 mg/kg of SR144528 when the transitions between the chambers was significantly enhanced in the male mice. These effects of CB2 cannabinoid receptor ligands in in vivo behavioral tests are provided as functional evidence of CB2 cannabinoid receptors in the brain that plays a role in motor function and emotionality tests.

CANNABINOIDS, SCHIZOPHRENIA AND GENDER

PRENATAL EVENTS MODULATE CB1R AGONIST-INDUCED IMPAIRMENT OF PRE-PULSE INHIBITION (PPI) DIFFERENTLY IN MALES AND FEMALES

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Rationale. Several lines of evidence suggest that prenatal stress exposure may be conducive to long-term neurobehavioral impairments in executive and attentional domains. Such sensorimotor alterations might be related to impaired gating functions, increasing vulnerability to develop schizophrenia. Marijuana exposure is thought to be associated with schizophrenia in humans, especially when consumed at a young age. In this study, we used a mouse model of sensorimotor gating to explore whether CB1 receptor system stimulation after prenatal stress (PS) results in behavioral changes consistent with psychopathological states like schizophrenia.

Objectives. To measure the effect of pre-pulse inhibition (PPI) of the acoustic startle reflex on PS offspring and/or CB1 receptor activation: a) whether the potent cannabinoid receptor agonist HU-210 decreased PPI as might be expected from such a psychotomimetic agent, through a well-validated in human and animals sensorimotor gating paradigm b) whether PS influences the effect of HU-210 on PPI c) whether male and female offspring were differently affected

Methods. Pregnant mice (Sabra strain) were subjected to a regimen of ultramild prenatal stress throughout gestation. The offspring at adulthood were assessed for PPI of the acoustic startle reflex, using standard methodologies, with or without an acute exposure the cannabinoid receptor agonist HU-210 (0.1 mg/kg i.p.).

Results.
1) PS significantly enhanced PPI in male, but had no effect on female offspring.
2) HU-210 significantly reduced PPI in male non PS offspring, which is compatible with similar studies in male rats. However, HU-210 did not affect PPI in females.
3) PS had a protective effect on the HU-210 induced disruption of PPI in males
4) In PS female offspring, HU-210 enhanced PPI compared to non-stressed control mice without HU-210 or to female mice exposed only to HU-210.

Conclusions. Ultramild prenatal stress has a “hardening” effect on the male offspring, preventing the schizophrenia-like effects seen after CB1 receptor activation. In contrast, females are not influenced by either PS or HU-210 alone but increase sensorimotor gating being exposed to both together. Thus early (prenatal) environmental experience as well as sex should be considered, when studying the role of the endocannabinoids CB1 receptor system in the expression of the schizophrenic symptoms.

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INVOLVEMENT OF 5-HT$_{1A}$ SEROTONERGIC RECEPTOR ON Δ^9-TETRAHYDROCANNABINOL (Δ^9-THC)-INDUCED EMOTIONAL RESPONSE IN RATS

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The major psychoactive constituent of cannabis, Δ^9-THC, affects emotional reactivity in humans (Porter and Felder, 2001) and laboratory animals by activating brain cannabinoid receptors (Onaivi et al. 1990; Berrendero and Maldonado 2002). The 5HT system plays a key modulatory role in CNS processes that appear to be dysregulated in psychiatric disorders such as anxiety, fear, depression or aggression (Griebel, 1995). The role of 5HT$_{1A}$ serotonergic receptor, located in serotonergic patways projecting from mid-brain raphe nuclei to limbic areas, in the modulation of anxious states has been particularly well studied (Handley, 1995; Barnes and Sharp, 1999). To date, there is only one report on the involvement of 5HT$_{1A}$ serotonergic receptor in anxiogenic-like response induced by CP 55,940 (Marco et al., 2004). The aim of the present work was to further elucidate the role of 5-HT$_{1A}$ serotonergic receptor on emotional reactivity induced by cannabinoids in rats using the elevated plus-maze (EPM) and forced swimming test (FST). Δ^9–THC (0.015-3 mg/kg), was studied in a EPM apparatus according to Pellow et al. (1985). The test length was 5 min, the total time spent in each arm and the number of arm entries were scored by trained observers in male Sprague-Dawley rats, 30 min after treatment. The FST, evaluated according to Porsolt et al., (1977), consisted in two swimming sessions where the time of immobility during the 2nd 5-min session was an indicator of antidepressant activity. Δ^9–THC showed a biphasic effect being anxiolytic at a low (0.75 mg/kg) and anxiogenic at a high (3 mg/kg) dose. Lower doses as 0.015 and 0.075 mg/kg significantly reduced the immobility time in the FST, showing an antidepressant activity. Pre-treatment with the 5-HT$_{1A}$ serotonergic receptor antagonist, WAY 100635 (0.3 mg/kg) given s.c. 1h before Δ^9–THC, significantly reversed its anxiolytic effect. A synergistic action on anxiolytic effect, when the 5-HT$_{1A}$ serotonergic receptor agonist 8-OH-DPAT (0.0075 mg/kg) was given in combination with Δ^9–THC, was observed. These findings support a key role of 5-HT$_{1A}$ serotonergic receptor in the regulation of Δ^9–THC-induced emotional states.

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SEX DIFFERENCES IN SENSITIVITY TO ANTIPSYCHOTIC EFFECTS ON CANNABINOID CB_1 RECEPTOR FUNCTION IN RATS

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Converging lines of research from a variety of scientific disciplines have suggested that the endocannabinoid system may play a major role in neuropsychiatric disorders, including schizophrenia. Alterations in regional brain cannabinoid (CB_1) receptor densities and endocannabinoid levels have been reported in a number of patients with schizophrenia, as has a genetic polymorphism of the CNR1 gene that encodes for CB_1 receptors. The degree to which antipsychotics, the primary pharmacological treatment for schizophrenia, affect CB_1 receptors, however, is not clear. This study examined the effects of sub-chronic administration of the antipsychotics, haloperidol and clozapine, on CB_1 receptor number and function. Adult male and female Long-Evans rats were injected with saline, 0.3 mg/kg haloperidol, or 10 mg/kg clozapine twice daily for 10 days. Following sacrifice on the morning of the 11^{th} day, membrane homogenates were prepared from the prefrontal cortex, striatum, and ventral midbrain and CB_1 receptor levels and function were assessed using [^{3}H]SR141716A and agonist-stimulated [^{35}S]GTPγS binding, respectively. Whereas changes in [^{3}H]SR141716A binding were not found in any region of male or female drug-treated brains (as compared to control), significant differences in CB_1-mediated G-protein activity were produced by both clozapine and haloperidol in the brains of drug-treated female rats. Treatment with the conventional antipsychotic haloperidol significantly attenuated maximal stimulation of CB_1-mediated G-protein activity in the prefrontal cortex of female rats, but did not affect activity in the striatum or midbrain. Similar to haloperidol, the atypical antipsychotic clozapine also attenuated CB_1-mediated G-protein activity in the prefrontal cortex of female rats; however, unlike haloperidol, clozapine decreased CB_1-mediated G-protein activity in the striatum of these rats as well. Agonist-stimulated [^{35}S]GTPγS binding in the striatum of male rats was not affected by treatment with either antipsychotic. No significant changes were seen in cannabinoid stimulated G-protein activity in the midbrain of male or female rats. These data show that antipsychotic treatment produces sex- and region-specific modulation of CB_1 receptor function and that these effects differ for clozapine versus haloperidol. While it is not clear whether this effect contributes to the therapeutic efficacy of these drugs, reports that CB_1 receptors and endocannabinoids are increased in schizophrenia suggest that this effect might be one component of antipsychotic actions, at least in females.

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EFFECT OF SOCIAL ISOLATION ON CB_1, D_2 AND FAAH EXPRESSION IN SPRAGUE-DAWLEY RATS

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Rearing rats in isolation has been shown to produce behavioural and neurochemical alterations similar to those observed in schizophrenia. In addition, a dysregulation in the endocannabinoid system has been implicated in the pathogenesis of schizophrenia. The aim of these experiments was to determine if there are differences in the amounts of CB_1 receptor protein and fatty acid amide hydrolase (FAAH) expression, as well as D_2 receptor expression in different brain regions in rats reared in different environmental conditions. Twenty-one day old male Sprague-Dawley rats were either reared in individual cages (isolated rats) or in group cages of 6 per cage (grouped rats) for 8 weeks. Preliminary experiments using Western blotting determined that significant decreases in CB_1 receptor protein occurred in the cingulate cortex and the hippocampus of socially isolated rats compared with grouped rats. Immunohistochemistry followed by quantitative fluorescent detection employing the LI-COR Odyssey Imaging® System was then used to determine the expression of CB_1 receptors, D_2 receptors and FAAH. Integrated intensities expressed as counts per mm^2 were determined and compared between the two groups of animals. There was a significant decrease in CB_1 receptor expression in the caudate putamen, the amygdala and the hippocampus of isolated rats compared with grouped rats. A significant increase in D_2 receptor expression was observed in the caudate putamen and the nucleus accumbens core of isolated rats compared with grouped rats. FAAH expression was significantly increased in the caudate putamen, nucleus accumbens core and the nucleus accumbens shell of isolated rats compared with grouped rats. These results are in agreement with previous studies indicating that the dopaminergic system is altered in socially isolated rats. In addition, these results suggest that the endocannabinoid system is altered in socially isolated rats. Alterations of the endocannabinoid system in rats reared under different housing conditions (i.e. isolated vs. grouped rats) may provide new insight into the role of the endocannabinoid system in schizophrenic individuals.

CB_1 RECEPTOR MEDIATES THE POTENTIATION OF PENTOBARBITAL-INDUCED SLEEP BY DELTA-9-TETRAHYDROCANNABINOL

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Delta-9-Tetrahydrocannabinol (THC), a major psychoactive constituent of marijuana, is known to exhibit multitude pharmacological effects such as catalepsy, hypothermia, analgesic effects, immobility, and motor incoordination including barbiturate synergism. Cannabinoid receptors were found in the mammalian brain (CB_1) and the peripheral organs (CB_2). The CNS effects of cannabinoids are inhibited by coadministration of CB_1 receptor antagonist such as SR141716A and AM251, but not a CB_2 receptor antagonist, SR144528, and those CNS pharmacological effects seemed to be mediated through CB_1 receptor. We reported prolongation effects of THC and cannabidiol (CBD) on pentobarbital-induced sleep. The mechanism of sleep prolongation of CBD was resulted from inhibition of pentobarbital metabolism, i.e., inhibition of hepatic drug-metabolizing enzymes, while THC might act for CNS.[1,2)] Therefore, the potentiation mechanism of THC with barbiturate was examined in this study. SR141716A, AM251, or SR144528 (2 mg/kg, i.v.) was administered 10 min before injection with THC (10 mg/kg, i.v.). Sodium pentobarbital (40 mg/kg) was injected i.p. 15 min after the injection of THC and the sleeping time was then measured as the time between loss of the righting reflex and the recovery. Cannabinoid receptor antagonists themselves did not affect the pentobarbital-induced sleeping time. CB_1 antagonists, SR141716A and AM251, reversed the potentiation of pentobarbital-induced sleep by THC, while SR144528 did not. However, pretreatment of SR141716A 2 or 5 mg/kg, i.v., did not inhibit CBD-enhanced pentobarbital-induced sleep. Since the prolonging effect of CBD on pentobarbital-induced sleep was due to an inhibition of hepatic drug-metabolizing enzyme, the CB_1 antagonist did not affect the synergistic effect of CBD with pentobarbital. The affinities of antagonists for CB_1 receptor were evaluated using a specific CB_1 ligand, [^{3}H]CP55940, and mouse brain synaptic membrane. The Ki values of THC and CBD were 6.62 nM and 2.01 uM, respectively, showing low affinity of CBD for the CB_1 receptor binding. The Ki values of CB_1 receptor antagonists, SR141716A and AM251, were 9.54 and 2.58 nM, respectively, indicating a high affinity to CB_1 receptor binding site as well as THC. CB_2 antagonist SR144528 did not have a high affinity to CB_1 receptor binding site, showing 266 nM of Ki value. High concentration of pentobarbital 1 mM did not affect specific [^{3}H]CP55940 binding to mice brain synaptic membrane. These results suggest that the interaction site of THC with pentobarbital might be downstream from the CB_1 receptor, while the receptor plays an important role as a trigger for potentiation of pentobarbital-induced sleep by THC.

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EFFECTS OF ADMINISTRATION OF THE CB_1 RECEPTOR ANTAGONIST SR141716A INTO THE RIGHT BASOLATERAL AMYGDALA ON CONDITIONED FEAR AND ASSOCIATED ANALGESIA IN RATS

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Fear-conditioned analgesia (FCA) is the profound suppression of pain during exposure to conditioned aversive stimuli and is an important survival response. Evidence supports a role for the basolateral amygdala (BLA) in the expression of FCA and conditioned fear *per* se. Recent evidence suggests a role for the endocannabinoid system in mediating analgesia expressed following exposure to conditioned (Finn *et al*., 2004, Eur J Neurosci) or unconditioned (Hohmann *et al*., 2005, Nature; Connell *et al*., 2005, Neuroscience Letters) aversive stimuli. Moreover, the endocannabinoid system plays a role in the extinction of conditioned aversive behaviour (Marsicano *et al*. 2002, Nature; Finn *et al*. 2004, Eur J Neurosci). The present study investigated the effects of administration of the CB_1 receptor antagonist, SR141716A (50 μg/500 nl), or DMSO vehicle, into the right BLA on FCA and conditioned aversion in male Lister-hooded rats (290-340 g, Charles River). The fear-conditioning paradigm used was footshock paired with context (10 x 1 s footshocks, 0.4 mA, at 1 minute intervals; non-footshocked controls included). The formalin test (intra-plantar injection of 50 μl, 2.5 % formalin or 0.9% saline into right hindpaw) was used to assess nociceptive behaviour (30-45 min post-formalin) during re-exposure to the conditioned context 24 hours post-footshock. Re-exposure to the context previously paired with footshock, significantly reduced formalin-evoked nociceptive behaviour, as indicated by the reduction in the composite pain score (CPS) compared with non-footshocked formalin-treated rats (Fig. 1a). SR141716A microinjected (via a previously implanted guide cannula) into the right BLA 10 minutes prior to re-exposure to context did not attenuate FCA with the exception of the first minute of the trial. SR141716A had no significant effect on formalin-evoked nociceptive behaviour in rats not receiving footshock with the exception of the first minute where it reduced nociceptive behaviour. Intra-BLA SR141716A significantly prolonged the duration of freezing behaviour (Fig. 1b) and 22 kHz ultrasonic vocalisations in footshock-conditioned rats receiving intra-plantar saline.

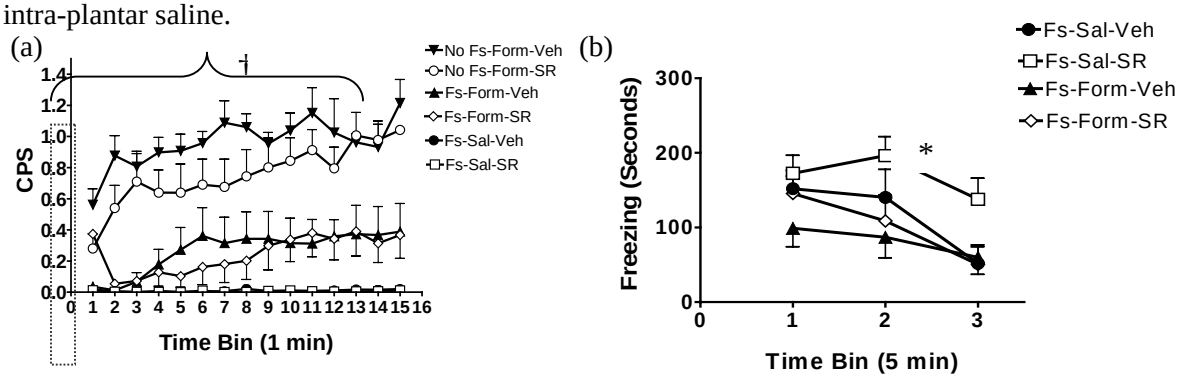

Fig 1 Effects of the CB_1 receptor antagonist, SR141716A, administered into the right BLA on (a) FCA and (b) contextually-induced freezing. Data are means ± SEM (n = 6-11). $^{‡}P< 0.01$ for Fs-Form-Veh vs Fs-Form-SR during first minute; $^{§}P<0.01$ for No Fs-Form-SR vs No Fs-Form-Veh during first minute. $^{†}P< 0.05$ for Fs-Form-Veh vs No Fs-Form-Veh over entire 15 minute trial. $^{*}P < 0.05$ for FS-Sal-SR vs FS-Sal-Veh during last 5 minutes of trial. Two-way ANOVA & Bonferroni *post-hoc*. FS (footshock); Form (formalin); Sal (saline); Veh (vehicle); SR (SR141716A).

These data suggest an important role for CB_1 receptors in the right BLA in mediating short-term extinction of conditioned aversive behaviour. The data also suggest that FCA is not exclusively mediated by CB_1 receptor activation in the right BLA.

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ROLE OF ENDOCANNABINOIDS IN THE MODULATION OF ANXIOUS STATES IN RAT PREFRONTAL CORTEX

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Fear and anxiety are crucial and adaptive components of the overall behavioral and autonomic "stress" response to dangerous situations which threaten to perturb homeostasis. Transient anxiety proportional to the challenge encountered elicits an appropriate response and is of fundamental importance as a survival strategy for all higher animals, including man. The aberrant operation of mechanisms controlling mood and response to stress can provoke a perturbation of equilibrium, an abrupt or gradual shift to a new "stasis" and, in pathological cases, a clinically-defined anxiety disorder.
There is an increasing interest in the role of endocannabinoids in anxiety. A very recent study (Kathuria *et al.*, 2003) provided support to the hypothesis that endocannabinoids, and anandamide in particular, exert an anxiolytic tone in rodents.
Different brain areas seem to be involved in the modulation of anxious states. Among them the prefrontal cortex seems to play an important role since both stress and anxiety have been shown to activate the medial prefrontal cortex in rats. In order to clarify in this brain region the role of the endocannabinoid tone in the modulation of anxiety, we adopted a variety of complementary techniques including local anandamide microinjection, FAAH pharmacological blocking and regulation of the endocannabic tone by lentivirus-mediated *in vivo* gene-transfer techniques.
Our results show that meta-anandamide microinjected into the prefrontal cortex exerted a biphasic effect on anxiety: low doses (0.1 – 1 μg) induced an anxiolytic response in the elevated plus maze, whereas a higher dose (10 μg) produced an anxiogenic effect.
Microinjections into the prefrontal cortex of URB597, a specific and irreversible FAAH inhibitor, at a low dose (0.01μg) significantly decreased anxiety.
Finally we used lentivirus-mediated *in vivo* gene transfer to stably overexpress FAAH in prefrontal cortex thus decreasing anandamide levels. First of all we tested the pWTP/FAAH/GFP construct on C6 rat glioma cells: correct FAAH expression was analyzed by western immunoblotting whereas its functionality by FAAH activity assay. Through microinjections of the lentiviral particles in the prefrontal cortex of rats we then studied the *in vivo* consequences of the lack of the endocannabic tone. We obtained successful FAAH overexpression as demonstrated by immunohystochemical experiments and altered behavioural manifestations related to anxious states.
Our data reveals that in the prefrontal cortex the endocannabinoid tone plays a key role in the regulation of emotional states .

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CHARACTERIZATION OF CANNABINOID RECEPTORS INVOLVED IN THE MODULATION OF [^{3}H]5-HT RELEASE FROM THE HIPPOCAMPUS

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The major cellular effect of exo- and endocannabinoids in the brain is to presynaptically control the release of neurotransmitters. Although the action of cannabinoids on the release of the major excitatory and inhibitory neurotransmitters are well characterized, relatively less is known about the cannabinergic regulation of central 5-HT release. The aim of this study was to analyse the effect of various exo- and endocannabinoids on the electrical stimulation induced release of [^{3}H]5-HT in the hippocampus and to identify the underlying cannabinoid receptor subtype. Rat hippocampal slices were preloaded with [^{3}H]5-HT and then superfused and subjected to electrical field stimulation, which resulted in a reproducible efflux of tritium under control conditions (S2/S1=1.02±0.02, n=6). The cannabinoid agonist WIN55212-2 (1 μM) by itself had no effect on electrically induced release of [^{3}H]5-HT. However, when measured in the presence of glutamate receptor antagonists (CNQX+AP-5), WIN55212-2 slightly, but significantly decreased the stimulation-evoked [^{3}H]5-HT efflux. Under identical conditions, another cannabinoid agonist CP55940 (1 μM) and the exocannabinoid delta^9- tetrahydrocannabinol (delta^9-THC, 1 μM) also acted in a similar way. The CB1 receptor selective antagonists SR141716A (1 μM) and AM251 (1 μM) prevented the inhibitory effect of WIN55212-2 on the stimulation evoked release of [^{3}H]5-HT. To confirm the involvement of CB1 cannabinoid receptor in this effect, mice, genetically deficient in CB1 cannabinoid receptors ($CB1^{-/-}$) and their wild type counterparts ($CB1^{+/+}$) were also utilized. In wild type mice, the S2/S1 ratio was 1.13±0.08 (n=4) under control conditions, and WIN55212-2 exerted a similar inhibitory action on [^{3}H]5-HT release to that observed in rats. This effect was completely absent in hippocampal slices derived from $CB1^{-/-}$ mice. In conclusion, our results suggest that the release of [^{3}H]5-HT is subject to inhibitory modulation by CB_1 cannabinoid receptors in the hippocampus, however this modulation is masked by the excitatory neurotransmission and is relatively modest, indicating that only a subpopulation of serotonergic afferents express CB1 receptors.

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FACILITATION OF CONDITIONED-FEAR EXTINCTION BY I.C.V. ADMINISTRATION OF AM404, A POTENT INHIBITOR OF ENDOCANNABINOID RE-UPTAKE

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Introduction: Fear conditioning models are useful to assess the role of aversive memories in important anxiety disorders such as the posttraumatic stress. In contextual fear conditioning, the animal is shocked in a conditioning chamber, so that re-exposure to this context elicits defensive responses, including freezing behavior. Repeated non-reinforced re-exposures to the context result in a progressive decrease of freezing (i.e. conditioned-fear extinction). Endocannabinoids are released during extinction of conditioned-fear (Marsicano et al, 2002) and administration of a cannabinoid agonist facilitates this process (Pamplona and Takahashi, 2005). The present study evaluated the effects of i.c.v. administration of AM404, a potent inhibitor of endocannabinoids re-uptake, on the contextual fear extinction in rats.

Methods: Male Wistar rats were implanted with a guide cannulae aimed at the third ventriculae (AP:-0.8, LL:-1.6, DV:-3.6). After 7 days, the rats were placed into the conditioning chamber and 3 min later received an electric-footshock (1.5 mA, 1 s) and remained in the chamber for another 1 min. The next day, they were re-exposed to the conditioning chamber for 9 min. This extinction protocol was executed on 3 consecutive days. The % of freezing was used as an index of fear memory. AM404 (0.2, 1.0 μg / 1 μL) or vehicle (10% DMSO in saline) was injected i.c.v 5 min before each extinction trial. An additional group was injected i.p. with rimonabant (0.2 mg/kg, i.p.), a selective CB_1 antagonist, 20 min before i.c.v. injection of AM404.

Results: The animals treated with AM404 (1.0 μg / 1 μL, i.c.v.) exhibited a reduced % of freezing during the 2^{nd} and 3^{rd} extinction trials compared to the control group ($p<0.05$). This effect was reversed by the pre-administration of rimonabant in a dose that *per se* do not affect the % of freezing ($p<0.05$).

Conclusion: The present results suggest that inhibition of endocannabinoid re-uptake facilitates extinction of aversive memories by the activation of CB_1 receptors. Therefore, it is likely that this enhancement of endocannabinoid effects could be used to ammeliorate behavioral symptoms of anxiety disorders related to unpleasant fear memories.

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ACQUISITION OF AN INSTRUMENTAL OPERANT TASK IS FACILITATED FOLLOWING INTRA-HIPPOCAMPAL INFUSION OF WIN 55, 212-2

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Systemic infusions of WIN 55,212-2 (WIN-2),a potent CB_1 receptor agonist has revealed dose/delay-dependent deficits in working/short-term memory performance with the radial arm maze and delayed non-match to sample (DNMTS) tasks. In each of these particular forms of memories, multiple processes such as acquisition; consolidation and retrieval are involved. To date, systemic Δ^9 – THC and WIN-2 studies have shown a reduction in hippocampal cell ensemble firing during the sample (acquisition) but not the non-match (retrieval) phase of the DNMTS task. Systemic administrations can affect many if not all areas of the brain. Thus, the site directed intra-hippocampal infusion technique was employed to examine whether WIN-2 effects on acquiring several stages of the DNMTS task were hippocampal specific.

18 adult, male Long-evans rats received WIN-2 (0.5µg); the inactive isomer, WIN-3 (0.5µg) and the CB_1 receptor antagonist, SR141716A (0.5µg) via bilateral cannulae directed to dorsal hippocampus by means of 28 day osmotic minipumps (*n=6* per group).The number of days taken to acquire different stages (Criterion I-V) during learning the DNMTS task and day 1 performances for Criterion III, IV and V were recorded and analysed. Preliminary data show that neither WIN-2 nor SR groups were significantly different to WIN-3 controls in days taken to accomplish criterion I-V at the given infusion rate of 0.75ng/hr. In contrast, the WIN-2 group showed a significant facilitation on day 1 performance during criterion III ($P<0.05$) that was fully reversed by SR ($P<0.05$).

These findings suggests that chronic WIN-2 infusions at the given dose, selectively enhances the early (day 1) learning of a particular criterion of the DNMTS task, possibly by activating CB_1 receptors expressed in terminals of the CCK containing GABA inter-neurons to reduce inhibition and augment excitation of hippocampal principal cells.

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THE CB1 RECEPTOR ANTAGONIST AM251 AMELIORATES COGNITIVE DEFICTS ASSOCIATED TO HYPERDOPAMINERGIA

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A large body of neuroanatomical and neurochemical data demonstrates that the cannabinoid system closely interacts with the dopaminergic system regulating dopaminergic signaling in the synaptic and the receptor level, while psychotropic drugs similar to those provoking or exacerbating psychosis in humans modulate endocannabinoid contents.

An important emerging concept in the nosography of schizophrenia is that the disease is characterized by profound trait cognitive deficits for which there is an unmet medical need. Interestingly, the cannabinoid system and in particular CB1 receptor signaling has been shown to play a critical role in cognitive processes.

Here we have used mice invalidated for the dopamine transporter (DAT-KO) to study the role of the cannabinoid system in hyperdopaminergia-related cognitive impairment. DAT-KO mice not only are hyperactive and display perturbed sensorimotor gating but also show deficits in habituation and diminished performance in cognitive tasks. These impairments could parallel profoundly perturbed cognitive functions in subjects suffering from schizophrenia. Namely, we have used a cued version of the Morris water maze in which the platform is visible to test the effects of the CB1 antagonist AM251 on cognitive performance in DAT-KO mice.

For wild-type mice latency to escape (i.e. time to find the platform) decreased significantly with trials. After five trials wild-type mice have reached optimal performance in the task. On the contrary DAT-KO mice failed to learn the task as their latency to escape did not change between trials. Without any treatment latencies to find the platform during the fifth trial were 19+/-3 and 82+/-4 sec for wild-type and DAT-KO mice respectively.

AM251 significantly ameliorated the performance of DAT KOs in the task. During the last trial, latency to escape was reduced by 50% in AM251-treated DAT-KOs as compared to their saline-treated littermates (latency to escape 82+/-4 for saline treated DAT-KOs; 40+/-5 for AM251 DAT-KOs; 19+/-3 for saline treated WT and 20+/-9 for AM251 treated WT). Latency to escape significantly diminished within successive trials in DAT-KOs treated with AM251 (3mg/kg), indicating that AM251 helps these mutants to learn this task.

We suggest that the cannabinoid system has an important role in hyperdopaminergia-related cognitive dysfunction and that CB1 antagonists could be a novel tool in the therapeutics of cognitive deficits associated with schizophrenia and/or ADHD.

EARLY ADOLESCENT THC ALTERS BEHAVIOR IN AVOIDANCE PARADIGMS IN SEX-SPECIFIC WAYS

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Marijuana is the most widely used abuse drug in the US today. Tetrahydrocannabinol (THC), the major active constituent of marijuana, has been found to alter several types of behaviors including cognitive behaviors. We hypothesized that THC administered during a time when the brain was developing would produce long-term alterations in behaviors which rely on the hippocampus, a brain region known to contain cannabinoid receptors. Therefore, we dosed Sprague-Dawley rats with 0, 1 or 5 mg/kg THC during postnatal days 22-40, a time equivalent to early adolescence, and tested behavior in adulthood. At 60+ days, we conducted Active Place Avoidance testing using the new Bio-signal Group Corp. equipment, at 132+ days, passive avoidance testing using the Accuscan Shuttle Box and at 140+ days, active avoidance testing in the same shuttle box. Results show that at 60 days, while both doses of THC improved performance in the active place avoidance paradigm, the learning curves were different for male and female rats. There were no effects of THC on latency to cross to dark compartment on test day for passive avoidance. However in the active avoidance test in the shuttle box, control females showed a greater percentage of avoidances compared to THC-treated females while in males, the high dose THC group performed better than the other groups. These data suggest that a brief exposure to THC during early adolescence has lasting effects on avoidance learning that vary depending on the sex of the subject and the testing modality utilized.

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SPONTANEOUS CA^{2+} SPIKES IN DENDRITES OF CEREBELLAR PURKINJE CELLS TRIGGER 2-ARACHIDONOYLGLYCEROL-MEDIATED RETROGRADE SYNAPTIC SIGNALING

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Activation of CB_1 cannabinoid receptors leads to presynaptic inhibition of neurotransmission in many brain regions (Szabo & Schlicker, Handbook of Experimental Pharmacology, 168: 318 –356, 2005, Springer, Heidelberg). This presynaptic inhibitory mechanism can also be activated by endocannabinoids released from postsynaptic neurons - a form of retrograde synaptic signaling. A typical trigger for the synthesis of endocannabinoids is an increase in calcium concentration in postsynaptic neurons (Freund et al., Physiol Rev 83:1017-1066, 2003). Depolarization-induced calcium increases in cerebellar cortical Purkinje cells lead to endocannabinoid-mediated retrograde synaptic signaling. Spontaneous Ca^{2+} spikes frequently occur in Purkinje cells. Our hypothesis was that these Ca^{2+} spikes trigger endocannabinoid-mediated retrograde synaptic inhibition.

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded with patch-clamp techniques in Purkinje cells of mouse cerebellar slices. In 60-70 % of Purkinje cells, spontaneous Ca^{2+} spikes occurred. In our preparation these spikes are elicited by depolarizing sIPSCs. Fluorescence imaging experiments with the Ca^{2+}-sensitive dye Oregon Green-488 BAPTA-5N indicated that the calcium spikes were accompanied by spatially restricted Ca^{2+} increases in dendrites. After Ca^{2+} spikes, the cumulative amplitude of sIPSCs was suppressed to 81 ± 5 % of the initial reference value (solvent group in fig. 1). Such a decrease did not occur in the presence of the CB_1 receptor antagonist rimonabant (10^{-6} M; fig. 1), indicating involvement of endocannabinoids and CB_1 receptors. In order to determine the chemical identity of the endocannabinoid mediating suppression of sIPSCs, we carried out experiments with orlistat, inhibitor of diacylglycerol lipase, the enzyme responsible for production of 2-arachidonoylglycerol (Bisogno et al., J Cell Biol. 16(3):463-8, 2003). In the presence of orlistat (10^{-5} M), Ca^{2+} spikes did not suppress subsequent sIPSCs, suggesting that the Ca^{2+} spikes triggered the release of 2-arachidonoylglycerol. Inhibition of fatty acid amid hydrolase by URB597 (5 x 10^{-7} M) did not change the Ca^{2+} spike-induced suppression of sIPSCs.

The data indicate that spontaneously occurring Ca^{2+} spikes in Purkinje cells are due to Ca^{2+} influx into restricted regions of the dendrites. Such Ca^{2+} spikes can trigger the synthesis of 2-arachidonoylglycerol. Subsequently, 2-arachidonoylglycerol diffuses to terminals of GABAergic axons and inhibits the release of GABA by activating CB_1 receptors.

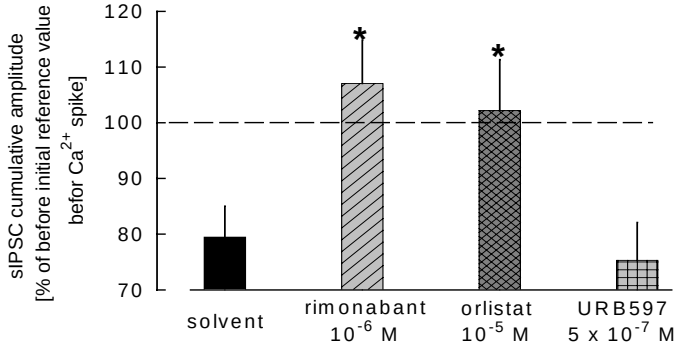

Figure 1.
In the presence of solvent, the cumulative amplitude of sIPSCs after Ca^{2+} spikes was suppressed to 81 % of the initial reference value. Such a Ca^{2+} spike-evoked suppression did not occur in the presence of rimonabant and orlistat. The suppression was not changed by URB597.

METABOTROPIC GLUTAMATE RECEPTOR MEDIATED LTD IN THE CA1 REGION: A ROLE FOR THE LIPOXYGENASE PATHWAY AND ENDOCANNABINOIDS

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The group 1 metabotropic glutamate receptor agonist DHPG induces a form of long-term synaptic depression in the CA1 region (DHPG-LTD). DHPG-LTD is thought to be induced *via* a postsynaptic mechanism, yet it is likely to be expressed *via* altered presynaptic transmitter release. Arachidonic acid and its metabolites have been suggested as potential retrograde messengers. Recently, endocannabinoids have been shown to act as retrograde messengers involved in modulating inhibition in the hippocampus. Here we have investigated the role of the arachidonic acid signaling cascade and endocannabinoid antagonists AM251 on DHPG-LTD. Extracellular excitatory postsynaptic potentials (EPSPs) in the CA1 were evoked by stimulation of the Schaffer collateral pathway in adult rat hippocampal slices (350 μm). RS-DHPG (100 μM), was bath applied for 20 min during which time, the EPSP slope was significantly reduced. On washout of DHPG, classical DHPG-LTD could be recorded with the synaptic potential being reduced to aprox. 70% of baseline (60 min wash). We have shown that the phospholipase A_2 inhibitor, 4-(4-Octadecylphenyl)-4-oxobutenoic acid (OBAA, 10 μM), when applied 60min prior to DHPG causes a significant reduction of DHPG-LTD. Inhibition of COX-2 by SC-236 prior to DHPG application also has no effect on DHPG-LTD. However the 12-lipoxygenase inhibitor, cinnamyl-3, 4-dihydroxy-α-cyanocinnamate (CDC, 10 μM) or the 5-lipoxygenase inhibitor (AA-861, 10 μM and 50 μM) applied for 30 min prior to DHPG, both inhibited DHPG-LTD. The CB_1 cannabinoid receptor antagonist, AM-251 [(N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)], when bath applied at a concentration of 1 μM or 5 μM does not significantly alter the base-line ESPS slope, however when applied for 20 min prior to DHPG, AM-251 inhibited DHPG-LTD at both concentrations tested (1 μM: 89.6 ± 6.0%, n=6 and 5 μM: 97.5 ± 4.3%, n=7, $P<0.05$). To examine the potential long-term effects of the lipoxygenase pathway, AA861 was perfused only during the washout period following DHPG application. Under these condition, there was no attenuation of DHPG LTD (65.4± 5.8, n=4). Our results indicate that lipoxygenase pathways and endocannabinoid signalling are likely to be involved in mGluR mediated depression of the EPSP in the CA1 region.

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DOSE DEPENDENT EFFECTS OF CANNABINOID CB_1 RECEPTOR ACTIVATION OR ANTAGONISM ON BEHAVIORAL FLEXIBILITY

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Cannabinoid CB_1 receptors are expressed in the prefrontal cortex, but their role in mediating executive functions such as behavioral flexibility, is unclear. The present study examined the effect of pharmacological activation or blockade of the cannabinoid CB_1 receptors on behavioral flexibility using a strategy set-shifting task conducted on a cross maze. In Experiment 1, rats initially were trained to turn left or right while ignoring the visual-cue to obtain a food; on the second test day, rats had to inhibit the previously learned rule and approach the cue to obtain the food. In Experiment 2, the order of discrimination training was reversed. Administration of the cannabinoid CB_1 receptor agonist HU-210 prior to the set-shift on Day 2 elicited dose-dependent effects on performance. A 20 ug/kg dose of HU-210 increased perseverative errors, whereas the effects of a lower, 5 ug/kg dose caused differential effects depending on whether rats were required to shift from a response to a visual-cue discrimination strategy or vice versa. Conversely, administration of a 2 mg/kg, but not a 5 mg/kg dose of the CB_1 receptor antagonist AM251 reduced perseverative errors. These data demonstrate a biphasic and dose sensitive role for the cannabinoid system in behavioral flexibility, which in turn may have clinical implications for the role of the endocannabinoid system in psychiatric disorders, such as schizophrenia, where behavioral flexibility is compromised.

A-796260: A CB_2-SELECTIVE FULL AGONIST EXHIBITING ANALGESIC ACTIVITY IN RODENT MODELS OF NOCICEPTIVE PAIN

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There is mounting evidence supporting the potential utility of CB_2-selective agonists in the treatment of pain. The CB_2-selective ligand AM1241 has been reported to be active in rat models of inflammatory and neuropathic pain (Ibrahim *et al.*, 2003, PNAS, 100, 10259), and a variety of studies have implicated an indirect interaction with the opioid system as an important contributor to the analgesic effects of AM1241 (Ibrahim, *et al.*, PNAS, 2005, 102, 3093).

A-796260 is a novel indole derivative with structural similarities to AM1241. It exhibits high potency and selectivity for the CB_2 receptor. In radioligand binding experiments using recombinant human CB_1 and CB_2 receptors expressed in HEK cells, A-796260 exhibited high affinity (K_i = 0.77 nM) for the CB_2 subtype and displayed a 430-fold selectivity vs. the CB_1 binding site. A similar level of potency and selectivity was observed in a functional assay measuring inhibition of forskolin-stimulated adenylyl cyclase activities, where A-796260 exhibited an EC_{50} of 0.75 nM at the CB_2 receptor and 820 nM for the CB_1 site, with maximal efficacies comparable to the non-selective full agonist CP55,940 observed at both the CB_1 and CB_2 receptors. By contrast, AM1241 exhibited no apparent agonist efficacy in the CB_2 forskolin-stimulated adenylyl cyclase assay, and appeared to act as a neutral antagonist under these assay conditions (Yao, *et al*., 2005, ICRS Symposium on the Cannabinoids, 95).

In the Complete Freund's Adjuvant (CFA) model of inflammatory pain, A-796260 exhibited full efficacy (ED_{50} = 5 mg/kg, i.p.). The analgesic effect was fully reversed with the CB_2-selective antagonists SR144528 and AM630, and was unaffected by the selective CB_1 antagonist SR141716A, supporting a CB_2 mechanism of action. A-796260 was further demonstrated to be highly efficacious in additional models of inflammatory pain and in models of moderate to severe post-operative pain.

To explore the potential opioid contribution to CB_2-mediated analgesia, a series of studies were conducted comparing the opioid dependency of AM1241 and A-796260. Consistent with reports that the antinociceptive effects of AM1241 were blocked by the μ-opioid antagonist naloxone in a model of acute thermal pain and the lack of efficacy in AM1241 in μ-opioid knockout mice (Ibrahim, *et al.*, PNAS, 2005, 102, 3093), we observed that the analgesic effects of AM1241 in the CFA model of inflammatory pain were also fully antagonized by naloxone. In contrast to these findings, naloxone had no effect on the analgesic activity of A-796260.

These studies support the conclusion that CB_2 agonists may represent a potentially useful new class of analgesic agents, and that significant differences in the mechanism of action may exist for different members of this class.

EFFECT OF THE CANNABINOID CB_1 RECEPTOR ANTAGONIST RIMONABANT ON NOCICEPTIVE RESPONSE AND SEVERITY OF ADJUVANT-INDUCED ARTHRITIS IN OBESE AND LEAN RATS

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Introduction: Rimonabant is a cannabinoid CB_1 receptor antagonist that could help reduce obesity and metabolic risk factors in overweight patients with dyslipidemia (Després et al., N Engl J Med 353, 20, 2121-2134). Rimonabant also has anti-inflammatory effects in rodents: it prevented the increase in TNF-alpha serum levels induced by *E. coli* LPS, and reduced indomethacin-induced small-intestinal lesions (Smith et al., J Pharmacol Exp Ther 293, 136–150, 2000, Croci et al., Eur J Pharmacol 450, 77-83) and neuropathic pain (Costa et al., Pain, 116, 52-61). Since obesity is a risk factor for several inflammation-based diseases including arthritis (Mehrotra et al., Am J Prev Med, 27(1), 16-21), we investigated the therapeutic effect of rimonabant on adjuvant-induced mono-arthritis and the associated hyperalgesia in lean and diet-induced obese (DIO) rats.

Methods: Adult female SD rats aged 48 weeks, weighing either 320±3.3g (lean) or 500±12g (obese) were injected into the right hind-paw with a suspension of 150 µg of complete Freund's adjuvant (CFA) in 0.15 mL saline. Controls in each group received an intra-plantar injection of the same volume of saline. On the 7th day after the injection, the paw edema volume was measured with a plethysmometer and hyperalgesia and allodynia were tested by thermal plantar and mechanical Von Frey tests. Both groups were then given rimonabant daily for seven days (3 and 10 mg/kg, p.o.) or its vehicle (10% Tween 80 in water). On the 14th day, the response to rimonabant was evaluated by measuring the pain, the edema and the severity of arthritis (global score: arbitrary macroscopic rating plus ankle-joint width) by comparing the inflamed hind-paw and the healthy contralateral paw and the hind-paw of the controls injected with CFA or saline. ANOVA plus Newman-Keuls test were used for statistical analysis. The results are expressed as mean±se of 8-16 rats per group.

Results: On the 7th day, the inflammatory response to CFA was significantly higher in DIO than in lean rats (edema volume, 2.3 ± 0.09 vs. 1.7 ± 0.11 mL, $p < 0.05$; global arthritis score: 2.9 ± 0.14 vs. 2.3 ± 0.05, $p < 0.01$) but the pain response was similar. The response to CFA-induced pain remained much the same on day 14 in drug-free rats though there was a slight reduction in the edema. After 7 days' treatment with either 3 or 10 mg/kg (day 14), rimonabant dose-dependently reduced thermal hyperalgesia in lean rats (recovery respectively 44± 4.1% and 62±8.3% of controls) and DIO rats (53±10%, 69±11%); on mechanical hyperalgesia (allodynia test), it was more effective in DIO than lean rats (68±11%, 78±9% and 39± 3.9%, 59.5±11.6% of controls, $p<0.01$). The global arthritis score was slightly, not significantly, better only in DIO rats; unlike in lean animals, in DIO rats rimonabant significantly reduced the ankle-joint width, with similar efficacy at 3 and 10 mg/kg (Δ mm vs. healthy contralateral paw 0.66 ± 0.09, 0.67 ± 0.10 vs. control-CFA rats 1.36 ± 0.13, $p < 0.01$). The drug had no effect on paw edema in either lean or DIO rats.

Conclusion: In this study rimonabant improved experimental CFA mono-arthritis, particularly in DIO rats, in which the symptoms are more severe. The drug had considerably more effect on pain than on other parameters of arthritis. This cannabinoid CB_1 antagonist might therefore have therapeutic potential in obesity-associated inflammatory diseases and to relieve arthritic pain.

DETERMINING THE ROLE OF A THIRD CANNABINOID RECEPTOR, GPR55, IN INFLAMMATORY AND NEUROPATHIC PAIN

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Recently it has been suggested that GPR55 is a third cannabinoid receptor, a site of action for endocannabinoid ligands which is distinct from CB1 and CB2 (see Baker et al., 2006 for review). Given the therapeutic potential of CB1 and CB2 compounds as analgesic agents (see Fox and Bevan, 2005 for review) and the GPR55 expression pattern (central and peripheral nervous systems and immune cells), we have postulated that GPR55 compounds may also have analgesic activity. However, as there are no selective GPR55 compounds available, controversy remains over the function of GPR55. For example, the mixed CB1/CB2 agonist CP55940 has been shown to be a GPR55 antagonist, inverse agonist or agonist. In order to investigate the potential role of the GPR55 receptor in pain, we have generated a GPR55 knockout mouse line for testing in models of inflammatory and neuropathic pain.

We will report data on the behavioural analysis of GPR55 knockout and wildtype mice following a hot-plate test, inflammatory pain using an established Freund's complete adjuvant (FCA) model and neuropathic pain using the Seltzer model of partial nerve ligation. In addition, we will also examine the effect of knocking out GPR55 on the production of a number of inflammatory cytokines to gain further information about a role for GPR55 in inflammatory pain. Data from all these studies will be discussed to determine the role of GPR55 in inflammatory and neuropathic pain.

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Baker D. et al. (2006) TIPS 27:1-4.

EFFECT OF A CANNABIS SATIVA EXTRACT ON NOCICEPTIVE BEHAVIOUR IN A RAT MODEL OF NEUROPATHIC PAIN

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In recent years, many reports suggested that combinations of cannabinoids may provide benefits that surpass treatment with single ones and may be potentially useful in the treatment of many diseases, such as chronic pain. On these bases, the aim of this research was to develop a novel analgesic regimen using low dose combinations of cannabinoids to treat neuropathic pain, a debilitating chronic pain refractory to actual drugs, induced in the rat through the chronic constriction injury of sciatic nerve (CCI) model. We used an high CBD extract (H-CBD) containing 96% of CBD plus 4% of Δ^9-THC in comparison to pure CBD or pure Δ^9-THC. We have previously demonstrated the antihyperalgesic properties of CBD when orally given to CCI rats (Costa B. et al., Symposium on the Cannabinoids, Burlington, Vermont, International Cannabinoid Research Society, 25, 2004). The present study wanted to investigate whether a combination of a dose of CBD that did not reach a total effect (10mg/kg) with a low dose of Δ^9-THC was able to abolish nociceptive behaviour. Rats were orally treated daily for a week, starting from the 7th day following the injury. A group received CBD (10mg/kg), a second group received H-CBD (CBD 10mg/kg plus Δ^9-THC 0.42mg/kg) and a third group received Δ^9-THC at the corresponding mixture dose (0.42mg/kg). As expected, CBD treatment partially relieved thermal and mechanical hyperalgesia and mechanical allodynia. When CBD was administered through H-CBD, we observed a total relief of thermal and mechanical hyperalgesia and no change of partial effect on mechanical allodynia, in spite of Δ^9-THC chronic treatment reduced neither thermal and mechanical hyperalgesia nor mechanical allodynia. These data suggested that the chronic treatment with H-CBD evoked pain relief in CCI rats improving the effects of single cannabinoids. Subsequently, we investigated whether an alteration in pharmacokinetic phase was responsible for H-CBD synergic effect observed by us. As known, cannabinoids were able to modulate the metabolic system of cytochrome P450, a superfamily of isoforms that play an important role in their oxidative metabolism. The data obtained by us revealed an inhibition of cytochrome P450 total content in liver by H-CBD and new studies are clarifying the isoforms implicated in this mechanism. Another protein, topic of our current study, responsible for the low or high oral bioavaibility of many drugs is represented by the intestinal P-glycoprotein (P-gp), an ATP-dependent efflux transporter coded by the MDR1 gene whose activity could be inhibited by CBD, as shown in a recent report (Zhu et al., JPET, 2006). In conclusion, our data showed a synergic effect between CBD and Δ^9-THC, probably due to a modulation of CBD metabolism by Δ^9-THC or to an inhibition of P-gp. We cannot exclude that such synergism can occur during pharmacodynamic phase. Works are in progress to determine whether the antihyperalgesic effect of this preparation can be mediated via CB1, CB2 and/or TRPV1 receptors.

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DORSAL ROOT GANGLIA (DRG) AND SPINAL CORD AS THE SITES OF ACTION FOR CB_2 RECEPTOR MEDIATED ANALGESIC ACTIVITIES

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Cannabinoid CB_2 receptor activation by receptor selective agonists, such as AM1241, HU-308 and GW405833 has been demonstrated to produce analgesic effect in preclinical models of inflammatory and neuropathic pain. CB_2 receptor mediated analgesia can offer a significant advantage, as the undesirable psychoactive side effects associated with non-selective cannabinoids (such as THC) are known to be mediated by the activation of the CB_1 receptor subtype.

The CB_2 receptor is expressed extensively in various immune tissues, such as spleen, tonsil, thymus, lymphocytes and PMNs. Recent reports have indicated that CB_2 receptors are also expressed in the central and peripheral nervous systems. However, the mechanisms underlying the analgesic effects of CB_2 agonists remain largely unknown. In order to gain further understanding of CB_2-mediated analgesia, CB_2 gene expression was profiled using quantitative PCR in various tissues obtained from animals under both chronic inflammatory and neuropathic pain conditions. We found that CB_2 gene expression was significantly up-regulated in the ipsilateral DRGs following L5-L6 spinal nerve injury compared to that of sham animals. There was no significant change in the level of CB_2 expression in the brain stem, thalamus, cerebellum and cortex. Similar expression profiles of the CB_2 receptor gene were also observed in tissues from animals under inflammatory pain conditions, although the level of increase in the CB_2 gene expression was not as extensive as that observed following nerve injury. In all tissues tested, the level of CB_1 gene expression remained largely unchanged in both injured and sham animals.

To further support a role of CB_2 receptors in the DRGs and spinal cord in CB_2-mediated analgesia, we evaluated the effects of CB_2-selective agonist AM1241 following intra-DRG or intrathecal administration in rats of nerve injury models. AM1241 exhibited full efficacy upon intra-DRG or intrathecal administration at doses well below those required to produce a comparable effect upon systemic administration, suggesting that the DRGs and spinal cord are the sites contributing to CB_2 receptor mediated analgesia.

In summary, increased CB_2 gene expression in the DRGs and spinal cord in animal models of inflammatory and neuropathic pain is a likely mechanism to regulate pain perception.

THE ROLE OF CANNABINOID CB_1 RECEPTORS IN PAIN THRESHOLD OF MICE STUDIED IN CHEMICAL AND THERMAL PAIN

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Opioids and cannabinoids are among the most illegally consumed drugs. Previous studies have demonstrated that both types of drugs produce similar pharmacological effects such as hypothermia, sedation, hypotension, inhibition of locomotor activity, intestinal motility and, in particular, analgesia. In the present study we investigated the changes of the pain threshold of CB_1 knock out (CB_1-/-) and wild mice (CB_1+/+) in three different analgesiometric tests using chemical (acetic acid induced writhing) or heat (hot plate and tail flick) stimulus and the sensitivity of CB_1-/- animals to the antinociceptive action of morphine. Writhing, tail-flick and hot plate tests were done as described previously (Koster et al., 1959, Tulunay et. al, 1982, Loh et al., 1976). All drugs were administered subcutaneously (s.c.). The numbers of writhes were 15.67 ± 2.33, (n=24) in CB_1-/- mice, and 22.83 ± 1.45 (n=23) in CB_1+/+ animals ($p< 0.05$, t test). To verify this tendency we studied and compared the latency of pain reaction in tail-flick and hot plate tests in CB_1-/- and CB_1+/+ mice. In the tail-flick test the latency times were 3.80 ± 0.23 sec (n=57) and 2.30 ± 0.10 sec (n=55) in CB_1-/- and CB_1+/+ mice, respectively, similarly to hot plate test, where the latency times were found to be 58.72 ± 7.47 sec (n=9) and 20.70 ± 4.22 sec (n=9) in CB_1-/- and CB_1+/+ mice, respectively. In all tests, statistical analysis revealed that deletion of CB_1 receptor significantly increased the pain threshold in mice. Subcutaneous naloxone restored the pain threshold of CB_1-/- mice. Moreover, s.c injections of 0.5 mg/kg morphine reduced the number of writhes to 7.73 ± 3.54, (n=11) in CB_1-/- mice and to 18.67 ± 2.49, (n=12) in CB_1+/+ mice, demonstrating a higher sensitivity of CB_1-/- mice toward morphine analgesia. Our present data indicate that the lack of CB_1 receptor in mice increased the pain threshold to chemical agent and heat induced pain, accordingly augmented the antinociceptive effect of morphine. Our results support the hypothesis on the interaction of cannabinoid and opioid systems in antinociception.

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CANNABINOIDS SUPPRESS CHEMOTHERAPY-EVOKED PAINFUL NEUROPATHY THROUGH ACTIVATION OF CB_1 AND CB_2 RECEPTORS

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Chemotherapeutic treatment can induce several severe side-effects including neuropathic pain. The present study was conducted to evaluate the efficacy of cannabinoids in suppressing behavioral sensitization to mechanical stimulation (tactile allodynia) induced by treatment with the anti-tumor agent vincristine in rats. Mechanical hypersensitivity developed over the course of ten once-daily injections of vincristine relative to groups receiving saline at the same times. WIN55,212-2 (0.75, 1.5 and 2.5 mg/kg i.p.), a potent cannabinoid CB_1/CB_2 agonist, induced a dose-dependent suppression of mechanical hypersensitivity in vincristine-treated animals. By contrast, WIN55,212-3 (2.5 mg/kg i.p.), the receptor-inactive enantomer of WIN55,212-2, did not alter mechanical withdrawal thresholds relative to vehicle. The CB_1 antagonist SR141716 (2.5 mg/kg i.p.) and the CB_2 antagonist SR144528 (2.5 mg/kg i.p.) both blocked the anti-allodynic effects of WIN55,212-2. To further confirm a role for CB_2 receptors in the WIN55,212-2-induced attenuation of vincristine-induced painful neuropathy, we compared the effects of the CB_2 selective agonist AM1241 and the opioid analgesic morphine on vincristine-induced tactile allodynia. AM1241 (2.5 mg/kg i.p.) induced a time-dependent suppression of vincristine-induced mechanical hypersensitivity relative to vehicle, and this effect was blocked by the CB_2 antagonist SR144528 but not by the CB_1 antagonist SR141716. The WIN55,212-2-induced suppression of tactile allodynia was greater and of longer duration than that induced by AM1241. By contrast, morphine (2.5 mg/kg i.p.) did not alter vincristine-induced mechanical hypersensitivity. The anti-allodynic effects of the cannabinoids in this model were independent of any effects on motor behavior. Both AM1241 and WIN55,212-2 failed to induce catalepsy at doses that suppressed vincristine-induced mechanical hypersensitivity. The present results demonstrate that cannabinoids effectively reduce vincristine-induced mechanical hypersensitivity through activation of both CB_1 and CB_2 receptors.

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NOVEL CANNABINOID RECEPTOR AGONISTS : IN VITRO AND IN VIVO CHARACTERIZATION OF IMINOTHIAZINE DERIVATIVES

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Besides THC, the cannabinoid receptor agonists have also been shown to have antinociceptive activity in rodents. It has been considered that CB1 is mainly responsible for this antinociceptive activity of the cannabinoid agonists, but, recently, there have been many reports indicating that the activation of CB2 also produces antinociception. In this study, to clarify the involvement of these two types of receptors for antinociception, we synthesized a series of iminothiazine derivatives as orally available novel cannabinoid agonist, and characterized their activity in vitro and in vivo. Compound **1** showed CB1/CB2 non-selective activity, with Kd values of 30 nM for human CB1 and 6 nM for human CB2. Compound **1** inhibited forskolin induced cyclic AMP elevation in CHO cells that expressed human CB1 or CB2 at IC50 values of 10 nM and 1.7 nM, respectively. In the formalin test in mice, orally administered Compound **1** caused significant dose-dependent inhibition of both the early (acute pain) and the late (inflammatory pain) phases of formalin induced licking; ED50 was 1.5 mg/kg and 1.0 mg/kg, respectively. This effect was reversed by CB1 antagonist SR141716A, but not CB2 antagonist AM630. On the other hand, the ED50 of CB1 mediated side effect (catalepsy and rotarod performance) was more than 10 times higher than that of the antinociceptive effect, suggesting that Compound **1** acts mainly on peripheral CB1. Compound **1** also reduced the Seltzer model of neuropathic pain in rats (ED50, 0.6 mg/kg, p.o.). Compound **2** was a CB2 selective agonist, with Kd values of 9 nM for human CB2 and >5000 nM for human CB1. In the formalin test in mice, Compound **2** also caused significant inhibition of both the early and late phases of formalin induced licking when coinjected with formalin. This effect was reversed by CB2 antagonist SR144528, but not SR141716A. In conclusion, we discovered a novel series of cannabinoid agonists that possess antinociceptive activity through CB1 or CB2.

REPEATED TREATMENT WITH SELECTIVE INHIBITORS OF THE ENZYME FATTY-ACID AMIDE HYDROLASE ALLEVIATES NEUROPATHIC PAIN

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Inhibitors of enzyme fatty-acid amide hydrolase (FAAH) have been suggested as a novel therapeutic strategy for the treatment of several diseases including pain (Lichtman et al., 2004). Here we have used a model of neuropathic pain, consisting of rats with chronic constriction injury (CCI) of the sciatic nerve, to investigate the effect of repeated treatment with selective inhibitors of the FAAH enzyme on thermal hyperalgesia and mechanical allodynia. Repeated treatment with URB-597 (3 mg/kg, s.c.), OL-135 (3 mg/kg, s.c.), and arachidonoyl serotonin (AA-5-HT, 5 mg/kg, s.c.) throughout the development of neuropathic pain all reverted both thermal hyperalgesia and mechanical allodynia 7 days after surgery. The doses employed of URB-597, OL-135 and AA-5-HT did not affect pain response of the contralateral paw neither they changed the thermal withdrawal latency and mechanical withdrawal threshold in sham-treated rats. The anti-hyperalgesic effects of AA-5-HT were prevented by daily co-administration with AM251 (1 mg/kg, s.c.), a selective CB_1 receptor antagonist. Conversely, AM630 (1 mg/kg, s.c.), a selective CB_2 receptor antagonist, did not change the effects of AA-5-HT when daily administered in combination with the FAAH inhibitor. Furthermore, capsazepine (CPZ, 2.5 and 10 mg/kg, s.c.), a selective TRPV1 receptor antagonist, blocked AA-5-HT-induced reversion of mechanical allodynia at the highest dose used. Finally, CPZ, when daily administered *per se* throughout the development of neuropathic pain, reversed thermal hyperalgesia at both doses used. In conclusion, the increase of endocannabinoid tone obtained by inhibiting endocannabinoid metabolism relieves neuropathic pain following CCI of the sciatic nerve. The anti-hyperalgesic action of AA-5-HT appears to involve both CB_1 and $TRPV_1$ receptors.

Lichtman A.H, Leung D., Shelton C.C., Saghatelian A., Harduin C., Boger D.L., Cravatt B.F. The Journal of Pharmacology and Experimental Therapeutics 311: 441-448, 2004.

CHANGES IN ENDOCANNABINOID AND PALMITOYLETHANOLAMIDE LEVELS IN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS IN AN ANIMAL MODEL OF NEUROPATHIC PAIN

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The analgesic properties of exogenous and endogenous cannabinoids have been investigated, and suggest a regulatory role for the endocannabinoid system in models of either acute nociception or inflammatory and neuropathic pain. Pharmacological studies have shown that activation of the CB_1 receptor by synthetic agonists suppresses hyperalgesia in several rat models. Also administration of palmitoylethanolamide (PEA), a cannabinoid-receptor inactive analogue of the endocannabinoid anandamide (AEA), has been shown to produce analgesic effects, particularly on inflammatory pain. However, in animal models of neuropathic pain, the concentrations of endocannabinoids in the nervous tissues involved in pain transmission have never been assessed. Here we have determined, in the chronic constriction injury (CCI) model in the rat, the levels of AEA, 2-arachidonoylglycerol (2-AG), and PEA in three brain areas involved in nociception, i.e dorsal raphe (DR), ventrolateral periaqueductal grey (PAG) and rostral ventral medulla (RVM), as well as in the spinal cord (SC).

Experiments were conducted with male Wistar rats and CCI was performed according to the method described by Bennet and Xie. The same procedure was performed in control animals except that the placement of the ligatures around the nerve was omitted (sham-operated animals). The animals were sacrificed after 3 and 7 days from CCI and the tissues were immediately removed and stored at –80° until lipid extraction. Tissues concentrations of AEA, 2-AG and PEA were then measured by means of isotope dilution LC-MS and expressed as pmol or nmol/g wet tissue weight.

After 3 days from CCI, AEA and 2-AG levels were significantly enhanced only in the SC and PAG, respectively. After 7 days from CCI, AEA levels were enhanced in the DR and SC, and 2-AG levels were enhanced in the SC and RVM. A maximal increase of the both anandamide and 2-AG levels was observed in the PAG at this time point, when thermal hyperalgesia and mechanical allodynia are maximal. On the contrary PEA levels were significantly decreased in the SC after 3 days from CCI, and in the DR and RVM after 7 days from CCI.

These data suggest that both AEA and 2-AG, operating at the level of both central and peripheral nervous systems, may be enhanced to inhibit chronic pain, and that a decrease of PEA levels might instead contribute to chronic pain. Our data also suggest that inhibitors of the proteins involved in endocannabinoid inactivation, particularly if they enhance also PEA and 2-AG levels, might be useful new analgesic drugs against chronic pain.

CANNABINOID RECEPTORS CB1 AND CB2 EXPRESSION BY OSTEOARTRITHIC AND RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTES

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Several cannabinoids have been demonstrated to exert an anti-inflammatory effect and to reduce histological damage in animal models of arthritis. At present, at least two cannabinoid receptors are well known and characterized, namely CB1 and CB2. In the light of the fact that synoviocytes are known to play an active role in the pathogenesis of rheumatoid synovitis, we aimed at demonstrating fibroblast-like synoviocytes (FLS) expression of cannabinoid receptors

Patients and Methods: FLS were obtained from 3 patients with erosive rheumatoid arthritis, and from 3 patients with knee osteoarthritis. In both groups, synovial samples were excised during knee replacement surgery. FLS were used for the experiments at the third passage.

mRNA and protein expression corresponding to CB1 and CB2 were evaluated by quantitative Real Time-PCR (RT-PCR) and western blot assay, respectively, in basal conditions, and after 1 hour incubation with 0.5 ng/ml IL-1β.

Results: RT-PCR showed the constitutive presence of CB1 mRNA and CB2 mRNA in both rheumatoid and osteoarthritic FLS, without any relevant difference between the two groups. Western blot analysis demonstrated that cells from rheumatoid and osteoartrhitic patients express CB1 receptor either in basal conditions and after stimulation with IL-1β. Conversely, we did not find any evidence of CB2 expression in the two cell lines neither in basal conditions nor after stimulation with IL-1β.

Conclusion: Our results show that FLS express the cannabinoid receptor CB1, as demonstrated by the studies of mRNA synthesis and protein expression. On the other hand, cannabinoid receptor CB2, which is known to be deeply involved in several non-rheumatoid immuno-inflammatory responses, does not seem to be expressed on osteoarthritic as well as rheumatoid FLS. In addition we did not found any relevant different in CB1 and CB2 mRNA levels among osteoarthritic and rheumatoid FLS.

IMMUNOLOCALISATION OF THE ANANDAMIDE DEGRADING ENZYME: FATTY ACID AMIDE HYDROLASE (FAAH) IN RAT DORSAL ROOT GANGLION AND SPINAL CORD TISSUE

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Introduction: Activity of the FAAH enzyme is critical for the degradation of endocannabinoids such as anandamide. Pharmacological inhibition or genetic deletion of FAAH increases endocannabinoid levels in nervous tissue and reduces pain sensitivity in animal models via cannabinoid receptor activation (Cravatt & Lichtman, J. Neurobiol. 2004 61:149-60). The aim of this study was to map the distribution of FAAH immunoreactivity in DRG and spinal cord tissue as a method to indicate the sites of metabolism for endocannabinoids implicated in modulating nociceptive transmission.

Methods: Immunohistochemistry: Naïve adult rats were perfused with 4% paraformaldehyde in 0.01M phosphate buffer. Cryosections of lumbar DRG or spinal cord were incubated with 10% normal donkey serum, then affinity purified polyclonal antibody raised against ΔTM–FAAH for 48hrs. DRG neurone populations and nuclei were co-stained and tissue processed for signal amplification and antibody revelation. FAAH-immunoreactive (ir) cells containing a NeuN or DAPI stained nucleus were counted and areas measured using computerised pixel intensity threshold detection methods. Immunoblotting: Samples of rat DRG, spinal cord and sciatic nerve tissue (as well as DRG tissue from FAAH knockout (-/-) and wild type (+/+) mice) were homogenised in lysis buffer, incubated on a roller at 4 degrees for 1hr and then centrifuged. Supernatant samples were normalised for protein content and subjected to electrophoresis on SDS-PAGE gels, transferred to a nitrocellulose membrane and blotted with the ΔTM–FAAH antibody followed by HRP-conjugated secondary antibodies. Visualization using chemiluminescence was followed by immunoblotting for a loading control protein.

Results: FAAH-ir was detected in 32.8 ±1.0 % of NeuN-ir naïve rat L4 DRG neurones (mean ± s.e.m, 1402 cells, n=4). The area of FAAH-ir soma 385.7 ±6.4 μm^2 was significantly smaller than NeuN-ir soma 510.2 ±9.7μm^2 (mean ± s.e.m p=0.002 Mann Whitney rank sum test). 64.5 ±3.2 % of FAAH-ir neurones co-labelled with TRPV1-ir representing 58.2 ±9.4% of the TRPV1-ir population. 64.2 ±2.5 % of IB4 labelled neurones and 30.1 ±10.8% of weakly CGRP-ir soma contained FAAH-ir. <1% of FAAH-ir neurones co-stained for NF200-ir. In spinal cord, FAAH-ir was found in nucleated cells from white and grey matter including NeuN-ir neurones and non NeuN-ir cells from dorsal and ventral horns. Western blotting using FAAH antibodies detected the presence of immunoreactive bands (approximately 60kDa in size) from tissue samples of rat and mouse DRG and also rat spinal cord, cerebellum and sciatic nerve. FAAH immunoreactivity was not detected in DRG tissue from FAAH (-/-) mice using either immunohistochemistry or immunoblotting methods.

Conclusions: FAAH-ir is found in neuronal and non-neuronal cells in the rat spinal cord and in small neurones of L4 DRG. Most FAAH-ir DRG soma are IB4 positive, marking the peptide-poor sub-population of primary afferent C-fibres. Around 60% of FAAH-ir neurones co-stained for TRPV1, a marker of nociceptive C-fibres.

CHRONIC DELIVERY OF WIN55,212-2 TO THE SITE OF A PERIPHERAL NERVE INJURY ATTENUATES MECHANICAL AND COLD ALLODYNIA

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Introduction: Cannabinoids are reported to reduce reflex responses in rodent neuropathic pain models when applied locally to peripheral tissues at systemically inactive doses (Fox et al., Pain. 2001. 92: 91-100; Guindon & Beaulieu, Neuropharmacol. 2006. Epub. January 23). This effect is reversed by local application of cannabinoid receptor antagonists, indicating that the analgesic mechanism involves peripheral cannabinoid receptors activated independently of those in the CNS. In these studies, compounds were injected into the hind paw skin. The hind-paw was also used to deliver sensory stimuli for testing reflex behaviours, thus introducing difficulties in distinguishing between a possible antinocieptive action of cannabinoids on peripheral nerve endings in hind paw skin. The aim of this study was to deliver cannabinoids directly to the site of a nerve injury, away from the sensory testing site, at systemically inactive doses and to examine effects on pain hypersensitivity measured using reflex behavioural paradigms.

Methods: WIN55,212-2 (WIN-2) or a vehicle solution was delivered at a rate of 0.6-2.8µg/µl/hr to the site of a partial ligation injury to the sciatic nerve by means of a silastic catheter connected to a mini-osmotic pump (Alzet model 2001). The pump and supply catheter were implanted at the time of nerve injury and kept in place for six days during the testing of reflex behavioural responses to mechanical and cold stimulation applied to the plantar surface of both injured and uninjured hind limbs. Testing took place at day 2, 4 and 6 after surgery by an investigator that was blinded to the treatments.

Results: For the treatment group receiving WIN-2 at 1.6µg/µl/hr (n=7) the mean (± s.e.m) force of mechanical stimulation required for reflex withdrawal of the injured hind limb was (34.9 ± 3.5 grams) on day 4 after injury (n=7). These values were significantly elevated compared to those of the vehicle-treated control group on the same day (20.5 ± 1.0g, $P<0.05$ One Way ANOVA, Tukey test). The response of injured limbs to a cold (acetone drop) stimulus also fell significantly compared to the vehicle treated animals on both day 2, 4 and day 6 after injury (Kruskal-Wallis One way ANOVA on ranks $P<0.05$). When a double dose of WIN-2 2.8µg/µl/hr was applied (n=6) there was a significant effect of treatment on day 2 after surgery for both pain measures. To control for a possible systemic analgesic action of WIN-2 in these experiments, both doses of WIN-2 were delivered by a catheter to the opposite side to nerve injury (n=6). This had no significant effect on pain behaviour measured on the injured or uninjured side, compared to vehicle treated controls. The maximum total daily dose of WIN-2 (0.02mg/kg/day) was lower than the range reported for systemic analgesic doses of WIN-2 used in neuropathic rats (0.4mg/kg – 2.5mg/kg: Fox et al., 2001; Bridges et al., Brit. J. Pharmacol. 2001. 133:586-594).

Conclusion: These data suggest that the cannabinoid WIN55,212-2 can reduce pain hypersensitivity that develops after a partial injury to the sciatic nerve when it is supplied directly and continuously the site of the nerve injury by means of a perineural catheter connected to a mini-osmotic pump. This effect is likely to be mediated by activity at peripheral cannabinoid receptors in the injury area rather than by systemic absorption of the drug, or an antinocieptive action on peripheral nerve endings in the hind paw skin area receiving the test stimulus.

IMMUNOLOCALIZATION OF THE ENDOCANNABINOID AND PROSTAGLANDIN SYSTEMS IN THE MOUSE SPINAL CORD

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The endocannabinoid system mediates analgesia in the CNS. In the spinal cord, nociceptive information is relayed through Aδ and C fibers that run through the zone of Lissauer (adjacent to lamina I) and terminate in the substantia gelatinosa (lamina II). It is well known that Lamina I exhibits immunoreactivity (IR) for substance P (Mantyh et al. Science 1997 278:275-279) and the TRPV1 receptor (Caterina et al. Science 2000 288: 306 - 313). Here, we investigated the immunolocalization patterns of other receptors and enzymes mediating endocannabinoid signaling within the termination field of pain fibers in mouse spinal cord. In addition, immunoreactivity (IR) of cyclooxygenase-2 (COX-2), an enzyme responsible for both the production of prostaglandins and the metabolism of endocannabinoids into prostamides (Yu et al. J Biol Chem. 1997 272:21181-6; Kozak et al. J Biol Chem. 2000 275:33744-9), and EP_2, the receptor mediating prostamide signaling, was examined.

Methods: Polyclonal antibodies to the C-terminus of human cannabinoid receptor 1 (CB1), amino acids 584-598 of murine cyclooxygenase-2 (COX-2), aa 335-358 of the human EP_2 receptor, aa 1-14 of monoglyceride lipase (MGL) were purchased from Cayman Chemical, and an antibody generated against aa 561-579 of rat fatty acid amide hydrolase (FAAH) was purchased from Alexis Biochemicals. A standard immunocytochemistry protocol was utilized. Briefly, cryostat sections of WT and FAAH KO spinal cord were blocked for 20 min in 2% normal goat serum then overnight in diluted primary antibody at 4°C. After washing in PBS for 30 min, tissue was blocked again and incubated with 1:800 donkey-anti-rabbit-Alexa 594 (Molecular Probes) for 35 min at 37°C. Sections were observed with a Zeiss epifluorescence microscope with a filter set optimized for Alexa 594 viewing.

Results: CB1-IR and FAAH-IR exhibited prominent staining in lamina I-II. MGL-IR in lamina I was robust, with moderate IR also observed in lamina II. While COX-2-IR was diffuse, moderate EP_2-IR was observed in lamina I-II. Immunolocalization in other spinal cord regions, and any observed compensatory changes in IR patterns in FAAH knockout spinal tissue will be discussed. These data support previous rat spinal cord immunohistochemical studies, and provide further evidence of endocannabinoids and prostamides mediating nociception in the mouse.

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THE EFFECT OF THE TRPV1 RECEPTOR ANTAGONIST, CAPSAZEPINE ON THE ANTINOCICEPTIVE POTENCY OF ANANDAMIDE AT SPINAL LEVEL

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Several studies suggest that the endogenous cannabinoid receptor ligand, anandamide activates the TRPV1 receptors too, but there is no *in vivo* study indicating the role of the TRPV1 receptor activation in the effect of anandamide at spinal level. The goal of this study was to investigate the effect of the TRPV1 receptor inhibition by capsazepine on the antinociceptive potency of anandamide.

Intrathecal catheters were implanted into male Wistar rats. After recovery, acute nociceptive sensitivity was assessed by tail-flick test. Unilateral inflammation of a hindpaw was produced by carrageenan (1.5 mg intraplantarly), and the pain threshold was assessed by paw withdrawal (PWD) test. Dose-dependent effects were determined for anandamide (1-100 µg) and for its combinations with 10 or 20 µg capsazepine. The capsazepine was administered 30 min before anandamide. Groups were compared by ANOVA with $P<0.05$ considered significant.

Anandamide by itself caused a slight antinociception at the normal side on the PWD test. It produced dose-dependent antihyperalgesia and at higher doses a prolonged effect was also observed. However, the highest dose caused temporary excitation, roaring, expressing pain sensation. Capsazepine by itself did not influence the pain sensitivity at normal and inflamed sides, but it dose-dependently decreased the antinociceptive effect of the highest dose of anandamide. In contrast, capsazepine increased the antinociceptive potency of 100 µg anandamide on the tail-flick test.

Our results suggest that the TRPV1 receptor activation plays a significant role in the antinociceptive effects of anandamide at spinal level. The effect of the inhibition of TRPV1 receptor depended on the pain test. We suppose that the activation of TRPV1 may cause the release of antinociceptive endogenous ligands at spinal level providing increased antinociception during inflammation.

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NEUROPSYCHOPHARMACOLOGICAL CHARACTERIZATION OF A SULFONAMIDE COMPOUND [4-(3-CYCLOPENTYL-INDOLE-1-SULFONYL)-N-(TETRAHYDRO-PYRAN-4-YLMETHYL)-BENZAMIDE], AS A NOVEL, POTENT, SELECTIVE AND ORALLY ACTIVE CB1 CANNABINOID RECEPTOR ANTAGONIST

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A structurally distinct sulfonamide compound [4-(3-cyclopentyl-indole-1-sulfonyl)-N-(tetrahydro-pyran-4-ylmethyl)-benzamide] is presented as a novel, potent, selective, and orally active antagonist of the CB1 cannabinoid receptors. In a functional GTP-γ-35S binding assay, this sulfonamide is a potent antagonist at human CB1 receptors (Kb = 0.89 nM) expressed in Sf9 cell membranes, whereas it is weakly potent at human CB2 receptors (Kb > 5 μM); in cerebellar membranes, the compound displays nanomolar potency at rat CB1 receptors (Kb = 3.86 nM). Furthermore, it shows little affinity for a number of receptors, transporters and enzyme targets tested (IC50 > 5 μM). *In vivo*, 3 hr after oral administration, this compound exhibited high levels of CB1 receptor occupancy in the frontal cortex of the rat (ED50 = 0.74 mg/kg). Oral admininistration (2.5, 5 and 10 mg/kg, 90 min pretreatment) of this sulfonamide dose-dependently attenuated the hypothermic effects of the direct full CB1 receptor agonist CP55,940, indicating functional CB1 receptor antagonism *in vivo*. Oral administration of this compound (0.5, 2.5 and 10 mg/kg; once daily for 14 days) to diet-induced obese rats reduced food intake and produced a significant reduction in body weight in a dose-dependent manner. Analysis of changes in body composition indicated that the reduced body weight resulted from a significant reduction in fat mass. This sulfonamide (1-10 mg/kg, p.o.) in mice reduced immobility in the forced swim test of antidepressant-like activity in a dose-dependent manner, an effect that was retained after 5 days of administration (once daily). Finally, acute oral administration of this compound (3 and 10 mg/kg) dose-dependently increased efflux of dopamine, norepinephrine and acetylcholine in the medial prefrontal cortex of the rat. These data portray this structurally distinct sulfonamide compound, as an orally active CB1 receptor antagonist with efficacy in animal models of obesity and neuropsychiatric disorders.

THE ENDOCANNABINOID SYSTEM IN HUMAN ADIPOSE TISSUE FROM HEALTHY SUBJECTS

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The two best studied endocannabinoids, anandamide (*N*-arachidonoyl- ethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the endogenous ligands of the central and peripheral cannabinoid receptors. Furthermore, AEA binds to the transient receptor potential vanilloid type-1 (TRPV1), a capsaicin-sensitive, non-selective cation channel. The synthesis of these endocannabinoids is catalyzed by the *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and the *sn*-1-selective diacylglycerol lipase (DAGL), whereas their degradation is accomplished by the fatty acid amide hydrolase (FAAH) and the monoglyceride lipase (MGL), respectively. Taken together, these proteins form the "endocannabinoid system".

We have investigated the presence of a functional endocannabinoid system in human adipose tissue from seven healthy subjects. Subcutaneous abdominal adipose tissue underwent biochemical and molecular biology analyses, aimed at testing the expression of this system and its functional activity. AEA and 2-AG levels were detected and quantified by HPLC. Real time PCR analyzed the expression of the endocannabinoid system, and immunofluorescence assays showed the distribution of its components in the adipose tissue. Furthermore, binding assay for the cannabinoid and vanilloid receptors and activity assay for each metabolic enzyme of the endocannabinoid system gave clear evidence of a fully operating system.

The data presented herein show for the first time that the human adipose tissue is able to bind AEA and 2-AG and that it is endowed with the biochemical machinery to metabolize endocannabinoids.

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ASSOCIATION BETWEEN CANNABINOID TYPE-1 RECEPTOR POLYMORPHISM AND BODY MASS INDEX IN A SOUTHERN ITALY POPULATION

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Endocannabinoids control food intake via both central and peripheral mechanisms and cannabinoid 1 receptor (CB1) modulates lipogenesis in primary adipocyte cell cultures and in animal models of obesity. We aimed to evaluate the frequency of a genetic polymorphism of CB1 and to study its correlation with body mass index and serum biochemical and lipid profiles in human. Healthy subjects from a population survey carried out in the southern Italy examined in 1992-93 and older than 65 years (N=419, M= 237 , F=182), were divided in quintiles of body mass index (BMI) and 210 subjects were randomly sampled from the first, third and fifth quintile of BMI (BMI, respectively: 16.2-23.8=normal, 26.7-28.4=overweight, 31.6-49.7=obese), 70 per each quintile. We found a clear trend of increasing relative frequency of the CB1 wild type genotype with the increase of BMI (p=0.03), and, using multiple logistic regression model, wild type genotype, female gender, age, glycemia and triglycerides were directly associated either with overweight (third quintile of BMI) or obesity (fifth quintile of BMI). The data indicate that presence of the CB1 polymorphic allele was significantly associated with lower BMI. Although performed in a limited number of subjects, our results support the hypothesis that polymorphism in the CB1 gene could mediate weight gain and contribute to specific differences in the responsiveness to endocannabinoids or to anti-obesity drugs..

INTESTINAL LEVELS OF ANANDAMIDE AND OLEOYLETHANOLAMIDE IN FOOD-DEPRIVED RATS ARE REGULATED THROUGH THEIR PRECURSORS

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The anorectic lipid oleoylethanolamide (OEA) and the orexigenic lipid anandamide (AEA) both belong to the group of *N*-acylethanolamines (NAEs) that are generated by the enzyme *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). The previously reported diverging levels of OEA and AEA during starvation were investigated in rat intestines after 24 hours of starvation as well as after re-feeding.

Total levels of precursor phospholipids (NAPEs) and NAEs as measured by mass spectrometry were decreased upon food-deprivation whereas the level of the AEA precursor molecule was significantly increased. The level of 2-arachidonoyl-glycerol was unchanged as was the activity of *N*-acyltransferase, NAPE-PLD, and fatty acid amide hydrolase (FAAH) upon starvation and re-feeding. The effect of oral administration of linoleoylethanolamide (LEA), which accounted for more than 50 mol% of the endogenous pool of NAEs, was compared to oral administration of OEA with continuous registration of food intake, water intake and activity for 18h after drug administration.

It is concluded that remodeling of the amide-linked fatty acids of NAPE is responsible for the opposite effects on levels of AEA and OEA in intestines of food-deprived rats and not an alternative biochemical route for AEA synthesis. Furthermore, LEA did not have the same inhibitory effect on food intake as did OEA.

ANANDAMIDE INDUCES THE DIFFERENTIATION OF RAT PREADIPOCYTES THROUGH THE INDUCTION OF PPARγ

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White adipose tissue (WAT) is the major energy store in the body. The enlargement of this tissue during the development of obesity is attributed to the larger number and size of adipocytes, the main cell type of adipose tissue. The increase in the number of adipocytes proceeds through the differentiation of fibroblast-like preadipocytes into mature spherical cells.The ability of adipose tissue to increase its mass even during adulthood through the differentiation of preadipocytes to adipocytes has been shown. Arachidonyl ethanolamide, (anandamide, AEA), regulates the body weight mainly through feeding modulation and exhibits a lipolytic effect on adipocytes but not via CBl and CB2 receptors. Oleoylethanolamide, a structural analogue of AEA, has been identified as PPARγ (Peroxisomal Proliferator Activated Receptor gamma) ligand. Recently, it has been reported that AEA induces transcriptional activation of PPARγ and differentiation of 3T3-L1 preadipocytes. Confluent primary preadipocyte cultures from rat epididymal adipose tissue, were incubated in the presence or absence of AEA and the degree of differentiation was measured by triglyceride Oil Red O staining and by semiquantitative determination of PPARγ gene expression. The presence of CB2 receptors in preadipocytes was monitored by western blotting. Adipocyte differentiation was increased by AEA in a dose- and time-dependent manner, reaching a plateau at 50 μM and at 312 hours. In order to exclude the possibility that AEA exhibited this effect through its conversion to arachidonic acid, the homogenate of preadipocytes was incubated with [^{3}H]AEA, but activity of the enzyme responsible for AEA hydrolysis, FAAH (fatty acid amide hydrolase), was not detected under the testing conditions. In addition VDM11, an inhibitor of the putative anandamide membrane transporter, caused a reduction on the effect induced by anandamide on the differentiation process. Furthermore, AEA induced PPARγ expression in primary cultures of preadipocytes from rat epididymal adipose tissue as it has been reported for 3T3-L1 cells. Finally, the differentiation process seems to affect CB2 expression levels.

CANNABINOIDS ALTER NFκB BINDING IN MICROGLIA

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Microglia, as resident macrophages of the brain, play a major role in remodeling and regeneration of the central nervous system (CNS). However, a variety of neuropathies, including AIDS dementia, Alzheimer's disease and Parkinson's disease, are associated with microglial persistent elicitation of pro-inflammatory cytokines, chemokines and reactive nitrogen intermediates that may be contributive, if not causative, of brain dysfunction. *In vitro* experiments have demonstrated that the partial cannabinoid agonist Delta-9-tetrahydrocannabinol (THC) and the potent synthetic cannabinoid agonist CP55940 down-regulate the robust production of pro-inflammatory cytokines elicited in response to bacterial lipopolysaccharide (LPS). These compounds bind to CB_1 and CB_2 cannabinoid receptors found on microglia, initiating a signaling cascade that modulates cytokine expression apparently at the promoter and transcriptional levels. NFκB is a universal transcription factor involved in regulating gene expression of pro-inflammatory cytokines. While this transcription factor can be comprised of several different subunits (p65, Rel B, c-Rel, p50, p52), the classical NFκB protein involved in immune regulation is the p65/p50 heterodimer. For our *in-vitro* studies, we employed the murine microglia-like cell lines BV-2 and EOC-20 to perform Electrophoretic mobility shift assays (EMSA). These assays were used to assess the effects of THC and CP55940 on the binding interactions of NFκB to its cognate promoter binding site. Results obtained from dose-response experiments indicated that binding of NFκB in LPS-induced BV-2 cells was down-regulated by both THC and CP55940. The down-regulation of NFκB occurred in a dose-related, biphasic manner. These results are consistent with reports that cannabinoid agonists suppress pro-inflammatory cytokine gene expression at the transcriptional level. Similar results were obtained using the EOC-20 cell line, indicating that the effects of cannabinoids on NFκB expression were not unique to a single microglia-like cell type. Western immunoblot analysis of cytoplasmic and nuclear protein extracts from drug-treated cells revealed that cannabinoid agonists, at levels indicative of receptor-mediated effects, altered NFκB components (p65) in the cytoplasm. These results suggest that cannabinoids modulate NFκB binding in the nucleus through events taking place in the cytoplasm such as protein synthesis, degradation, misfolding and/or phosphorylation. Collectively, these observations suggest that cannabinoids have the potential to serve as selective therapeutic agents for selective interaction with CB_1 and/or CB_2 cannabinoid receptors, which may serve as molecular targets for ablating chronic brain inflammation.

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EFFECTS OF WIN 55,212-2 ON CD4+ T-CELL PROLIFERATION AND CYTOKINE SECRETION AT NANOMOLAR CONCENTRATIONS

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Cannabinoids have been shown to exhibit immunomodulatory effects. More specifically, it has been demonstrated by others that cannabinoids can alter T-cell differentiation, proliferation, and cytokine production. *In vivo* studies have demonstrated that cannabinoids can cause a Th1 to Th2 shift as evidenced by the suppression of IFN-γ and IL-12. As CD4+ T-cells play a major role in regulating Th1/Th2 responses, we want to investigate whether cannabinoids can exert a direct effect on their function. Additionally, as CD4+ T-cells express CB2, it is believed that these effects may be mediated through through this receptor. This leads us to our present study, where we investigated the immunomodulatory effects of WIN 55,212-2 in wildtype and CB2 knockout mice derived CD4+ T cells. Spleens were extracted from c57BL/6 mice aged 8-12 weeks and placed into single cell suspension. CD4+ T-cells were separated using a MACS immunomagnetic separation column, activated with immobilized anti-mouse CD3 and anti-mouse CD28, and treated with WIN 55,212-2. CD4+ T-cells assayed for proliferation were labeled with CFSE prior to treatment and collected at day 5 for acquisition. IFN-γ, IL-2, and IL-4 secretions were quantified from supernatant at 72h using an ELISA. Preliminary findings suggest that cannabinoids do in fact modulate CD4+ T-cell proliferation and cytokine production.

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ARVANIL AND ANANDAMIDE UP-REGULATE CD36 EXPRESSION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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In this study we analysed the regulation of gene expression by arvanil and anandamide in human peripheral blood mononuclear cells (PBMCs) to clarify their immunosuppressive properties. We have previously demonstrated that the proliferative response of mitogen-activated human PBMCs was inhibited by arvanil and anandamide. In particular, arvanil more efficiently than anandamide and capsaicine inhibited the proliferation of CD3-CD28-activated PBMCs. On the basis of these findings we performed microarray assays using total RNA obtained from CD3-CD28-activated PBMCs in the presence and/or in absence of the two drugs. The percentage of inhibition of cell proliferation at the concentration of 10μM in two different experiments was 60.6%±4.9 with arvanil and 11%±2.7 with anandamide, thus confirming data previously obtained on human PBMCs proliferation. We used microarray technology to identify a regulatory pattern associated with cell proliferation in the presence of both substances. CD3-CD28-stimulated PBMCs showed a pattern of up-regulated and down-regulated genes after treatment with these substances, in particular 83 genes were up-regulated and 22 genes were down-regulated in anandamide treated cells, whereas 30 genes were up-regulated and 35 genes were down-regulated in arvanil treated cells, furthermore 10 genes were up-regulated and 3 genes down regulated in both arvanil and anandamide treated cells in comparison to the cells stimulated with CD3-CD28. We selected and analysed several genes chosen by their function in the regulation of cell proliferation. We observed that the gene transcript, and the normalized concentration for CD36 transcript displayed a concentration of around 10 times higher when compared to the normal sample, confirming the expression levels detected with Affymetrix arrays. Furthermore we detected an increased CD36 protein expression in the presence of both substances, with a higher expression induced by arvanil compared to anandamide in CD3-CD28 activated PBMCs. Our results suggest a possible role of CD36 in the pathways targeted by both these drugs.

THE ACTIVATION OF CENTRAL CANNABINOID RECEPTORS INHIBITS SALIVARY SECRETION

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It is known that Δ^9-tetrahydrocannabinol modulates autonomic neurotransmission. Therefore, we hypothesized that the central endocannabinoid system would also modulate salivary secretion by acting on the autonomic nervous system. Several studies suggest that the lateral preoptic-hypothalamic region is an important center for eliciting salivary secretion through pre-ganglionic parasympathetic nerves that project to the salivary glands in the rat. Moreover, in this study we found by immunohistochemical techniques that type 1 cannabinoid receptors (CB1-r) are densely distributed in the lateral hypothalamic area of adult male Wistar rats, supporting the hypothesis of autonomic modulation by the endocannabinoid system. In addition, we observed that the endocannabinoid anandamide (AEA, 50 ng/5µl vehicle) injected into the lateral cerebral ventricle (i.c.v) reduced significantly the salivary secretion induced by increasing doses of metacholine injected i.v. This inhibitory effect was partially but significantly blocked by previous i.c.v injection of the CB1-r antagonist, AM251 (500 ng/5µl vehicle), and totally blocked by previous injection of bicuculline (25 ng/5µl saline), a $GABA_A$ receptor antagonist. The i.c.v injection of saline or vehicle had no effect on salivary secretion. These results suggest that AEA inhibits saliva secretion by increasing GABAergic activity that inhibits the parasympathetic neurotransmission, since when the rats were decentralized by cutting the chorda tympani nerve, AEA injection was without effect. Moreover, the parasympathetic decentralization *per se* caused an inhibition of saliva secretion equivalent to that produced by AEA. On the contrary, when the superior cervical ganglion, a sympathetic ganglion, was removed, the inhibitory effect of AEA injection remained unaltered. (Supported by PICT 03-14264)

LIPOPOLYSACCHARIDE (LPS) ACTIVATES THE ENDOCANNABINOID SYSTEM IN THE SUBMANDIBULARY GLAND OF THE RAT

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It was shown previously (Lomniczi et al Am J Physiol 281:405-411, 2001) that LPS inhibits saliva secretion by increasing PGE_2 production. Also, it is well known that TNFα is rapidly released after LPS administration and mediates a number of the endotoxin functions. On the other hand, it is known that marihuana use decreases saliva secretion. Furthermore, we have previously demonstrated that type 1 and 2 cannabinoid receptors (CB-rs) are localized in the submandibulary gland (SMG) and their activation mediates the inhibition of salivation. On the basis of previous studies that showed an interaction between LPS responses and the endocannabinoid system, the aim of the present work was to investigate whether the endocannabinoid system could be activated during inflammation in the salivary glands of adult male Wistar rats. We found that anandamide (AEA) synthase activity measured by the radioconversion of ^{14}C-arachidonic acid and ethanolamine to ^{14}C-AEA was increased significantly ($p<0.01$) in SMG 3h after ip administration of LPS (5mg/Kg). Also, taking account that CB-rs are coupled to Gi proteins that respond by inhibiting adenylyl cyclase activity, we measured cAMP content 3h after LPS administration, and found that it was diminished significantly ($p<0.05$) in the SMG, which suggested CB-rs activation. Furthermore, we found that the *in vitro* incubation of SMGs slices in the presence of TNFα ($2.9x10^{-9}$M) for 30 min markedly reduced ($p<0.01$) the forskolin-induced increase of cAMP content. This effect was blocked significantly ($p<0.01$) by AM251 (10^{-5}M) but not by AM630 (10^{-5}M) (CB1-r and CB2-r antagonists, respectively), indicating that CB1-r, but not CB2-r, are implicated in the TNFα activation of cannabinoid receptors in the SMG. In summary, the present results demonstrate that the endocannabinoid system is activated by proinflammatory signals in the submandibular glands. (Supported by PICT 03-14264)

RELAXATION OF THE GASTROINTESTINAL TRACT OF *SUNCUS MURINUS* INDUCED BY CANNABIDIOLIC ACID (CBDA) AND CANNABIDIOL (CBD)

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We have previously shown that cannabidiolic acid (CBDA) and cannabidiol (CBD) cause a relaxation response in the isolated intestine of *Suncus murinus* (Cluny *et al.* **2005** ICRS 156). The aim of the present study was to investigate the possible involvement of the cannabinoid receptors in mediating a relaxation response to CBDA and its decarboxylation product, CBD.

Segments (1 cm length) taken from the intestine (2-4 cm (proximal) and 5-7 cm (central) distal to the pyloric sphincter and 2cm proximal to the anal region (terminal)) of adult male Japanese House musk shrew, Suncus murinus (77.8 ± 2.3 g) were mounted in 20 ml organ baths containing Krebs' solution (37 °C, aerated with 95 % O_2, 5 % CO_2) and left to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and contractions were recorded isometrically using a paired experimental design. Cumulative concentration response curves to CBD and CBDA (10 nM – 30μM), with a contact time of 30 min, were established in the presence and absence of the cannabinoid CB_1 receptor antagonist, AM 251 (1 μM) and the CB_2 receptor antagonist, AM 630 (1 μM). CBDA and CBD were dissolved in 2% DMSO plus 1% Tween 80. The antagonists were dissolved in DMSO and were applied to the tissues 1 hr before CBD or CBDA. Changes in tension (g) were expressed as the mean ± s.e.mean (n = 4) and analysed using the paired Student's t-test.

In all areas of the intestine neither AM 251 (1 μM) nor AM 630 (1 μM) reversed the relaxation effect of CBD or CBDA (10 nM – 30μM). Tissue responses induced by the phytocannabinoids in the presence of the antagonists were comparable to those recorded in the absence of the antagonists. Furthermore, pre-treatment with AM 251 or AM 630 alone did not have any effect on the basal tension of the tissues examined.

In conclusion the relaxation of Suncus murinus intestine induced by CBD and its cannabinoid carboxylic acid precursor, CBDA, appears unlikely to be mediated by CB1 or CB2 receptors.

STUDIES ON THE RECEPTORS MEDIATING RELAXANT RESPONSES TO THE ENDOCANNABINOID ANANDAMIDE IN THE PORCINE CORONARY ARTERY

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Anandamide is an endocannabinoid that acts as a partial agonist at CB_1 receptors, with low efficacy at CB_2 receptors (Pertwee 1999) and there is also evidence that it activates vanilloid receptors (Randall et al., 2002). Due to the presence of anandamide in the vascular endothelium it has been proposed that it may act as a bio-regulator in the cardiovascular system. The vascular pharmacology of anandamide is highly complex, with responses depending upon the species and arterial bed under study (Pacher et al., 2005). The aims of this present study were (i) to compare the relaxant responses to anandamide with those of the known CB_1/CB_2 (WIN 55,212) and CB_1 (ACEA) agonists in endothelium intact and denuded porcine coronary arteries precontracted with 5-HT (10^{-6} M) and (ii) to determine the role of hydrolyzed metabolites of anandamide, nitric oxide and vanilloid receptors in the responses to anandamide. Cumulative dose response curves to anandamide (10^{-10}-10^{-6}M) in intact arteries demonstrated that responses were only obtained in the presence (E_{max} 42.0±1.5%) but not the absence (E_{max} 5.0±1.0%; $P<0.001$) of the fatty acid amine hydrolase inhibitor phenyl methyl sulfonyl fluoride (PMSF; 1mM). Thus all subsequent experiments with anandamide were performed in the presence of PMSF. ACEA (10^{-9}-$5x10^{-6}$M) and WIN55,212 (10^{-9}-$5x10^{-6}$M) also induced concentration-dependent relaxations in intact arteries (E_{max} 40.0 ± 12.0% and 59.3±15.2%, respectively). Endothelial denudation abolished the response to ACEA and markedly attenuated the response to anandamide (Emax 17.0±3.0%; $P<0.001$), but had no effect on the response to WIN55,212 (E_{max} 59.7±13.2%). Furthermore L-NAME (10^{-3}M) abolished the response to anandamide. The relaxant response to anandamide was only partly reduced in the presence of the selective CB_1 antagonist AM251 (1μM; E_{max} 33.3±4.0%; $P<0.05$) or the vanilloid receptor antagonist capsazepine (10μM; 25.5±2%; $P<0.05$). The results of these studies suggest that in the porcine coronary artery the relaxant response to anandamide is endothelium and nitric oxide dependent. However, while part of the response to anandamide appears to be mediated by the CB_1 and the vanilloid receptor, most of the response is mediated through a non-CB_1/CB_2/vanilloid receptor.

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CORRELATIONS BETWEEN SERUM CONTENTS OF ENDOGENOUS CANNABINOIDS AND BLOOD PRESSURE IN WOMEN WITH DEPRESSION

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Depression is known to be a risk factor for cardiovascular diseases but the underlying mechanisms remain unclear. Recent animal studies have shown that endogenous cannabinoids (eCBs), anandamide (AEA) and 2-arachidonylglycerol (2-AG) can modulate cardiovascular function and are associated with depression and anxiety disorders. These data suggest that eCBs could be a link between depression and cardiovascular function. To explore this hypothesis, we examined the relationship between serum contents of eCBs and resting blood pressure in depressed patients.

A total of 55 adult women from Saint Louis, MO, USA participated in the study. Depressed subjects (n = 28, age 28.0 ± 1.8) met DSM-IV diagnostic criteria for clinical depression whereas their matched control subjects (based on age and ethnicity; n = 27, age 29.0 ± 1.6) had no lifetime history of psychiatric illness. None of the subjects had prescribed medication in the past six months, with the exception of oral contraceptives. Serum eCBs contents were determined using solid phase extraction followed by liquid chromatography/mass spectrometry. Data are given as mean±SEM and analyzed using bivariate or partial correlation tests and Student's unpaired *t*-tests.

Women with depression had significantly higher systolic blood pressure than control subjects (Depressive: 115.1 ± 2.1 mmHg; Control: 108.6 ± 1.5 mmHg; $p = 0.02$). There was no significant difference in diastolic pressure (Depressive: 69.8 ± 1.3 mmHg; Control: 67.9 ± 1.5 mmHg; $p = 0.36$). In depressed women, serum AEA (0.8 ± 0.1 pmol/ml) and 2-AG (18.5 ± 2.7 pmol/ml) contents were positively correlated with diastolic (AEA: r = .46, $p = .01$; 2-AG: r = .62, $p < .01$) but not significantly with systolic pressure (AEA: r = .35, P = .07; 2-AG: r = .22, $p = .26$). Very similar correlations were also obtained after controlling for a third variable; body mass index (BMI), waist-to-hip ratio, serum total cholesterol levels, smoking, alcohol consumption, or oral contraceptives. In contrast, no correlations between eCBs and blood pressure were found in the control subjects (AEA: 0.7 ± 0.1 pmol/ml, diastolic r = -.05, $p = .81$, systolic r = -.17, $p = .40$; 2-AG: 19.0 ± 2.4 pmol/ml, diastolic r = -.04, $p = .84$; systolic r = .04, $p = .83$). It was noted that whilst BMI was larger in depressed subjects (Depressive: 31.5 ± 1.8 kg/m^2; Control: 25.4 ± 1 kg/m^2; $p < 0.01$) and was positively correlated with systolic pressure (Depressive: r = .41, $p = .03$; Control: r = .38, $p = .05$), it was not correlated with eCBs. Interestingly, further analysis of data among the depressives revealed that AEA and 2-AG were positively correlated with diastolic pressure in major (AEA: r = .55, $p = .03$; n = 16) and minor (2-AG: r = .74, $p = < .01$; n = 12) depression respectively.

This study shows that resting, diastolic blood pressure is positively correlated with a serum content of eCBs, AEA and 2-AG in women with depression. Given the potential roles of endogenous cannabinoids in patho/physiological cardiovascular and neuronal functions, interrelationships among these agents, cardiovascular parameters and depression warrant future research attention.

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CANNABINOID CB_1 RECEPTORS MAY MEDIATE GASTRIC MUCOSAL PROTECTION AND INHIBITION OF STIMULATED GASTRIC MOTILITY IN RATS AND MICE

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Cannabinoid receptors were described to modulate numerous gastrointestinal functions: Δ^9 -tetrahydrocannabinol inhibited gastric ulcer formation in acid dependent ulcer model (Sofia et al., Pharmacology 1978, 17: 173 – 177), decreased intragastric pressure and the pyloric contractility (Krowicki et al., Eur. J. Pharm.1999, 371: 187 – 196) and CB_1 receptor agonists decreased the stimulated gastric acid secretion (Adami et al., Br J. Pharm.2002, 135: 1598 – 606). Our aim was to analyze the effect of endogenous cannabinoids (anandamide and methanadamide) on acid-independent ulcer model and stimulated gastric motility. Gastric mucosal damage was induced by acidified ethanol in rats as well as in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. The gastric ulcer index was evaluated 60 and 30 min after ethanol challenge in rats and mice, respectively. Gastric motility was tested by using the balloon method as described by LeFebvre et al. (Brit. J. Pharmacol. 1992, 105: 305 - 312) in the rats. Gastric motility was stimulated centrally by insulin (5 IU.). The substances were given intravenously (i.v.), subcutaneously (s.c.) and intracerebroventricularly (i.c.v.). Our results indicate that:
1. Anandamide and methanandamide inhibited the ethanol-induced gastric mucosal damage in a significant manner in the doses range of 1.3-5.2 μmol/kg i.v. and 12.5-110 nmol/rat i.c.v. This gastroprotective effect was antagonized by the cannabinoid CB_1 receptor antagonist SR 141716A (21.5 nmol/rat, i.c.v. and 4.32 μmol/kg, i.v.) and the opioid receptor antagonist naloxone (27.5 nmol/rat i.c.v., 2.75 μmol/kg s.c.). The effect is likely to be central, since SR 141716A given i.c.v. antagonized the effect of intravenously injected methanandamide. 2. The gastroprotective effect of opioid peptides DAGO (19.5 pmol/mouse) and deltorphin II (11.3 pmol/mouse) was significantly reduced in $CB_1^{-/-}$ mice. 3. The insulin-induced enhanced gastric motor activity was inhibited in a dose-dependent manner by anadamide and methanandamide (1.3-5. 2 μmol/kg, i.v.). This effect was reversed by SR 141716A (4.32 μmol/kg, i.v.) but not by naloxone (2.75 μmol/kg, i.v.).

Conclusion: 1. Cannabinoid CB_1 receptors are likely to be involved in gastric mucosal defense and correlation between opioid and cannabinoid system in gastric mucosal protection may be raised. 2. Activation of CB_1 receptors results in inhibition of increased gastric motor activity.

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VASCULAR EFFECTS OF O-1918, A PUTATIVE RECEPTOR ANTAGONIST AT A NOVEL CANNABINOID RECEPTOR

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Apart from the two designated CB_1 and CB_2 receptors, a novel endothelial cannabinoid receptor has been suggested to be responsible for the direct vasodilatory action of cannabinoids. The two analogs of the non-psychoactive cannabidiol, abnormal cannabidiol (abn-cbd) and O-1918, have been suggested to be, respectively, a selective agonist and antagonist for this receptor (Offertáler *et al.* 2003). Recent findings show that O-1918 also inhibits vasorelaxation induced by the endocannabinoid-like *N*-arachidonoyl-L-serine in isolated endothelium-denuded rat mesenteric artery preparations (Milman *et al.* 2006). Here, the antagonistic effect of O-1918 was investigated against a number of endocannabinoids (anandamide, 2-arachidonoyl glycerol, virodhamine, oleamide) and some well-characterized synthetic cannabinoids (CP55940, HU210, JWH015, WIN55,212-2) in rat isolated small mesenteric arteries with an intact endothelium. Studies on contraction to methoxamine and U44619 were done in the presence and absence of endothelium. Segments (2mm) of third generation (internal diameter, 313±5μm; 126 vessels) from male Wistar rats (300-400g) were mounted in a Mulvany-Halpern type myograph for isometric tension recording. Vessels were bathed in Krebs-Henseleit buffer solution (composition, mM: NaCl, 118; KCl 4.7; $MgSO_4$, 1.2; KH_2PO_4 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 5.5) equilibrated with 95% O_2:5% CO_2 at 37˚C with 10µM indomethacin. Cumulative concentration-response curves were constructed and responses were expressed as the percentage relaxation of the submaximal contraction induced by methoxamine (10µM). It was found that O-1918 inhibited all the relaxation responses induced by endocannabinoids. Increasing concentration of O-1918 (1, 10 and 30µM; 30 min) induced progressive rightward displacement of the concentration-relaxation curves for anandamide in an almost parallel manner with a Schild plot of slope=1.1 and an equilibrium constant (K_B)=2.2µM (control: EC_{50}=0.13±0.02µM, n=7; O-1918, 1µM: EC_{50}=0.18±0.03µM, n=4; O-1918, 10µM: EC_{50}=1.3±0.1µM, n=5; O-1918, 30µM: EC_{50}=1.9±0.2µM, n=3). Significant inhibitions by O-1918 were also seen with 2-arachidonoyl glycerol (control: EC_{50}=1.7±0.49µM, n=3; O-1918, 10µM: EC_{50}=42.3±4.0µM, n=3), virodhamine (control: EC_{50}=0.13±0.02µM, n=9; O-1918, 10µM: EC_{50}=5.9±1.6µM, n=3), and oleamide (approximately 25-fold shift in the concentration-relaxation curve; n=7). O-1918 also inhibited relaxation induced by HU210 (control: $EC_{50\%}$=4.2±1.1µM; O-1918, 10µM: $EC_{50\%}$=31.4±9.7µM; n=4) and WIN-55212-2 (control: $EC_{50\%}$=0.3±0.1µM; O-1918, 10µM: $EC_{50\%}$=16.3±1.4µM; n=3; P<0.05) but not CP55940 or JWH015. Interestingly, a slight increase in vessel tone was observed with cannabinoid agonists after incubation with the highest concentration of O-1918 (30µM). This was overtaken by relaxation at higher agonist concentration (>1µM). Although the lowest concentration of O-1918 (1µM) did not have any significant effect on the contractile responses induced by methoxamine or U46619, 10µM O-1918 tended to shift the curves of both agonists to the left (methoxamine: control: EC_{50}=3.1±0.1µM, n=4; O-1918, 10µM: EC_{50}=1.5±0.1µM, n=4; U46619: control: EC_{50}=0.34±0.03µM, n=4; O-1918, 10µM: EC_{50}=15.9±3.3nM, n=4). While 10µM O-1918 did not affect carbachol-induced relaxation, 30µM O-1918 reduced the maximum relaxation (control: EC_{50}=64.8±3.7nM, n=4; O-1918, 30µM: EC_{50}=0.18±0.01µM, n=4). These results imply that O-1918 at higher concentrations (>10µM) might have effects other than at the novel endothelial cannabinoid receptor.

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CANNABINOID-MEDIATED REGULATION OF ADULT RAT MESENCHYMAL STEM CELL DIFFERENTIATION ALONG THE OSTEOGENIC LINEAGE

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Adult mesenchymal stem cells (MSCs) are a mulitpotent population of stem cells that can differentiate along the osteogenic, chondrogenic and adipogenic lineages. They offer a potentially exciting source of cells for engineering skeletal tissues for the treatment of osteochondral defects. The cannabinoid system has recently been reported to play a role in bone remodeling (Idris *et al*., 2005; Ofek *et al*., 2006). In this study we examined the influence of the CB1 receptor in regulating MSC osteogenesis *in vitro*. MSCs were isolated from the bone marrow of adult Wistat rats and expanded in culture. To induce osteogenesis the cells were exposed to osteogenic growth factors [dexamethasone (0.68 nM), ß-glycerol phosphate (10mM) and ascorbic acid (50 μM)] for a period of 3, 4 or 5 weeks, in the presence or absence of Δ^9-tetrahydrocannabinol (THC, 5μM) or the CB1 antagonist, AM251 (10μM). When the MSCs were exposed to the osteogenic factors a time-dependent increase in expression of the bone-specific proteins, collagen I and osteocalcin, was observed. Furthermore evidence of extracellular matrix deposition was evident, as assessed by von Kossa and alizarin red staining. This pattern of protein expression and mineralization is indicative of osteogenesis. THC had no effect on the osteogenic process. In contrast, exposure of cells to AM251 alone resulted in increased expression of collagen I, osteocalcin and mineralization, albeit to a lesser extent than that observed when MSCs were exposed to the osteogenic growth factors. This result demonstrates a stimulatory effect of AM251 on MSC osteogenic capacity, suggesting that endocannabinoids may exert a tonic suppression of osteogenesis. This suggests that blockade of the CB1 receptor may increase bone deposition by stimulating MSC differentiation along the osteogenic lineage, as well as inhibiting osteoclast formation (Idris *et al*., 2005). As such, this data supports the suggestion that CB1 antagonists may have therapeutic potential for the treatment of osteoporosis.

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STUDIES ON THE EFFECTS OF ABNORMAL CANNABIDIOL AND THE CB_1 AGONIST ACEA ON VASCULAR FUNCTION AND SMOOTH MUSCLE CELL GROWTH IN THE PORCINE CORONARY ARTERY

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Restenosis is the re-narrowing of an artery after angioplasty or stent implantation in an atherosclerotic artery and can be reduced using anti-proliferative and anti-inflammatory drug-eluting stents. There is evidence that cannabinoids have anti-proliferative and anti-inflammatory effects that reduce the progression of atherosclerosis (Steffens *et al.,* 2005) and may therefore also have beneficial properties to target restenosis. Abnormal cannabidiol is a cannabinoid that has been reported to induce relaxation via a novel non-CB_1 or CB_2 receptor (Offertaler *et al.,* 2003), whereas ACEA is a synthetic cannabinoid thought to act via the CB_1 receptor (Sterin-Borda *et al.*, 2005). The aims of this study were (i) to determine the relaxant responses to abnormal cannabidiol and ACEA in endothelium intact porcine coronary arteries precontracted with U46619 ($5x10^{-7}$M); (ii) to investigate the nature of the receptors mediating their actions and (iii) to assess the effects of abnormal cannabidiol and ACEA on porcine coronary artery smooth muscle cell (PCVSMC) growth using the Alamar Blue cell proliferation assay. Cumulative dose response curves indicated that ACEA (10^{-9} – 10^{-4}M) and abnormal cannabidiol (10^{-9} – 10^{-4}M) induced significantly greater relaxation (E_{max} 45.57 ± 8.5% and 52.83± 7.8%, respectively) in endothelium intact arteries in the presence of phenyl methyl sulfonyl fluoride (PMSF; 1mM) compared to those in the absence of PMSF (E_{max} 33.12 ± 9.13% and 40.48 ± 6.2%, respectively; $p<0.05$; one-way ANOVA and Tukey's post-test). However, in the presence of the selective FAAH inhibitor, URB 597 (10nM) the relaxations induced by ACEA and abnormal cannabidiol in endothelium intact arteries (E_{max} 35.39 ± 6.3%) were not significantly different from the control (E_{max} 38.56± 6.5%). These observations suggest that the PMSF-induced enhancement of the effects of ACEA and abnormal cannabidiol that we observed does not depend on the known ability of PMSF to inhibit fatty acid amide hydrolase (FAAH). In the presence of PMSF, the relaxations induced by ACEA and abnormal cannabidiol were significantly attenuated by both the selective CB_1 antagonist AM251 (1μM; E_{max} 7 ± 6.5% and 12 ± 5.1% maximum, respectively; $p<0.05$) and the transient potential vanilloid receptor (TPVR1) antagonist capsazepine (10μM; E_{max} 15 ± 5.7% and 12 ± 7.3% maximum, respectively; $p<0.05$), suggesting that the agonists induce relaxation via both the CB_1 and the TPVR1 receptor. The results from the Alamar Blue assay indicated that ACEA and abnormal cannabidiol, in the absence of PMSF, concentration-dependently increased PCVSMC growth (by 0.3 ± 0.6 fold and 2.4 ± 1.5 fold, respectively at 1μM) determined after 24 hours of incubation. Further studies on cell growth in the presence of selective cyclooxygenase and lipoxygenase inhibitors are currently underway to determine the contribution of metabolites of these drugs to the observed responses.

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THE EFFECT OF CANNABINOIDS ON SMOOTH MUSCLE CONTRACTILITY OF THE RAT PROSTATE

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Cannabinoids are capable of affecting the male reproductive system via a number of different mechanisms. Immunoreactivity for the CB_1 receptor has previously been shown in the epithelium of the human prostate. The aim of this study was to investigate the effect of WIN55,212-2, anandamide and methanandamide on nerve mediated contractions of smooth muscle in the rat prostate. Isolated rat prostates were suspended in 10ml organ baths filled with Krebs-Henseleit solution maintained at 37ºC, bubbled with 95% O_2 : 5% CO_2. Tissues were stimulated using electrical field stimulation (EFS; 2s train, 0.5ms, 60V, 10Hz, once every minute) and increasing concentrations of WIN55,212-2, anandamide and methanandamide (1nM-0.3µM) were tested on the subsequent contractile responses. WIN55,212-2 inhibited EFS ($P<0.001$) induced contractions in a concentration dependent manner, whereas anandamide ($P=0.445$) and methanandamide ($P=0.790$) had no effect. The inhibition WIN 55,212-2 produced was blocked by the CB_1 antagonists SR141716 (1µM; $P < 0.001$) and LY320135 (1µM; $P=0.002$), but not the CB_2 antagonist SR144528 (1µM; $P=0.824$). L-NAME (0.01-1mM) and capsaicin (10µM) had no effect on the inhibition produced by WIN55,212-2 ($P>0.571$), whereas indomethacin (0.1µM) reversed the effect ($P=0.041$). These results indicate that WIN55,212-2, but not anandamide and methanandamide inhibit contractions of the rat prostate. Inhibition by WIN55,212-2 is via by a CB_1 receptor mechanism, which is dependent on the cyclooxygenase pathway.

ARE CANNABINOID CB_1 AND OPIOID RECEPTORS RELATED IN THE CONTROL OF GASTROINTESTINAL MOTILITY IN MICE?

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The endocannabinoid system has repeatedly been implicated in the control of gastrointestinal motility. The present study investigated whether (a) cannabinoid CB_1 receptor knockout ($CB_1^{-/-}$) mice displayed an altered gastrointestinal transit when compared to homozygous $CB_1^{+/+}$ ($CB_1^{+/+}$) mice, and (b) the well-described functional interaction between the cannabinoid CB_1 and opioid receptors extends to the regulation of gastrointestinal transit. Gastrointestinal transit was assessed using two experimental procedures: the Whole Gastrointestinal Transit, which measures the time to excretion of an intragastrically administered non-absorbable marker (whole intestine), and the Upper Gastrointestinal Transit, which measures the distance covered by the non-absorbable marker from pylorus to caecum (small intestine). The results of the study indicated that $CB_1^{-/-}$ and $CB_1^{+/+}$ mice did not differ in basal levels of time of whole gut transit and distance travelled by the marker (0.3 ml/mouse carmine) in the small intestine. $CB_1^{-/-}$ and $CB_1^{+/+}$ mice were equally responsive to the inhibitory effect of morphine (10 mg/kg, i.p.) and loperamide (3 mg/kg, i.p.) on time of whole gut transit. Additionally, in CD1 mice the cannabinoid CB_1 receptor antagonist, rimonabant (0-0.5 mg/kg, i.p.), failed to block the inhibitory effect of morphine (0-1.25 mg/kg, i.p.) and loperamide (0-0.5 mg/kg, i.p.) on transit in small and whole intestine. Similarly, the opioid receptor antagonists, naloxone (0-1 mg/kg, i.p.) and naltrexone (0-10 mg/kg, i.p.), failed to block the inhibitory effect of the cannabinoid WIN 55,212-2 (0-3 mg/kg, i.p.) on transit in small and whole intestine. These results suggest that: (a) compensatory mechanisms likely developed in $CB_1^{-/-}$ mice to overcome the lack of inhibitory function of endocannabinoid system on gastrointestinal motility; (b) cannabinoid and opioid receptor systems did not functionally interact in regulating gastrointestinal transit in mice.

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REGULATION OF OSTEOTROPIC CYTOKINE EXPRESSION IN OSTEOBLASTS BY THE ENDOCANNABINOID ANANDAMIDE

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Bone metabolism is regulated by both systemic factors as well as factors produced locally in the bone microenvironment. Two recent studies[1, 2] have shown that the cannabinoid receptors, CB1 and CB2, are important regulators of bone mass and have therefore been suggested as possible therapeutic targets in treatment of bone diseases such as osteoporosis.

To explore further the involvement of the endocannabinoid system in bone metabolism, we have investigated the possible regulation by anandamide of osteotropic cytokine expression in primary mouse calvarial osteoblasts, as well as the presence of CB1, CB2, fatty acid amide hydrolase (FAAH), and *N*-acyl phosphatidylethanolamine specific phospolipase D (NAPE-PLD) in bone cells.

Semi-quantitative RT-PCR demonstrated the presence of both CB1 and CB2 mRNA in primary mouse calvarial osteoblasts as well as in cells from the osteoblastic cell line MC3T3-E1. In the calvarial osteoblasts, CB2 mRNA expression increased over time for at least 120h, while CB1 mRNA expression was unchanged over time. In addition, both cell types expressed mRNA for NAPE-PLD and FAAH, enzymes involved in anandamide synthesis and hydrolysis. Similar to CB2, FAAH mRNA expression increased over time, whereas NAPE-PLD mRNA expression remained unchanged. Regarding FAAH activity, both primary calvarial osteoblasts and MC3T3-E1 cells showed a time-dependent increase of [^{3}H]-ethanolamine production, which could be blocked by the FAAH inhibitor URB597 in a dose-dependent manner.

To investigate the possible regulation of osteotropic cytokine expression by anandamide, isolated primary mouse calvarial osteoblasts were incubated in the absence (control) or presence of anandamide (1 μM), URB597 (1 μM), or the combination of anandamide and URB597 for 6 and 48h before RNA isolation. Semi-quantitative RT-PCR showed that anandamide increased the mRNA expression of receptor activator of NF-κB ligand (RANKL) and interleukin-6, two well-known stimulators of bone resorption, after 48h. Co-stimulation with URB597 resulted in even more profound effects compared to anandamide alone. Furthermore, the mRNA expression of osteoprotegerin (OPG), a protein functioning as a decoy receptor for RANKL, was also increased by anandamide, however less than that of RANKL.

In summary, these results suggest that anandamide is a possible endogenous stimulator of bone resorption through an osteoblast dependent mechanism, and further strengthen the role of the endocannabinoid system in the regulation of bone metabolism.

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CANNABINOID MODULATION OF VOLTAGE-GATED CALCIUM CHANNELS IN GUINEA-PIG MYENTERIC NEURONS

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Activation of CB_1 receptors present on myenteric neurons inhibits electrically evoked contractions in guinea-pig ileum (Pertwee *et al*., 1996, Br J Pharmacol. 118:2199-205; Coutts *et al*., 2002, J Comp Neurol. 448:410-22). In addition there is evidence for non-CB_1 receptor mediated effects of cannabinoids. Lopez-Redondo *et al*. (1997, Br J Pharmacol. 122:330-4) showed that the inhibition by cannabinoids of fast excitatory synaptic potentials in S-type myenteric neurons was reversed by the CB_1 receptor antagonist rimonabant in only 38% of neurons. We have recently reported that cannabinoid inhibition of nicotinic receptors in myenteric plexus-longitudinal muscle (MPLM) tissue and isolated myenteric neurons occurs via a mechanism sensitive to the non-CB_1 cannabinoid ligand WIN55,212-3 but not to rimonabant or pertussis toxin (PTX) (Sones *et al*., 2004, Br J Pharmacol. http://www.pa2online.org/Vol2Issue4abst005P.html). In other preparations, cannabinoid inhibition of voltage-gated calcium channels (VGCC) involves a βγ-subunit $G_{i/o}$-protein coupled CB_1 receptor (Caulfield & Brown, 1992, Br J Pharmacol. 106:231-2; Pan *et al*., 1998, Mol Pharmacol. 54:1064-72). The current study investigates the involvement of CB_1 receptors in cannabinoid inhibition of VGCC in isolated guinea-pig myenteric neurons.

Myenteric neuron primary cultures were prepared from ileal segments using a method adapted from that of Barajas-Lopez *et al*. (1993, Eur J Pharmacol. 250:141-5). In the whole-cell patch clamp configuration, Ca^{2+} currents were evoked by voltage-step protocols and isolated pharmacologically. Values are expressed as pA/pF to compensate for cell size and presented as the mean ± S.E.M ($n \geq 5$ for all data). Depolarizing voltage steps evoked inward currents peaking at 27.1 ± 2.3 pA/pF at 0 mV. CP55,940 inhibited this current with an IC_{50} of 747 nM. Rimonabant (1 μM) did not significantly inhibit the current, but 10 μM rimonabant reduced the current to 13.6 ± 3.4 pA/pF while O 2050 (10 μM), a putative neutral cannabinoid antagonist, reduced the current to 5.1 ± 1.4 pA/pF. Ca^{2+} current in the presence of 1 μM CP55,940 and rimonabant (10.6 ± 3.7 pA/pF) was not significantly different from the current seen with CP55,940 alone (13.1 ± 2.5 pA/pF). Inhibition with 10 μM rimonabant and 1 μM CP55,940 (3.0 ± 0.5 pA/pF) was greater than that with CP55,940 alone. PTX (100 ng/ml) significantly reversed CP55,940 inhibition ($P < 0.05$) whilst WIN55212-3 (10 μM) produced significantly less inhibition than WIN55,212-2 (10 μM; $P < 0.05$).

The results suggest the involvement of a $G_{i/o}$-coupled, non- CB_1 receptor in the inhibition of VGCC by cannabinoids in myenteric neurons.

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EFFECT OF ANANDAMIDE (AEA) AND LIPOPOLYSACCHARIDE (LPS) ON EARLY MURINE PREGNANCY

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Nitric oxide (NO) fulfils important functions during pregnancy and has a role in implantation, decidualization, vasodilatation and myometrial relaxation (Sladek et al., 1997; Chwalisz et al., 1999; and Garfield, 2000). However, at high concentrations, such as those that are produced in sepsis, NO has toxic effects as it is a free radical (Grisham et al., 1999).

Our previous results indicate that LPS, an integral part of the outer membrane of Gram negative bacteria, is capable of producing embryonic resorption in mice due to NO increased production not only in uterus but also in decidua (Ogando et al., 2003). Recent research has revealed that LPS induces AEA synthesis in murine macrophages (Liu et al., 2003).

The aim of the present work was to determine the effect of LPS and AEA on early murine pregnancy. On day 7 of pregnancy female mice were killed by cervical dislocation and uterus and decidua were separated in each implantation site. Uterus was then incubated for 24 h in the presence of a) LPS (1 ug/ml), b) AEA (10^{-7}M) and c) LPS + AM251 (20uM) (cannabinoid type 1 (CB1) receptor antagonist) and NO as NO_3^- plus NO_2^- was measured in culture supernatants. Both LPS and AEA were capable of increasing NO levels and, NO production induced by LPS was inhibited when uterus was incubated in the presence of AM251.

We also determined AEA synthesis (conversion of [^{14}C]-arachidonic acid and ethanolamine in [^{14}C]-AEA) and the expression of the fatty acid amide hydrolase (FAAH–the enzyme responsible for hydrolysis of AEA) by western blot in the presence or absence of LPS. Treatment with the endotoxin increased AEA synthase activity and seemed to decrease FAAH expression. These results and the inhibition of LPS-induced augmentation of NO synthesis by AM251 suggest that AEA could be an intermediate in LPS effect.

Prostaglandins (PG) and NO are intimately involved in the mechanism of parturition (Mitchell et al., 1995; Farina et al., 2001). NO maintains uterine quiescence during pregnancy (Yallampalli et al., 1993) whereas PGs are involved in eliciting contractions of uterine smooth muscle (Franchi et al., 1994). PG biosynthesis is catalized by ciclooxygenase (COX) which exists in tow isoforms: COX-1 and COX-2 (Kujubu et al., 1991; Simmons et al., 1993). Because LPS is known to induce high levels of PGs in several tissues, we determined the levels of PGE_2 in the uterus of female mice on day 7 of pregnancy in the presence of LPS and LPS+AM251. We observed that the high levels of PGE_2 induced by LPS were decreased by AM251. These results suggest that the synthesis of the two main molecules involved in LPS-induced embryonic resorption (NO and PGs) may be mediated by AEA.

CANNABINOIDS INFLUENCE THE VIABILITY OF NORMAL HUMAN COLONIC EPITHELIAL CELLS

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Important roles for the endocannabinoid system in the gastrointestinal (GI) tract under physiological and pathophysiological conditions have been demonstrated. We have shown that cannabinoids promote colonic epithelial wound closure through the CB1 receptor at nanomolar concentrations, which had no significant effect on proliferation. Previous work showed that low micromolar concentrations were anti-proliferative in DLD-1 and Caco-2 tumour cell lines. Interestingly, cannabinoids exert opposite effects on the survival of transformed and non-transformed neuronal cells, inducing apoptosis in tumour cells, but not in primary cells. In this study, we investigated whether cannabinoids influenced the proliferation of non-transformed colonic epithelial cells (NCM460), which are non-tumorigenic cells derived from normal human colonic epithelial tissue.

NCM460 cells express both CB1 and CB2 receptors under normal culture conditions. Proliferation experiments were performed in the presence or absence of synthetic and endogenous cannabinoids (10nM-5μM) or vehicle over a 96 hour period, which equates to 3 doublings. ACEA (CB1 agonist), JWH (CB2 agonist) and metAEA (methanandamide, endogenous CB1/CB2 agonist) had no significant influence on the proliferation of these cells, even under low-serum conditions. However, the CB1 receptor antagonist, AM251 (0.2-5μM) alone had a profound anti-proliferative effect on these cells. This implies that the activity of cannabinoid receptors, although not essential, is linked to the viability and survival of normal colonic epithelial cells. Further investigations using immunoblotting techniques revealed that cannabinoids impact on heme-oxygenase (HO)-1 expression, a protein thought to be important during restitution of inflammation.

These results support our suggestion that cannabinoids may have therapeutic potential during the healing phase of gastrointestinal inflammation.

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EFFECTS OF WIN 55,212-2 IN RAT ISOLATED HEARTS SUBJECTED TO ISCHAEMIA-REPERFUSION

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It has been described that cannabinoids could affect the cardiovascular system. Recent investigations suggest that endocannabinoids as 2-arachidonoylglycerol and palmitoylethanolamide could protect the heart against ischaemia-reperfusion (IR) injury. In contrast, anandamide failed to reduce IR injury probably because it was rapidly taken up and degraded, although it is able to reduce infarct size. Given the potential confusion about the effectiveness of endocannabinoids in mediating cardioprotection, we decided to asses whether a synthetic cannabinoid, Win 55,212-2, is able to protect the heart against IR injury.

Wistar rats (250-300g) isolated perfused hearts were mounted by the Langendorff technique and subjected to global, no-flow ischaemia (30 minutes or 45 minutes) and reperfusion (60 minutes). The effect of the synthetic cannabinoid Win 55,212 at two different concentrations, 300nM and 1µM, in ischaemic hearts were evaluated. The possible involvement of CB_1 cannabinoid receptor was also investigated, by administration of SR141716A (1µM) before treatment with the synthetic cannabinoid. The cardiac parameters analyzed were: Left Ventricular Developed pressure (LVDP), Coronary Perfussion Pressure (CPP) and End Diastolic Pressure (EDP).

Administration of Win 55,212-2 1µM impaired the cardiac function before the ischaemia period: reduced the LVDP in 58.73 ± 4.46 mmHg ($P<0.001$ vs control 3.24± 7.89 mmHg); increase CPP in 108.99 ± 12.30 mmHg ($P<0.001$ vs control 10.89 ±0.90 mmHg); and increase EDP in 32.06 ± 8.21 mmHg ($P<0.01$ vs control 6.75 ± 4.73 mmHg). Furthermore, the treatment with this cannabinoid did not improve cardiac parameters in hearts subjected to 45 minutes of ischaemia and impaired the cardiac parameters in hearts subjected to 30 minutes of ischaemia. SR141717A 1µM, a selective cannabinoid CB_1 receptor antagonist, was not able to prevent the deleterious effect of Win 55,212-2 on cardiac function in any of the experimental groups. Actually the implication of cannabinoid CB_2 receptors in the cardiac effects of Win 55,212-2 is carrying out.

These results suggest that the synthetic cannabinoid Win 55,212-2 does not protect hearts against IR injury. Further investigation should be carried out: 1) to determine if other synthetic cannabinoids exert similar effects on hearts subjected to IR injury; 2) to clarify if only endocannabinoids have the peculiarity to protect myocardium against ischaemia and the mechanism involved.

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SURVEY OF MEDICINAL CANNABIS USE AMONG CHILDBEARING WOMEN: PATTERNS OF ITS USE IN PREGNANCY AND RETROACTIVE SELF-ASSESSMENT OF ITS EFFICACY AGAINST 'MORING SICKNESS'

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A majority of women experience some nausea and/or vomiting during pregnancy. This condition can range from mild nausea to extreme nausea and vomiting, with 1-2% of women suffering from the life-threatening condition *hyperemesis gravidarum*. Cannabis (*Cannabis sativa*) appears to have potential in mitigating pregnancy-induced nausea and vomiting, but it has not been studied in a clinical setting. This paper presents the results of a survey of 84 female users of medicinal cannabis recruited through two compassion societies in British Columbia, Canada by means of an anonymous survey. The respondents ranged between 19 and 64 years of age; their median age was 42. The respondents had given birth to between 0 and 8 children (median 1.57), and 64 women (75% of the respondents) reported having at least one child. In general (not specific to pregnancy), the vast majority of our respondents considered Cannabis to be "extremely effective" or "effective" as a therapy for nausea (92%) and vomiting (75%), and as an appetite stimulant (95%). In the context of pregnancy, cannabis was rated as "extremely effective" or "effective" by 92% of the respondents who had used it as a therapy for nausea and vomiting (morning sickness). Our study suggests that cannabis therapy for severe nausea and vomiting related to pregnancy merits further investigation.

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A SINGLE DOSE OF CBD REDUCES THE DISSOCIATIVE EFFECTS PROVOKED BY (S)-KETAMINE IN HEALTHY VOLUNTEERS

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The psychotic-like symptoms induce by high doses of Δ9–tetrahydrocannabinol (Δ9-THC) in healthy volunteers are significantly reduced by cannabidiol (CBD). Antipsychotic-like properties of CBD have been investigated in animal models which suggested that CBD has a pharmacological profile similar to atypical antipsychotic drugs. Open case-reports of schizophrenic patients treated with CBD and a preliminary report of a controlled clinical trial comparing CBD with an atypical antipsychotic confirm that this cannabinoid can be a safe and well tolerated alternative treatment for schizophrenia. The mechanism(s) of action whereby CBD produces these effects remains obscure. Several lines of evidence have suggested that the glutamatergic N-methyl-D-aspartate (NMDA) receptor is involved in the mechanism of psychosis and schizophrenia itself. In animals, CBD antagonizes glutamate neurotoxicity and attenuated the hyperlocomotion induced by ketamine, a glutamate NMDA receptor antagonist, able to induce psychotic symptoms in healthy volunteers. The aim of this research was to test the hypothesis that a single dose of CBD would interfere with ketamine-induced psychopathology in healthy volunteers. In a double-blind crossover procedure, nine (9) healthy volunteers were randomly assigned, to the placebo or CBD (600 mg) groups in two experimental sessions, with a one-week interval. After fulfilling psychiatric-assess scales, the volunteers received orally placebo or the drug and rested for 65 minutes. Afterwards, an infusion pump was installed and an intravenous bolus of S-ketamine (0.26 mg/kg) was administered during one minute followed by a maintaining dose of 0.25 mg/kg for 30 minutes. A Clinician-Administered Dissociative States Scale (CADSS) was applied both at the beginning of the sessions and 90 minutes after the bolus injection, accordingly to the period in which they felt most symptomatic. The local Ethics Committee approved the study protocol. CBD attenuated the effects of ketamine significantly for the depersonalization factor, further reinforcing the idea that antipsychotic-like properties of CBD could be related to a glutamatergic mechanism.

STABILITY OF DELTA-9-THC AND OTHER CANNABINOIDS IN DIFFERENT CANNABIS PRODUCTS

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The stability of various Cannabinoids in different Cannabis products (Marijuana plant material, Marijuana cigarettes and Cannabis extract) stored at The University of Mississippi - National Center for Natural Products Research was studied under different storage conditions. These included room temperature (17˚C±4˚), refrigerator (4˚C± 2˚) and freezer (-20˚ C±2˚). The content of each individual cannabinoid was then determined periodically for up to 60 months for Cannabis biomass and Cannabis extract, and for 36 months for Cannabis cigarettes. The Cannabinoids monitored were: Δ^9-THC, CBD, CBC, CBG, THCV, and CBN.

Marijuana plant material of three different potencies was monitored: low potency (approx. 1.5 % THC), medium potency (approx. 2.5 % THC) and high potency (approx. 4.0 % THC), while marijuana cigarettes of approx. 8% THC were used for the study. The potency of Cannabis extract was approx. 26 % THC.

While the concentration of some cannabinoids (CBD and CBC) did not change over the course of the study under any of the storage temperatures, the concentration of Δ^9-THC and its C-3 homolog dropped significantly when the materials were stored at room temperature (e.g. the medium potency material dropped from 2.5 % THC to 0.91 % THC over the 60 months period and the THCV dropped from ~ 0.032 % to < 0.001 % over the same time frame). The concentration of CBN, on the other hand, showed a gradual increase over time under room temperature storage. For the same medium potency material, CBN rose from 0.1 % to 0.45 % over the 60 months period. It is obvious that the decrease in THC content results in increase in the CBN content, supporting the conclusion that CBN is an artifact resulting from the oxidation of THC. It is to be noted, however, that the conversion of THC to CBN is not quantitative and the plant material must therefore contain other intermediates in the THC to CBN pathway as previously proposed. The last cannabinoid monitored was CBG. While it appears that the concentration of CBG decreases over time at room temperature storage, the trend is not consistent and is not uniform. The apparent fluctuation in the CBG content might be related to sampling or peak integration, having a close retention time to CBN. The observations and trends for all cannabinoids tested were the same for the different potencies biomass, cigarettes and extract.

It is concluded that for long term storage of Cannabis products, storage should be under freezer or refrigerator conditions. Room temperature storage should be considered only for a short period of time (~ 3 months).

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INFLUENCE OF DEMOGRAPHIC CHARACTERISTICS ON QUITTING OF CANNABIS SMOKING

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We have previously reported on the self-reported characteristics of cannabis withdrawal in a convenience sample of 104 non-treatment-seeking adult cannabis smokers (mean [SD] age = 35.0 [11.3] years, 78% male, and 52% white, 40% African-American) recruited from two non-treatment research studies: 45 subjects participating in an observational lung health study at UCLA and 59 subjects in residential studies at the NIDA IRP (Boyd et al., Am J Addict 14:35-42, 2005; Copersino et al., Am J Addict 15:8-14, 2006). We report here the influence of subjects' demographic characteristics on their attempts to quit cannabis smoking. Subjects were primary cannabis users (age at first use = 16.6 [3.9] years, lifetime use = 19.0 [10.1] years, days used in prior 30 days = 23.9 [7.8]) with no other current substance use disorder (except tobacco) who reported at least one "serious" (self-defined) quit attempt without formal treatment. The influence of age (younger vs. same or older than median age of 34 years), gender, and race on quit attempt characteristics was evaluated by t-test for quantitative variables and by chi-square test for categorical variables. African-Americans, compared to whites, had a significantly younger age of first use (15.9 [3.6] vs. 17.6 [4.2] years, $p = 0.03$), shorter duration of lifetime cannabis use (10.7 [5.0] vs. 27.1 [6.3] years, $p = 0.0001$), and longer duration of their longest quit attempt (215 [400] vs. 51 [76] days, $p = 0.01$). There were no significant age or gender influences on number of quit attempts or duration of longest quit attempt. With regard to cannabis withdrawal symptoms, anxiety was reported more often by whites than by African-Americans (42.6% vs. 21.4%, $p = 0.04$), by men more than women (71.6% vs. 47.8%, $p = 0.03$) and by older than by younger subjects (45.3% vs. 17.6%, $p = 0.002$). Increased sex drive was reported more often by African-Americans (31.0% vs. 13.0%, $p = 0.03$), men (23.4% vs. 4.3%, $p = 0.04$), and younger subjects (27.4% vs. 11.3%, $p = 0.04$). Cannabis craving was reported more often by whites (72.2% vs. 52.3%, $p = 0.01$). With regard to coping strategies for quitting, there were no significant age, gender, or racial differences except that younger subjects were more likely to get rid of their cannabis paraphernalia (61.7% vs. 35%, $p = 0.01$). With regard to reasons for quitting, to get praise from others and workplace drug testing were reported more often by African-American (42% and 52.4%, $p = 0.005$ and 0.0001, respectively) and younger subjects (35.3% and 43.7%, $p = 0.03$ and 0.001, respectively) than by white (14.8% and 9.3%) and older subjects (17% and 13.2%). These findings suggest that there are significant age, gender, and racial differences in the experience of quitting cannabis use without formal treatment (so-called "spontaneous" quitting), although the clinical significance of these differences remains unknown.

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HUMAN SKIN PERMEATION OF JWH-203 AND O-2426, TWO SYNTHETIC CANNABINOIDS

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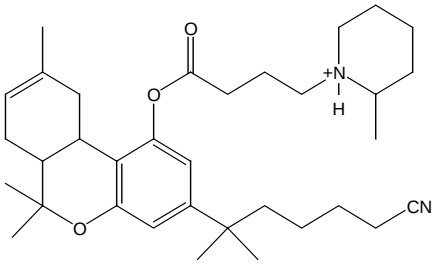
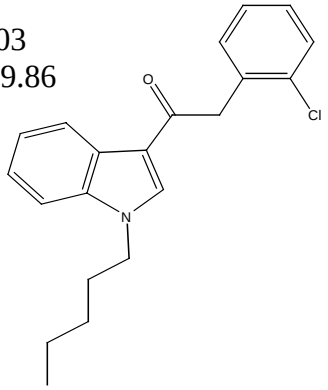
Cannabinoid drug delivery research is necessary in order to optimize the beneficial properties and minimize the negative drug side effects. Synthetic cannabinoids exhibiting high affinity for CB_1 and CB_2 receptors are the most promising for future therapeutic use. In this study, two synthetic cannabinoids, JWH-203 and O-2426, were investigated for their human skin permeation rates in order to evaluate their capabilities for transdermal delivery. JWH-203 is a 3-(2-substituted phenylacetyl)indole which has a very high affinity for the CB_1 receptor (Huffman et al., Bioorganic & Medicinal Chemistry Letters, 15 (2005) 4110-4113). O-2426 is a more water soluble derivative of O-2545, a compound investigated in a previous study.

O-2426
MW 535.77



JWH-203
MW 339.86



In vitro diffusion studies of JWH-203 and O-2426 were completed using split-thickness human abdominal skin in flow-through diffusion cells. The receiver solution consisted of HEPES-buffered Hanks' balanced salt solution with 40% polyethylene glycol 400. The two drugs were prepared in isopropyl myristate and isopropyl myristate with 30% ethanol. The drug disposition in the skin at the end of the 48 hour experiment was also determined. Samples were analyzed by high pressure liquid chromatography with UV detection.

The *in vitro* fluxes of JWH-203 without and with 30% ethanol and O-2426 without and with 30% ethanol in human skin were 0.31 ± 0.17, 0.24 ± 0.04, 0.00 ± 0.00 and 0.46 ± 0.01 nmol/cm^2/h, respectively. The mean drug contents of JWH-203 without and with 30% ethanol and O-2426 without and with 30% ethanol in the skin were calculated to be 5.54, 1.15, 1.51, and 6.14 µmol/g of skin, respectively. The target therapeutic flux (transdermal delivery rate) of Δ^9-THC is 9.6 nmol/cm^2/h, although the flux values of JWH-203 and O-2426 did not reach this target, it is highly likely that these more potent compounds would have a lower therapeutic flux target. O-2426, the more water soluble derivative of O-2545, diffused through the skin faster as compared to O-2545, which did not permeate the skin. The results indicated that significant levels of JWH-203 and O-2426 could be delivered via the transdermal route. Formulation modifications and the design of more water soluble derivatives may improve the transdermal delivery of the two compounds. This work was supported by the American Cancer Society (RSG-00-027-04-CDD).

USE OF SELF-REPORT AND URINE DRUG SCREEN RESULTS IN CLINICAL RESEARCH WITH MARIJUANA-DEPENDENT INDIVIDUALS

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The use of an objective outcome measure in substance abuse research is ideal. However, interpreting urine drug screen (UDS) results is complicated in a marijuana-dependent population. Use of creatinine normalization has been proposed as a method to help differentiate new marijuana use from residual drug excretion. The objective of this analysis was to examine the relationship between semi-quantitative cannabinoid screens, quantitative cannabinoid screens, and self-report data collected in a clinical trial. Data was collected from 28 subjects participating in two ongoing 12-week treatment trials. Subjects provided one urine sample weekly. For 21 subjects, semi-quantitative cannabinoid screens were obtained with a detection cut-off value of 50 ng/ml and a maximum reported value of 135 ng/ml. For 7 subjects, additional dilutions of urine samples were performed to provide quantitative values for samples >135 ng/ml. Creatinine concentrations of all urine samples were also obtained. A ratio of cannabinoid:creatinine concentration was calculated for each sample with a quantified cannabinoid level. Self-reported marijuana use was collected weekly using the Timeline Follow-Back (TLFB). For each of the three measures of marijuana use, a binary indicator variable was constructed to represent use vs. no use. For quantitative measures, a subject was considered to have used if the cannabinoid:creatinine ratio increased over the course of one week. For either the TLFB or the semi-quantitative measure, any day with reported use or a positive UDS at the end of the week were considered use. Using the two UDS-based measures, we computed an index to indicate the concordance of the interpretation of the UDS (i.e., positive/negative). This variable, in our data set, had three levels: qualitative positive, ratio positive; qualitative positive, ratio negative; qualitative negative, ratio negative. The mean TLFB-reported use over the week prior to the UDS was computed and compared across the three levels of the concordance index using PROC Mixed to account for the clustering of UDSs within subject. Subjects were predominantly male (n=24), and smoked an average of 77.9 (± 12.1) days in the three months prior to study entry. The mean age was 32 years (range 20-53 years). The model based means (SE) for the concordance levels were as follows: Neg/Neg=0.13 (0.14); Pos/Neg=1.30 (0.28); Pos/Pos=1.81 (0.31), and the overall Type III test was statistically significant (p=0.003). For this data, the point estimate use of Neg/Neg was not statistically different from zero which would suggest that when both the qualitative and ratio measure were both zero, no use per the TLFB occurred. However, when the qualitative UDS was positive, the mean use per day over the preceding week was statistically different from zero, and in circumstances of the ratio being negative, there was an observation of less use. A limitation of the ratio measure observed in this preliminary analysis is that the ratio result can be negative yet use is still present by TLFB. One explanation is that in heavy users of marijuana, one may reduce use over the course of the study, thereby causing the cannabionid:creatinine ratio to decrease, without eliminating use. In these cases, the qualitative UDS captures this use. Further laboratory based studies are needed better examine the excretion of marijuana in heavy users so that a more predictive model could be constructed to better identify deviations from an expected rate of decline in the presence of no use.

CASE REPORT: INSOMNIA LEADING TO IATROGENIC ILLNESS

Tod H. Mikuriya, M.D.

Alex P., accompanied by his mother, first visited my office in February 2005 at age 15 years, 6 months. At that time he had been prescribed and was taking Fioricet with codeine (30 mg, 3x/day); Klonopin (1 mg, 2x/day); Ativan (1 mg, 2x/day); and Dilaudid "as needed" to treat migraine headaches (346.1), insomnia (307.42), and outbursts of aggression to which various diagnoses —including bipolar with schizophrenic tendencies— had been attached by doctors in the Kaiser Healthcare system.

A history taken from Alex and a separate interview with his mother, Barbara P., were in accord. In an otherwise healthy childhood, Alex experienced persistent insomnia and reluctance to leave the house. At age 11, after teachers advised that Alex "had trouble concentrating and processing things," he was prescribed Ritalin. Soon thereafter, participation in a car theft led to a four-year sojourn through institutions of the Central Valley juvenile justice system and Kaiser-affiliated hospitals and clinics. In this period, Alex was prescribed Prozac, Paxil, Maxalt, Immitrex, Depacote, Phenergan, Inderal, Thorazine, Amitriptaline, Buspar, Vicodin, Seroquel, Risperdal, Zyperxa, Clozaril, Norco, and Oxycodone. These drugs did not quell his rage and in fact exacerbated it. At age 13 Alex made a serious suicide attempt by hanging himself from a tree outside his house. He reports making other attempts to overdose on pills.

Alex had known since age 11, when he first smoked cannabis, that it had a calming effect. His mother's request that a Kaiser physician prescribe Marinol for Alex was rejected. Through the internet she identified the author as a specialist in cannabinoid therapeutics and arranged an appointment for Alex. A prescription was written in February 2005 for Marinol (10 mg), along with a recommendation to use cannabis by means of a vaporizer.

Alex and his mother were seen by the author at a follow-up visit in February 2006. Alex had been medicating exclusively with cannabis for 12 months. He reported dramatically improved ability to concentrate and to sleep —and no episodes of rage. He has had only one migraine attack in the past year, not severe enough to require Dilaudid. He is in an independent study program at a small public school and getting straight A's and B's. His mother reports that Alex is assuming responsibilities within the household, has gotten his driver's license, and is beginning to look for a job.

The case of Alex P. suggests that employing pharmaceutical stimulants, antidepressants, and antipsychotics as first-line treatment exposes children gratuitously to harmful side effects in violation of Hippocratic principles. Given the benign side-effect profile of cannabis, it should be considered a first-line of treatment in certain mental disorders of childhood, including persistent insomnia.

THE RELATIVE TOXICITY, MUTAGENIC ACTIVITY AND CHEMICAL COMPOSITION OF TOBACCO AND CANNABIS SMOKE

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While the prevalence of tobacco use has decreased over the last decade, that of cannabis use has increased, particularly among youth. In addition, the use of cannabis for medical purposes is a reality and is currently legally allowed under certain circumstances in Canada. Smoking cannabis is often perceived as less harmful than smoking tobacco, but solid scientific evidence regarding the risk of adverse health effects associated with cannabis smoke is lacking, and the comparative risk of adverse effects from tobacco and cannabis smoke is currently debated. This study examined the chemical composition and toxicological properties of cannabis and Canadian flue-cured tobacco smoke. Condensates of main- and side-stream smoke from hand-rolled cannabis and tobacco were prepared using standard (i.e., ISO) conditions, as well as "extreme" conditions designed to reflect cannabis smoking habits. Biological analyses included cytotoxicity assessment via the neutral red uptake assay, and Salmonella mutagenicity analyses using the standard tester strains TA100, TA98, TA1537, TA1535 and TA102, in addition to metabolically enhanced versions of TA98 and TA1538 (i.e., YG1041 and YG5161). Chemical analyses included measurements of cannabinoids, tar, TPM, PAHs, and other toxic chemicals commonly tracked in tobacco smoke. Mutagenicity analyses showed significant positive responses on base-pair and frameshift strains, with maximum responses obtained in the presence of metabolic activation. Results for Salmonella YG1041 with metabolic activation showed a 2.5 to 4-fold increase relative to TA98, suggesting an important contribution from aromatic amines. Cytotoxicity analyses showed lower IC_{50} values for cannabis smoke, thus indicating higher toxicity. Chemical analyses indicate that many analytes (e.g., PAHs) are more abundant in mainstream tobacco smoke. Total PAHs in tobacco smoke are approximately 1.6-fold higher than levels in cannabis smoke; however, cannabis smoke contained higher levels of pyridine, acrylonitrile, 1,3-butadiene, aminonaphthalenes and aminobiphenyls. Continuing analyses are evaluating the degree to which targeted analytes can account for the differential biological activity, and the relative ability of cannabis- and tobacco-derived samples to induce chromosomal damage.

INVOLVEMENT OF CANNABINOID CB2 RECEPTOR IN MICE ALCOHOL PREFERENCE AND IN HUMAN ALCOHOLISM

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We tested the hypothesis that cannabinoid CB2 receptor (CNR2) in central nervous system may have a role in alcohol dependence using animal models, and then by examining an association between genetic variants of *CNR2* and alcoholism in a human population. In mice *Cnr2* gene expression was determined in brain regions after acute administration of ethanol, and development of alcohol preference. Mice that developed alcohol preference had reduced *Cnr2* gene expression, while ones without development of alcohol preference in same experiment showed no *Cnr2* gene regulation in ventral midbrain. To evaluate Cnr2 role in alcohol related behavior and non-genetics factor in difference of preference, ethanol consumption in mice subjected to chronic daily administration with CB2 agonist JWH015 on ethanol consumption, along with the effect of environmental effects from chronic mild stress, were measured. Development and enhancing of alcohol preference was observed in chronic treatment with JWH015 in stressed mice. The association between *CNR2* gene variation and Japanese alcoholic subjects was found using the non-synonymous polymorphisms, Q63R in the *CNR2* gene (p = 0.007; 95%CI: 1.25(1.06-1.47)). Cannabinoid CB2 receptors are involved with the effects of alcohol along with environmental factors, such as stressors, and may be targeted with CB2 ligands in alcoholism.

INHIBITION BY SR147778 OF ETHANOL CONSUMPTION ELICITED IN RATS AND MICE BY SCHEDULE-INDUCED POLYDIPSIA

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The cannabinoid system has been suggested to play a major role in drug dependence. Indeed, animal studies showed that cannabinoid receptor antagonists such as rimonabant and SR147778 reduce intake of various substances of abuse, including alcohol. The excessive drinking elicited by schedule-induced polydipsia (SIP) makes it an attractive paradigm for investigating ethanol over-consumption in rodents. This procedure elicits drinking of high quantities of ethanol, which may result after interruption of training in an alcohol deprivation effect (a measure of craving). The present study first investigated in rats the activity of SR147778 on SIP for ethanol, then on craving. Secondly, we determined whether CB1 receptors play a role in ethanol consumption during SIP using CB1 receptor KO mice. Finally, we evaluated whether deletion of CB1 receptors alters Fos protein expression, a marker of neuronal activation, in different limbic regions of mice trained in SIP for ethanol.

Male Wistar rats were trained in the SIP procedure for water (FT60s). Increasing concentrations of ethanol were substituted for water to reach a final training concentration of 10%. When liquid consumption reached a plateau, SR147778 dose response relationship was investigated. The alcohol deprivation effect and the activity of SR147778 were evaluated by determining alcohol consumption in a single test session after a 3-day interruption of training. SIP was also studied in WT and CB1 KO mice, then the effect of SR147778 was evaluated in these mice. After the last training session, brains of KO and WT CB1 mice were removed and processed for c-Fos immunohistochemistry. In all experiments, SR147778 was injected (ip) 1h prior to test sessions.

In rats, 1, 3 and 10 mg/kg of SR147778 significantly reduced ethanol (10% concentration) consumption in the SIP procedure, while water intake was not modified. After a 3-day ethanol deprivation, half of the vehicle–treated animals showed an increase in alcohol intake as compared to the preceding training sessions, an effect not seen in animals treated with SR147778 (1 mg/kg). This latter effect suggests that SR147778 may be effective in reducing craving. Both KO and WT mice acquired the SIP for water. Unlike WT mice, CB1 KO animals did not increase their ethanol consumption when ethanol concentrations were elevated from 10 to 14%. Furthermore, SR147778 (0.3 mg/kg ip) reduced alcohol intake in WT mice by 55%, while it did not further modify the already reduced alcohol intake (-33%) in KO as compared WT mice. Fos immunohistochemistry showed that CB1 KO mice trained in SIP for ethanol exhibited significantly less Fos positive cells than WT animals in several limbic regions, including the central nucleus of the amygdala, the prefrontal and cingular cortices.

These results show that SR147778 reduced alcohol consumption in a SIP procedure, and that CB1 receptors are critically involved in this effect. In addition, they provide evidence that CB1 receptors are implicated in the activation of interconnected limbic brain regions in mice subjected to the SIP procedure. Together with the SR147778 action on craving, these findings further confirm that blockade of the CB1 receptor is an attractive approach for the pharmacological treatment of alcoholism.

SUCCINATE ACTS ON BACLOFEN-INSENSITIVE GHB RECEPTORS IN THE NUCLEUS ACCUMBENS

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Gamma-hydroxybutyrate (GHB), a naturally occurring metabolite of gamma-aminobutyric acid (GABA), has been recently known as a recreational drug of abuse. Succinate – an intermediate of tricarboxylic acid cycle – is a common metabolite of GABA and GHB. In many brain areas GHB acts on its own receptor and also binds to $GABA_B$ receptors. In the *Nucleus Accumbens* (NA) – a brain area responsible for the development of reward properties of different drugs - the presence of GHB binding sites has been shown, but the cellular actions of GHB and the pharmacology of GHB binding are not yet characterised. Therefore we studied (1) the pharmacological profile of [^{3}H]GHB binding to synaptic membranes prepared from rat forebrain or human NA samples; (2) the effect of GHB and succinate on intracellular Ca^{2+} ion signalling in the NA cells of rat or $GABA_BR1$ knockout mice.

Affinity screening of the natural ligand GHB, the only known GHBR antagonist NCS-382, succinate, the $GABA_BR$ agonist (*R*)-baclofen and antagonist CGP 55845 were performed in [^{3}H]GHB displacement measurements. The synaptic membrane fraction isolated from rat forebrain was characterized by GHB binding inhibition constants: $K_{i,NCS\text{-}382}$ = 1.2 ± 0.2 µM, $K_{i,GHB}$ = 1.6 ± 0.3 µM and $K_{i,SUCCINATE}$ = 212 ± 66 µM. There was no significant binding interaction between (*R*)-baclofen or CGP 55845 and these [^{3}H]GHB-labeled sites. A similar profile, complete displacement of [^{3}H]GHB by GHB, NCS-382 and succinate but no inhibition by $GABA_BR$ agonist or antagonist was found in the human NA synaptic membrane fraction.

The effects of GHB (2 mM) or succinate (2 mM) on intracellular Ca^{2+} ion signalling was measured using the techniques of laser scanning confocal microscopy. NA slices prepared from neonate rats were loaded with the cell-permeant form of the Ca^{2+} ion indicator dye Fluo4. GHB or succinate application induced intracellular Ca^{2+} ion transients, which were not blocked by NCS-382 (0.3 mM) or CGP 55845 (20 µM). Under conditions, 25 µM (*R*)-baclofen was ineffective. These GHB- or succinate-induced Ca^{2+} ion transients were observed in slices from $GABA_BR1$ -/- mice also.

Our findings indicate the existance of a $GABA_BR$-independent, succinate-sensitive GHB binding site in the NA. NCS-382 does bind to these GHBRs, however it fails to antagonise the effect of GHB on intracellular Ca^{2+} ion transients.

Probably, these GHB- or succinate-induced Ca^{2+} ion transients are mediated by a NCS-382 and $GABA_BR$-insensitive GHB receptor subtype, which can be involved in the development of GHB abuse.

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ADOLESCENT EXPOSURE TO CANNABINOIDS AND LONG TERM CONSEQUENCES ON THE ADULT BEHAVIOUR

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Marijuana and hashish are the most used drugs during adolescence. The adolescence represents a critical phase for cerebral development and it is characterized by strong neuronal plasticity, with sprouting or pruning of synapses, mielinization of nervous fibers, variations of neurotransmitter concentration and of the number of their receptors. It's known from literature that cannabinoids are able to modulate the release and the action of different neurotransmitters; this brings out the possibility that the assumption of appropriate doses of cannabinoids for a prolonged period of time during adolescence may influence the neurobiological development of the brain and its functional, cognitive, affective and behavioural correlates with long term consequences. In order to investigate cerebral alterations induced by chronic THC administration during teenage years, adolescent male and female rats (35-45 PND) have been treated with increasing doses of THC twice a day for 11 days (2.5mg/kg day 1-4; 5 mg/kg day 5-8; 10 mg/kg day 9-11). Once reached adult age, the same rats (75-85 PND) have taken behavioural tests to evaluate locomotor activity, anxiety and depression. The results show that adolescence THC chronic treatment has different effects on male and female rats and only female rats displayed an altered behaviours in adult life. In fact THC female rats exhibited an increase of anxiety (tested with elevated plus maze), as demonstrated by the reduced number of entrances in the open arm and the time spent in that arm. THC female rats showed reduced locomotor activity and increased immobility (tested with open field) and exhibited in the swim test, a reduction of the time spent in "swimming" and "climbing" and an increase in "immobility"which reflect a state of despair. Cellular mechanisms and electrophysiological correlates are also investigated to parallel the observed altered behaviours with specific neurochemical substrates. Our data suggest that adolescence exposure to THC can alter the adult behaviour mainly in the females suggesting that female brain is more susceptible to long term neurobiological effects induced by cannabinoids.

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THE SITES OF SYNTHESIS AND ACTION OF ENDOCANNABINOIDS IN THE NUCLEUS ACCUMBENS

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Modulation of the brain's reward circuitry and the effects of all addictive drugs are based on the alteration of long-term synaptic plasticity. It is widely accepted that administration of cannabinoids occludes a new form of long-term depression induced by electrical stimulus at prefrontal cortical afferents to the nucleus accumbens (Acb). This excitatory synaptic transmission is modulated by presynaptically located CB_1, and/or postsynaptic type 5 metabotropic glutamate receptors (mGluR5), which play crucial roles in addiction-related behaviours. However, little is known about the precise distribution of the molecular components involved in this synaptic plasticity and retrograde cannabinoid signaling. Thus, we examined – using newly developed antibodies and immunogold techniques – the localization of CB_1 and diacylglycerol lipase alpha (DGL-α), the enzyme involved in generation of the endocannabinoid, 2-arachidonoyl-glycerol (2-AG) in the Acb. Our electron microscopic analysis reveals that DGL-α is located on the postsynaptic side of glutamatergic synapses, while the presynaptic terminals are equipped with CB_1 receptors. Thus, postsynaptically released 2-AG may influence glutamate release in a retrograde fashion, and change the conditions for synaptic plasticity. Furthermore, overlapping distribution of mGluR5 and DGL-α may suggest molecular interaction between the glutamatergic and cannabinoid systems in triggering endocannabinoid synthesis in association with addiction-related synaptic plasticity.

MEDIAL FOREBRAIN BUNDLE STIMULATION EVOKES ENDOCANNABINOID-MEDIATED EFFECTS ON DOPAMINE IN NEURONS IN VIVO

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Depolarization-induced suppression of excitation and inhibition (DSE/DSI) are the best characterized endocannabinoid-mediated short-term forms of synaptic plasticity. DSE/DSI have been demonstrated to occur *in vitro* in several brain regions, including the dopaminergic ventral tegmental area (VTA). It was earlier shown that depolarization-induced back-propagating action potentials are likely to play an important role in triggering the increases in intracellular Ca^{2+} necessary for DSI. Thus, we reasoned that depolarization of dopamine (DA) VTA neurons induced by antidromic action potentials evoked by stimulation of the medial forebrain bundle (MBF), could represent an ideal approach for the demonstration of DSE/DSI-like mechanisms *in vivo*. MFB is a large collection of axons, which includes those ascending from VTA DA neurons to the nucleus accumbens and other forebrain structures. Stimulation of the MFB is rewarding in animals, since it elicits an enduring potentiation of behaviour directed at obtaining additional stimulation (intracranial self-stimulation).

Single cell extracellular electrophysiological activity was recorded from VTA DA neurons in anaesthetized rats. DA neurons responded to MFB stimulation (1 s, 40 Hz, threshold intensity) with a short lasting (20 s) not significant increase in spontaneous firing rate and burst firing. The CB1 receptor antagonist SR141716A (SR, 1 mg/kg, i.v.) evoked an enhancement of post-stimulus firing rate and burst firing of the antidromic cell ($p<0.001$) which lasted for 30 s. Thus, endocannabinoids acting at CB1 receptors dampened MFB stimulation-induced increase in firing rate of DA neurons, an effect unmasked by the CB1 antagonist. In a separate set of experiments, we used sub-threshold stimulations of MFB, ineffective to evoke antidromic spikes in the recorded cells. Under these conditions, SR did not enhance post-stimulus firing rate and burst firing of DA neurons, indicating that no endocannabinoid tone was present. Opposite to SR, the CB1 receptor agonist WIN55212-2 (0.125 mg/kg, i.v) produced a decrease ($p<0.05$) in MFB stimulation-evoked firing of DA neurons. This suggests that the effects of CB1 receptor activation by endocannabinoids are additive to those induced by exogenous agonists.

Our results indicate that DA neurons may release endocannabinoids in response to the stimulation of the MFB. This action might be triggered by the excitation of the cell following antidromic action potential travelling to the somatodendritic region, in a DSI/DSE-like mechanism. Since we found that endocannabinoids predominantly depress excitation of the DA neuron *in vivo*, it is presumed that this may represent a form of synapse-specific modulation and a feedback mechanism to reduce over-excitation of DA neurons.

Our data further suggest that endocannabinoids likely play an important role in the ongoing regulation of brain functions *in vivo*, including those involved in mediating reward, pleasure, motivation, and perhaps addiction.

THE CANNABINOID RECEPTOR ANTAGONIST SR141716A BLOCKS THE INHIBITORY ACTIONS OF ALCOHOL ON BASOLATERAL AMYGDALA PROJECTION NEURONS *IN VIVO*

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A large body of evidence indicates that the limbic system is involved in the neural processing underlying drug addiction. Among limbic regions, the basolateral nucleus of amygdala (BLA) is implicated in some aspects of the neurobiological mechanisms of drugs of abuse, including alcohol and cannabinoids. Hence, inputs from the BLA to central nuclei and onto autonomic and neuroendocrine centers constitute an important pathway in the induction of different kinds of emotional, autonomic and neuroendocrine responses. It is recently emerging that the endocannabinoid system is involved in many pharmacological and behavioural effects of alcohol. The BLA possesses a very high density of CB1 cannabinoid receptors, whereas endocannabinoids modulate forms of synaptic plasticity in this region. The aims of our study were first to investigate the sensitivity of BLA neurons to alcohol and second to determine the role of the endocannabinoid system in the actions of alcohol.

We utilized extracellular single cell recordings in urethane anesthetized animals from BLA principal neurons, antidromically identified from their projection site in the nucleus accumbens.

Alcohol (0.25-2.0 g/kg i.v.) induced a marked decrease in the spontaneous firing rate of BLA projecting neurons (49.7±14% of baseline at 0.5 g/kg alcohol $n=7$, $p<0.0001$). The involvement of the endogenous cannabinoid system was investigated by administering the CB1 receptor antagonist SR141716A (SR) (1.0 mg/kg i.v.) before alcohol. SR *per se* did not significantly affect firing rate of BLA neurons, but it prevented the inhibitory effect of alcohol (100.8±23% of baseline firing at 0.5 g/kg $n=7$ $p>0.05$). Then, we studied the actions of alcohol following a chronic treatment with the CB1 agonist WIN55212-2 (WIN), which was previously shown to induce a reduction in CB1 receptor coupling efficiency. Animals were administered WIN for 7 days (2.0 mg/kg, i.p. once per day), then alcohol dose-response curves were carried out on firing rate of BLA neurons 24 h following the last injection of the cannabinoid agonist. Preliminary experiments indicate that the inhibitory effects of alcohol on BLA neurons were less pronounced in treated animals when compared to vehicle-injected animals.

Our results further support the hypothesis of an involvement of the endocannabinoid system in the effects of alcohol and highlight the possibility that pharmacological intervention targeting the endocannabinoid system might be useful in the treatment of alcoholism.

ENHANCEMENT OF DOPAMINE LEVELS IN THE RAT NUCLEUS ACCUMBENS SHELL DURING WIN 55,212-2 SELF-ADMINISTRATION

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The rewarding properties of cannabinoids are thought to be primarily mediated by the release of dopamine (DA) in the nucleus accumbens (NAcc). *Passive* (i.e. experimenter given) administrations of delta-9-tetrahydrocannabinol (THC) and synthetic CB1 receptor agonist WIN 55,212-2 are reported to enhance extracellular DA overflow in the NAcc and other reward-related forebrain areas. However, DA level variations in this brain region during *voluntary* intake (i.e. self-administration) of cannabinoids were still unknown. In the present study, accumbal DA level was monitored with "in vivo" microdialysis procedure during ongoing cannabinoid self-administration in rats to achieve a more detailed understanding of how DA mediates the reinforcing effects of cannabinoids. As rat strain used may be an important variable in self-administration studies, extracellular levels of DA were measured in the shell portion of the NAcc of either Long Evans and Lister Hooded rats trained to stably self-administer WIN 55,212-2 (12.5 μg/kg/inf) intravenously. During self-administration sessions, cannabinoid-induced changes in motor activity were monitored by means of 4 photocells enclosed in each SA box. An overall significant relationship between extracellular DA levels and bar-pressing rates for the cannabinoid was observed in both rat strains, as the release of DA increased in the NAcc shell in respect to basal pre-session levels during drug intake. However, the magnitude of the increases as well as the temporal fluctuations of DA levels across the 2h sessions were different in the two strains of animals, thus revealing strain-dependent differences in the neurochemical response to cannabinoid. Yet, neither motor activity nor the number of infusions gained nor the basal level of extracellular DA was significantly different. In comparison to the basal DA release, rises in DA extracellular levels ranged from 126 to 202 % of basal values in Long Evans rats but only from 120% to 154% in Lister Hooded rats, with a mean DA level of 141.5±2.4 % and 160.3±5.68%, respectively, occurring during cannabinoid self-administration. Moreover, Long Evans rats displayed more swinging variations than Lister Hooded rats, with maximal effects at approximately 80 min after the beginning of the session. The present results indicate that, similarly to other drugs of abuse, an enhanced activity of limbic DA transmission underlies cannabinoid-taking behaviour, providing a neurochemical basis for understanding the abuse liability of *Cannabis* derivates.

Δ^9-THC STIMULATES ALCOHOL INTAKE IN ALCOHOL-PREFERRING sP RATS

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Recent studies demonstrated that the synthetic cannabinoid receptor agonists, WIN 55,212-2 and CP 55,940, markedly stimulated alcohol intake in selectively bred Sardinian alcohol-preferring (sP) rats. Of interest, the stimulating effect of both drugs on alcohol intake was completely prevented by administration of the cannabinoid CB_1 receptor antagonist, rimonabant, or the opioid receptor antagonist, naloxone (both antagonists were given at doses that did not affect alcohol intake *per se*). The present study was designed to extend this investigation to Δ^9-tetrahydrocannabinol (Δ^9-THC; provided by the National Institute on Drug Abuse), the major psychoactive constituent of *Cannabis sativa*. Δ^9-THC was administered to alcohol-experienced sP rats exposed to the standard, homecage 2-bottle "alcohol (10% v/v) *vs* water" choice regimen with unlimited access for 24 hours/day. Δ^9-THC (0.3-3 mg/kg, i.p.) was acutely administered 20 min before lights off. As expected, Δ^9-THC stimulated alcohol intake: 60 min after lights off, the increase in alcohol intake in the rat groups treated with 0.3, 1, and 3 mg/kg Δ^9-THC averaged approximately 25%, 46%, and 42%, respectively. Pretreatment with either rimonabant (0.3 mg/kg, i.p.) or naloxone (0.05 mg/kg, i.p.), given at doses which were ineffective *per se*, completely blocked the stimulating effect of 1 mg/kg Δ^9-THC (i.p.) on alcohol intake. These results add further support to the hypotheses that (a) the cannabinoid receptor system is part of the neural substrate regulating alcohol intake in sP rats, (b) cannabinoid receptor agonists may fix to a higher level the hedonic set-point mechanism regulating alcohol drinking behavior in sP rats, and (c) the promoting effect of cannabinoid receptor agonists, including Δ^9-THC, on alcohol intake in sP rats is mediated by stimulation of the cannabinoid CB_1 receptor and require the activation of the endogenous opioid system.

RIMONABANT AMELIORATES NEUROCHEMICAL UNBALANCE AND BEHAVIORAL SYMPTOMS OCCURRING DURING ALCOHOL WITHDRAWAL

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Recent studies have postulated that the cannabinoid system might play a regulatory role in physiological processes altered in addictive states. Thus, there is evidence on an involvement of this system in genetic vulnerability, seeking behavior, dependence, relapse and other phenomena elicited by different types of habit-forming drugs, such as alcohol, opioids, cocaine or tobacco. Even, recent evidence suggest that the blockade of CB_1 receptors might serve to reduce craving for different substances such as nicotine or alcohol. However, there is less evidence concerning the possibility that the blockade of CB_1 receptors might be also beneficial to attenuate withdrawal symptoms elicited by the interruption of regular abuse of these substances. In the case of alcohol, this hypothesis is supported by the reduction of withdrawal responses observed in CB_1 receptor knockout mice [Racz et al., J. Neurosci. 23, 2453-2458 (2003)]. The present study have been designed to examine the potential of rimonabant (SR141716) to improve neurochemical unbalance and to reduce behavioral symptoms elicited by the interruption of chronic alcohol exposure (7.2% in the drinking water for 10 days) in male Wistar rats. We found that the administration of rimonabant to alcohol abstinent rats corrected the GABA/glutamate unbalance developed by these animals in various brain regions mostly related to emotional and motor responses. In addition, it reduced the stimulation of enkephalinergic transmission caused by alcohol abstinence in motor and limbic structures. Rimonabant also affected, although to a lesser extent, the dopamine deficits generated by alcohol abstinence, but it was unable to correct serotonin impairment. The result of these neurochemical effects was an amelioration of the anxiogenic state developed by the animals after the interruption of regular alcohol intake. In summary, rimonabant might be efficacious to attenuate withdrawal signs associated with the alcohol abstinence. This effect was presumably due to normalization of GABA, glutamate, opioid, and, to a lesser extent, dopamine transmission in emotion-related and motor-related areas. It is also possible that it is related to an attenuation of the stress response activated by the interruption of regular alcohol abuse.

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NICOTINE-INDUCED RELAPSE TO ALCOHOL AND THE NEW SPECIFIC CANNABINOID CB1 RECEPTOR ANTAGONIST SR147778

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In the last five years, preclinical and clinical studies have demonstrated the role of CB_1 cannabinoid antagonists and inverse agonists in potential strategies of treatment for drug addiction, such as alcoholism and smoking.

We have used the Non-Contingent Drug Model developed in our laboratory for the investigation of the new selective CB_1 cannabinoid antagonist SR147778 in the nicotine-induced relapse to alcohol in Wistar rats. The nicotine-induced shifts of responding for alcohol were evaluated in chambers of operant oral self-administration. Furthermore, nicotine's effects during alcohol deprivation were assessed.

SR147778 is able to modulate the nicotine-induced relapse to alcohol and it seems that oral administration has greater effect than intraperitoneal administration. Only oral withdrawal of SR147778 was followed by a rebound increase in alcohol consumption. However, animals not pretreated with SR147778 showed a long-lasting relapse to alcohol. In addition, nicotine-induced hypothermia, nicotine-induced hypolocomotion and the absence of rewarding or aversive effects of nicotine are also reported.

The present study provides the first evidence that SR147778 can modulate the relapse to alcohol even when it is nicotine-induced. This suggests that the cannabinoid system could be an interesting target for the control of alcohol-motivated behaviours.

CANNABINOIDS MODULATE SYNAPTIC TRANSMISSION IN THE RAT NUCLEUS ACCUMBENS IN VIVO

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Evidence suggests that the Nucleus Accumbens (NAc), part of the mesolimbic system is likely to play an important role in the acute reinforcing action of drugs of abuse. Biochemical studies suggest that endocannabinoids, through CB1 receptors, are candidates for retrograde messengers in short-term regulation of transmitter release, and that retrograde release of endocannabinoids are important for the establishment of long-term synaptic plasticity.

ANANDAMIDE UPTAKE INHIBITORS AFFECT CELL PROLIFERATION AND CAUSE CALCIUM INFLUX INTO C6 GLIOMA CELLS

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Evidence is accumulating to suggest that in addition to their actions upon the cellular accumulation of anandamide, acyl-based uptake inhibitors produce actions upon cell proliferation. In a previous study, it was found that preincubation of C6 glioma cells for ≥24 h with 10 μM AM404 or VDM11 reduced cell proliferation in a manner not blocked by a combination of the cannabinoid receptor antagonists AM251 and AM630 and the TRPV1 antagonist capsazepine (Jonsson et al., Arch Toxicol 77 [2003] 201-7). In the present study, the effects of these compounds upon C6 glioma cell function have been studied in more detail.

AM404 and VDM11 at concentrations of 10 μM greatly reduced the viability of C6 cells assessed by measuring their ability to take up and hydrolyse calcein-AM after an exposure time of as little as 3 h. Effects of AM404 and VDM11 upon cell viability were also observed using other assays (release of lactate dehydrogenase and total nucleic acid levels), although these measures were less sensitive and significant effects were first seen after 14 h and 9 h respectively. Calcein fluorescence of differentiated C6 cells was also reduced by these compounds, suggesting that the effects seen are due to cell toxicity rather than a slowing down of their rates of proliferation. Similar results were found with 10 μM OMDM-2. UCM707 was less potent, and took longer to produce its effect upon cell viability. Flow cytometry of propidium iodide-labelled cells suggested that under the conditions used, the loss of cell viability was not accompanied by widespread apoptosis. The effects of the compounds were not prevented by AM251, AM630 and capsazepine either separately or in combination, by the FAAH inhibitor URB597, or by the lipoxygenase inhibitor cinnamyl-3,4-dihydroxy-α-cyanocinnamate, but were reduced by the antioxidant N-acetylcysteine.

The ability of the compounds to affect calcium homeostasis was investigated using C6 cells and Fluo-4 as fluorophore. All four compounds produced a robust increase in calcium fluorescence that was prevented by removal of extracellular calcium. The increased intracellular calcium peaked ~1 min after addition of the uptake inhibitors and was still above basal after 6 min. Capsazepine pretreatment did not affect the calcium response, whereas the duration of the signal was shortened by N-acetylcysteine treatment.

It is concluded that the acyl-based uptake inhibitors AM404, VDM11, OMDM-2 and UCM707 produce dramatic effects upon C6 glioma viability and calcium homeostasis at concentrations that are needed to reduce the uptake of anandamide in these cells.

ANANDAMIDE ARRESTS CELL CYCLE PROGRESSION IN MDA-MB-231 HUMAN BREAST CANCER CELLS THROUGH INHIBITION OF CDK2 ACTIVITY AND CHK1 ACTIVATION

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We have previously reported that the endocannabinoid anandamide inhibits breast cancer cell proliferation by acting at cannabinoid receptor CB1, but little is known regarding the molecular mechanisms underlying these effects. Flow cytometric analysis revealed an S phase cell cycle blockade in the human breast cancer cell line MDA-MB-231 when treated with a stable analogue of anandamide (Met-F-AEA).We therefore examined the effect of this compound on components of the cell cycle machinery responsible for regulating cell cycle progression. The S phase arrest was associated with up-regulated cyclin-dependent kinases (Cdks) inhibitors $p21^{waf1}$ and $p27^{Kip1}$ levels, decreased protein expression of cyclin E and A, and with the inhibition of Cdk2 kinase activity *in vitro*. Indeed, immunoprecipitation experiments confirmed that cyclin E was complexed with Cdk2 and it was decreased in Met-F-AEA treated cells. Furthermore, the retinoblastoma protein (Rb), a Cdk2 substrate whose phosphorylation is necessary for cell cycle progression, is hypophosphorylated in Met-F-AEA treated cells. Furthermore, we show that Met-F-AEA induces an intra S phase checkpoint by activating Chk1 that in turn mediates the degradation of Cdc25A phosphatase inducing the S phase arrest.

INVESTIGATION OF CB1 AS A POTENTIAL DRUG TARGET IN ALVEOLAR RHABDOMYOSARCOMA

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Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma and can histologically be divided into two main subgroups, namely embryonal and alveolar rhabdomyosarcoma (eRMS and aRMS). The majority of the aRMS carry a specific PAX3/FKHR-translocation. Microarray analysis of biopsy samples revealed an expression signature specific for the translocation-positive rhabdomyosarcoma compared to the other subgroups. This signature is composed of several hundred genes highly upregulated in translocation-positive samples, among them the gene for the cannabinoid receptor 1 (CB1) *(M.Wachtel et al., Cancer Research, 2004*). CB1 activation has been shown to lead to apoptosis in a series of tumors, such as leukaemia, glioma, breast cancer and skin carcinoma (*Guzman, Nature Reviews Cancer, 2003*). Based on these facts, it is the aim of our project to investigate whether CB1 can also serve as a potential drug target in the treatment of aRMS.

First, we demonstrate that treatment of aRMS cells with an anandamide-related structure (eg. ACEA or Met-F-AEA) was able to specifically reduce the percentage of viable cells. However, short-term specific siRNA- as well as stable shRNA-mediated down-regulation of CB1, to about 20% of the original CB1 RNA expression, did not significantly rescue the inhibition of proliferation under drug treatment. Since the cells express very low levels of CB2 only, this suggests that a non-receptor mediated effect can not be ruled out at the moment. Alternatively, anandamide membrane transporter (AMT) may lead to the accumulation of anandamide in the cells, which is further metabolized by fatty acid amide hydrolase (FAAH) and cyclooxygenase (COX). To test the role of COX enzymes, we co-treated cells with a COX inhibitor, Ibuprofen, and indeed an enhanced anti-proliferative effect compared to ACEA treatment alone was observed. Our experiments demonstrate that cannabinoid receptor agonists might have a therapeutic potential in aRMS, however the mechanism of action has to be further investigated.

ANANDAMIDE RECEPTOR MEDIATED MATRIX METALLOPROTEASE ACTIVITY IN TUMOR CELLS IS PARTIALLY MEDIATED BY NITRIC OXIDE AND NEGATIVELY REGULATED BY HETEROTRIMERIC GI PROTEIN

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We have previously shown that activation of CB_1 receptor and non-CB_1/CB_2 anandamide receptor produced differential phosphorylation of endothelial nitric oxide synthase (eNOS) in endothelial and tumor cells (Johnson and Mukhopadhyay Proceeding in Am. Soc. Cell Biol 2005). In the present study we have shown that methanandamide produced an increase in matrix metalloprotease (MMP-2 and MMP-9) activity in the conditioned media from CB_1 cannabinoid receptor blocked LNCaP prostate cancer cells. The methanandamide-mediated increase in MMP activity was attenuated by anandamide receptor antagonist O-1918 suggesting the role of non-CB_1/CB_2 anandamide receptor in the process. We initially hypothesized that anandamide-mediated nitric oxide production acts as a regulatory switch for MMP-2 and MMP-9 activity in LNCaP cells. However in the present study we found that nitric oxide synthase inhibitor L-NAME only partially block anandamide-mediated increase in matrix metalloprotease activity. Further we found that treating the cells with pertussis toxin augmented anandamide-mediated increase in MMP-2 or MMP-9 activity in these cells. Thus results from this study suggest that anandamide-mediated increase in MMP-2 and MMP-9 activity is mediated by non-CB_1/CB_2 anandamide receptor and partially dependent on NO production in LNCaP cells. (This study was supported by AHA 0060377Z and pilot project CA-92077 to SM, NIDA U24 DA 12385 and R25-GM66332)

CYTOTOXIC EFFECTS OF CANNABINOIDS ON UNDIFFERENTIATED AND RETINOIC ACID-INDUCED P19 EMBRYONAL STEM CELLS

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Beside the well established neuromodulatory effects of the cannabinoids, evidence suggests that the endocannabinoid system may be involved in the regulation of neurogenesis (see Augado et al. *FASEB J* **19** [2005] 1704-6; Jiang et al. *J Clin Invest* **115** [2005] 3104-16). The role(s) played by the cannabinoid receptors in neural cell proliferation, determination and differentiation, however, are unclear and further investigation is necessary.

In the present study, we have examined the effects of anandamide, HU 210, WIN 55,212-2, methanandamide and arachidonoyl glycine, and the polyunsaturated fatty acids arachidonic acid and eicosapentaenoic acid upon mouse embryonal carcinoma P19 stem cell viability - before, during and after retinoic acid (RA)-induced neuronal differentiation. This cell model system shows native expression of functional CB_1 receptors, and both CB_1 mRNA and protein expression are significantly increased upon neuronal differentiation under serum-free culture conditions (Svensson et al. *J Neurosci Res* [2006], in press). In particular, the model allows the study of toxicity for the same cell type in undifferentiated conditions, in conditions where the neural developmental pathway has been initiated ("RA-induced cells"), and in neurons that are completely differentiated.

Cells were incubated with various concentrations of the cannabinoids and cell viability and proliferation were monitored for up to four days of exposure. All cannabinoids examined produced a time- and concentration-dependent decrease in P19 cell viability. HU 210 was the most potent compound with an IC_{50} value of ~1.3 µM in undifferentiated cells, and in the range 5-9 µM for both RA-induced cells and in differentiated neurons. Anandamide was less potent with IC_{50} value of ~10 µM in undifferentiated cells and did not reach 50% inhibition at concentrations below 30 µM in RA-induced cells and differentiated neurons. The cytotoxic effects of HU 210 (1 µM) and AEA (10 µM) were inhibited by pre-incubating with the cannabinoid CB_1 receptor antagonist AM251 (0.3 µM and 1 µM), the antioxidants α-tocopherol (10 µM) and *N*-acetyl cysteine (0.5 mM). In addition, HU 210 toxicity was prevented by the ceramidase synthase inhibitor fumonisin B_1 (10 µM) and the nitric oxide synthase inhibitor L-NAME (10 µM). Inhibitors of the enzymes COX, LOX, and FAAH, and TRPV1 antagonists did not prevent cannabinoid-induced effect on P19 cell viability.

The results support the concept that cannabinoids have antiproliferative effects on tumour cells, but induction of the neural pathway reduces the sensitivity to the cytotoxic effects of cannabinoids. The mechanism whereby synthetic and endogenous cannabinoids affect cell viability and proliferation may partly be dependent on CB_1 receptor activity, but the increased CB_1 receptor expression in P19-derived neurons suggests that other non-CB_1 receptor-mediated mechanisms are also present.

CANNABINOID CB_1 RECEPTOR ACTIVATION INHIBITS MPTP-INDUCED EXPRESSION OF S100B IN GLIAL CELLS THEREBY LEADING TO SURVIVAL OF CO-CULTURED NEURONS. MECHANISM OF ACTION AND INVOLVEMENT OF ENDOCANNABINOIDS

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S100B is an astroglial-derived Ca^{+2}-binding protein exerting a neurotrophic role on neighbouring neurons. However its overproduction has been described in several neuropathologies, including Parkinson's Disease. The mechanism underlying the neurotoxicity of S100B is still unclear. Natural and synthetic cannabinoids from the plant *Cannabis sativa,* exhibit a plethora of biological effects, including neuroprotection. In this study we investigated the mechanism of S100B toxicity, and the protection thereof by cannabinoids, in co-cultures of rat glioma C6 cells treated with the neurotoxin MPTP, and PC12 rat pheocromocytoma cells, differentiated into neurones. C6 cells were treated with MPTP for 24 h in the presence or absence of selective CB_1 (ACEA) or CB_2 (JWH-015) agonists. MPTP (1-100 μg/ml) concentration- and time-dependently increased S100B transcription and expression in C6 cells. This effect was followed by increased C6 cell proliferation and decreased cell viability of co-cultured PC12 cells. An S100B antibody, given to PC12 cells before co-culture, led to their survival. ACEA (10^{-8}-10^{-6}M), but not JWH-015 (10^{-8}-10^{-6}M), significantly inhibited MPTP-induced S100B expression in C6 cells and protected co-cultured PC12 cells from cell death. Since MPTP (100μg/ml) increased selectively the levels of the endocannabinoid anandamide in C6 cells, the involvement of endocannabinoid system was investigated by using: two selective inhibitors of endocannabinoid inactivation (cellular re-uptake or enzymatic hydrolysis), selective CB_1 and CB_2 receptor antagonists and by silencing CB_1 receptor. Our data suggest that selective activation of CB_1 receptors by either exogenous or endogenous cannabinoids might afford neuroprotection in MPTP-induced neurotoxicity also by controlling S100B up-regulation in activated glial cells.

CANNABIDIOL INHIBITS INDUCIBLE NITRIC OXIDE SYNTHASE PROTEIN EXPRESSION AND NITRIC OXIDE PRODUCTION IN β-AMYLOID STIMULATED PC12 NEURONS THROUGH P38 MAP KINASE AND NF-κB INVOLVEMENT

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Alzheimer's disease (AD) is the most common form of age-related dementia, characterized by a massive accumulation of beta-amyloid (Aβ) peptide aggregates in specific region within the brain. The toxicity of Aβ aggregates is due, at least in part, to enhancement of oxidative stress that is a trigger signal for a cascade of pro-inflammatory reactions implicated in the AD pathogenesis. Among the pro-inflammatory mediators nitric oxide (NO), deriving from the inducible NO synthase enzyme (iNOS), is released during AD. In view of the pro-inflammatory scenario observed in AD, in the recent years anti-inflammatory drugs have been proposed as potential therapeutic agents. We have previously shown that cannabidiol (CBD), the main non-psychotropic component from *Cannabis sativa*, possess a variegate combination of anti-oxidant and anti-apoptotic effects that protect PC12 cells from Aβ toxicity. In parallel, CBD has been described to have anti-inflammatory properties in acute models of inflammation but the possible inhibitory effect of CBD on iNOS protein expression and nitrite production in the nitrosative stress induced by Aβ in neuronal cell-line is un-investigated. CBD (10^{-6}-10^{-4} M) inhibited both nitrite production and iNOS protein expression induced by Aβ (1-42) (1μg/mL) in differentiated PC12 cells. CBD effect was mediated through the inhibition of p38 MAP kinase and transcription factor nuclear factor-κB (NF-κB) activation in a concentration-dependent manner. The here reported data increases our knowledge about the possible neuroprotective mechanism of cannabidiol, highlighting the importance of this compound to inhibit β-amyloid induced neurodegeneration, in view of its low toxicity in humans.

MODULATION OF THE ENDOCANNABINOID SYSTEM IS INVOLVED IN ESTROGEN-MEDIATED NEUROPROTECTION AGAINST FOCAL BRAIN ISCHEMIA

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Endogenous levels of the endocannabinoid anandamide (AEA), and the activity of the AEA-synthesizing enzyme NAPE-PLD and of the AEA-hydrolyzing enzyme FAAH were determined in the cortex (penumbra) and the striatum (core) of rats subjected to middle cerebral artery occlusion (MCAo) for 2 h. AEA content was markedly increased (~3-fold over controls; $P < 0.01$) in the ischemic core of MCAo rats, but not in the penumbra, and this elevation was paralleled by increased activity of NAPE-PLD (~1.7-fold; $P < 0.01$), and reduced activity (~0.6-fold; $P < 0.01$) and expression (~0.7-fold; $P < 0.05$) of FAAH. These effects of MCAo were further potentiated by 1 h reperfusion, whereas AEA-binding to CB1 and TRPV1 receptors was not affected significantly by the ischemic insult. Additionally, the CB1 receptor antagonist SR141716, but not the receptor agonist R-(+)-WIN55,212-2, significantly reduced (33%; $P<0.05$) cerebral infarct volume detected 22 h after reperfusion had been initiated. Interestingly, a neuroprotective dose of estrogen (0.20 mg/kg, i.p.), that reduced infarct size by 43%, also minimized the effect of brain ischemia on the endocannabinoid system, in an estrogen receptor-dependent manner.
In conclusion, we show that modulation of the endocannabinoid system may contribute to the neuroprotection afforded by estrogen against ischemia-induced brain damage.

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SPECIFIC CHANGES IN 2-ARACHIDONOYL-GLYCEROL MEDIATED ENDOCANNABINOID SIGNALLING AT EXCITATORY SYNAPSES IN TEMPORAL LOBE EPILEPTIC PATIENTS

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CB_1 cannabinoid receptors are expressed on the terminals of glutamatergic principal cells and GABAergic interneurons. Thus, any dysfunction of the endocannabinoid system in epilepsy may contribute to the instability of network activity. In animal experiments, synthetic agonists, antagonists, and natural ligands of CB_1 have been found to evoke or prevent epileptic seizures, whereas endocannabinoid levels were changed by seizures. To elucidate potential chronic changes in the activity of the endocannabinoid system in hippocampi of temporal lobe epileptic patients, we measured the expression level of all genes identified to date as components of the endocannabinoid system with quantitative real-time PCR. Experiments provided evidence that mRNA level of the CB_1 receptor is decreased to ~50% in non-sclerotic and to ~30% in sclerotic epileptic hippocampal tissue. Immunostaining for CB_1 receptors confirmed this reduction, which was most apparent in the inner molecular layer of the dentate gyrus. Furthermore, we found a significant reduction in the level of diacylglycerol lipase α (DGLα), the main enzyme responsible for the synthesis of the endocannabinoid 2-arachidonoyl-glycerol (2-AG) as well as in level of cannabinoid receptor interacting protein 1a (CRIP1a), a glutamatergic cell-specific anchoring protein of CB_1. In contrast, levels of the enzymes N-arachidonoylphosphatidylethanolamine phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) responsible for synthesis and degradation of the endocannabinoid anandamide, did not change significantly. Similarly, we did not observe significant alteration in the level of 2-AG synthetizing diacylglycerol lipase β (DGLβ), 2-AG degrading monoglyceride lipase (MGL) and cannabinoid receptor interacting protein 1b (CRIP1b). These findings demonstrate that those elements responsible for 2-AG-mediated endocannabinoid signaling at glutamatergic synapses are selectively reduced in the epileptic human hippocampus, which may contribute to the development of excitation/inhibition imbalance in the epileptic hippocampal network.

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AM 404 LEADS TO NEUROPROTECTION AGAINST ISCHEMIA-INDUCED NEURONAL INJURY

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Anandamide and 2-AG, the most endocannabinoids studied, are produced "on demand" after cerebral ischemia from membrane associated precursors (Baker et al., 2003). As soon as anandamide is released, the diffusion process is accelerated by a rapid and selective carrier system (Piomelli 2003). The development of a series of anandamide transport inhibitors, to slow its elimination and to magnify its beneficial effects, such as AM404, can provide a new tool to investigate the role of endocannabinoids (Piomelli, 2003). Since it is well known the protective role of anandamide in processes occurring during cerebral ischemia (van der Stelt et al., 2001; Berger et al, 2004), the aim of the present work was to investigate the effect of AM404 against neuronal injury *in vivo*. The animal model we used was the transient global cerebral ischemia induced by Bilateral Carotid Arteries Occlusion (BCAO) in mongolian gerbils. The compound was given i.p. 5 min after BCAO in a range of doses between 0.01 and 1 mg/kg. To quantify the ischemic damage we measured from 1 hour to 7 days after recirculation, electroencephalographic (EEG) mean total spectral power, spontaneous motor activity, cognitive function, rectal temperature and hippocampal neuronal count, all parameters known to be hardly influenced by BCAO (Peruche et al., 1995). AM404 antagonized hyperlocomotion, evaluated in an "activity cage" on Day 1 and the EEG flattening, on Day 7. AM 404 also induced a significant decrease of rectal temperature, within the first hour, and reversed ischemia-induced cognitive deficit, evaluated through the passive avoidance test, on Day 3. Finally, histological examination, carried out on Day 7 with cresyl violet staining, showed that AM404 protected against neuronal loss in CA1 hippocampal subfield. These results, taken together, demonstrate the anti-ischemic effect of AM 404 suggesting a protective role of endocannabinoids in events occurring during cerebral injury. Since it's well documented an affinity for vanilloid receptors, experiments are in progress to clarify the mechanism by which AM404 shows its protective effect.

SIMULTANEOUS CB1 AND CB2 RECEPTORS ACTIVATION SHOWS A SYNERGIC NEUROPROTECTIVE EFFECT AFTER OXYGEN AND GLUCOSE DEPRIVATION IN 7-DAY-OLD RAT FOREBRAIN SLICES

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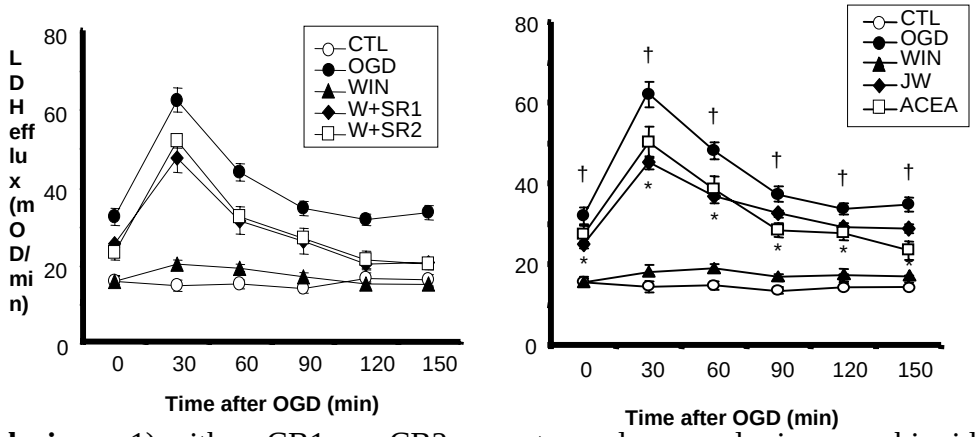
Introduction: we have demonstrated that the cannabinoid agonist WIN55212 affords neuroprotection both in an in vitro model of newborn hypoxic-ischemic brain damage. Since WIN is a non-specific CB1/CB2 receptor agonist, the aim of the present work was to discriminate the relative importance of these receptors in the cannabinoid-induced neuroprotective effect.

Methods: 500 μm brain slices obtained from 7-day-old Wistar rats were exposed to Oxygen-Glucose Deprivation (OGD) for 30 min. The effect of the incubation of OGD slices with the CB1/CB2 agonist WIN 55,212-2 (50 uM), the CB1 agonist ACEA (50 uM) or the CB2 agonist JWH-133 (50 uM), alone or with the CB1 or CB2 receptor antagonists Rimonabant (50 uM) and SR144528 (50 uM), respectively, was studied using the quantification of LDH efflux by spectrophotometry as indicator of hypoxic-ischemic damage in the brain.

Results: OGD led to severe tissue damage, as reflected by the increase in LDH efflux. Incubation of slices with WIN prevented that increase in LDH efflux; this prevention was reversed by the co-incubation of the slices with WIN in presence of SR1 or SR2. Incubation of the slices with ACEA or JWH-133 alone reduce LDH efflux, but lesser than WIN in both cases. This effect was receptor-specific, as SR1 reversed the effect of ACEA and SR2 did so with that of JWH-133. In addition, the effect of ACEA was similar to that of WIN+SR2, whereas that of JW-133 was similar to that of WIN+SR1.



Conclusions: 1) either CB1 or CB2 receptors play a role in cannabinoid-induced neuroprotection; and 2) simultaneous activation of both CB1 and CB2 receptors was more effective than exclusive CB1 or CB2 activation in modulating the factors involved in hypoxic-ischemic brain damage, suggesting a synergic neuroprotective role of both neural and glial receptors.

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CB2 RECEPTOR ACTIVATION ATTENUATES CEREBRAL ISCHEMIA/REPERFUSION INJURY

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Numerous studies have focused on the potential involvement of the CB1 receptor in altering the magnitude of injury following stroke. The potential involvement of the CB2 receptor in modulating ischemia/reperfusion injury has not received similar attention. Since attenuation of the inflammatory response to ischemia/reperfusion injury has been demonstrated to influence infarct size following stroke, and CB2 receptors are present on the inflammatory cells involved, we hypothesized that that activation of these receptors may influence outcome following stroke. The purpose of this investigation was therefore to investigate the potential protective effect of CB2 receptor activation following stroke. A mouse transient middle cerebral artery occlusion model was used. Transient middle cerebral artery occlusion was created in C56BL/6 mice by occluding the vessel with an intravascular suture introduced into a branch of the external carotid artery. The duration of occlusion was one hour. The suture was removed and the brain reperfused for 24 hours prior to sacrifice of the animals. The animals were treated with either vehicle alone, or with one of two selective CB2 agonists (0-3853 or 0-1966). In one component of the study the drugs were administered one hour prior to ischemia. In the second part of the study the drugs were administered after one hour of reperfusion. Infarct volume was evaluated following triphenyltetrazolium chloride staining. Motor function was analyzed after 24 hours of reperfusion. The evaluations were performed using a double blind protocol. Treatment with both drugs (both pretreatment and treatment after 60 of reperfusion) resulted in a significant reduction in infarct volume and a significant improvement in motor function. The results of this investigation provide the first demonstration that CB2 receptor activation is protective against ischemia/reperfusion injury in the brain.

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PPARα-MEDIATED NEUROPROTECTION BY OLEOYLETHANOLAMIDE

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Cannabinoids have neuroprotective actions in both *in vitro* and in *vivo* models but activation of CB_1 and CB_2 receptors might not account for all of their effects. Recently, it has become clear that some cannabinoids can also act as ligands of PPARs (peroxisome proliferator-activated receptors) which belong to the superfamily of nuclear hormone receptors acting via regulation of the transcription of many genes. We have previously shown that the delayed vasorelaxant effects of tetrahydrocannabinol (THC) are PPARγ-mediated and it has been demonstrated that the effects of the entourage endocannabinoid oleoylethanolamide (OEA) on feeding behaviour are PPARα-dependent. We have, therefore, examined the potential for a variety of cannabinoids to act as PPARα activators. We have also investigated the *in vivo* neuroprotective effects of the known PPARα activator, OEA.

To measure receptor activation *in vitro*, Hela cells were transiently transfected with a mouse PPARα expression plasmid together with a reporter gene containing three PPAR binding sites linked to a promoter controlling the gene for firefly luciferase. Transcription was significantly enhanced by μM range concentrations of anandamide, OEA, PEA, Win-55212-2, noladin ether and virodhamine; THC was without effect.

In the middle cerebral artery occlusion model of ischemic stroke in mice, cerebral lesion size was significantly reduced by a 3day pre-treatment with the PPARα ligand fenofibrate and also by OEA (both 10mg/kg i.p.). In transgenic PPARα knock-out mice the OEA-derived neuroprotection was absent. It remains to be determined whether neuroprotection due to cannabinoids other than OEA has a PPAR-dependent component.

REGULATION OF FATTY ACID AMIDE HYDROLASE AND N-ACYLPHOSPHATIDYLETHANOLAMINE PHOSPHOLIPASE D IN FOCAL CEREBRAL ISCHEMIA IN MICE

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Anandamide and other *N*-acylethanolamines (NAEs) are important signal molecules in the brain and may be involved in several pathophysiological conditions. The NAEs are formed from their corresponding *N*-acylphophatidylethanolamines by *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and are metabolized by fatty acid amide hydrolase (FAAH). In animal models of stroke, it has been demonstrated that NAEs accumulate, but the mechanism responsible remains unknown. To investigate whether upregulation of NAPE-PLD or downregulation of FAAH is involved in ischemic NAE accumulation, we induced permanent focal cerebral ischemia through middle cerebral artery occlusion (MCAO) in SJL mice and determined NAPE-PLD and FAAH mRNA levels with real-time PCR. In parallel, we measured the enzymatic activities of NAPE-PLD and FAAH. The mRNA levels of NAPE-PLD and FAAH after MCAO were unchanged during the experimental time period (1 hour to 10 days post MCAO). In contrast, the enzymatic activity of NAPE-PLD was significantly decreased 12 hours post ischemia and returned to control level 24 hours after MCAO. The enzymatic activity of FAAH in the cortex was unaffected by MCAO. Interestingly, the significant decrease in NAPE-PLD enzymatic activity was found both in the ischemic and the contralateral hemisphere. Our results indicate that NAPE-PLD and FAAH may not be responsible for maintaining high concentrations of anandamide and other NAEs following focal cerebral ischemia in mice. NAPE-PLD and FAAH are not transcriptionally regulated in mice following permanent MCAO and we found no variation in FAAH enzymatic activity. NAPE-PLD enzymatic activity decreased at 12 hours following MCAO, but this clearly can not explain the increase in NAE levels seen in stroke. These findings point to a possible role of other factors (such as transacylase activation) in NAE accumulation after cerebral ischemia.

CHANGES IN THE DISTRIBUTION OF SOME ELEMENTS OF THE ENDOGENOUS CANNABINOID SYSTEM IN DOWN'S SYNDROME

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Our group previously described changes in cannabinoid receptors and FAAH enzyme expression in brains from patients with Alzheimer's disease. CB_2 receptors were over-expressed in microglial cells within senile plaques enriched in β-amyloid peptide, and FAAH enzyme in astrocytes surrounding the plaques. CB_1 receptor distribution was the same in controls and in patients with Alzheimer's disease.

Down's syndrome is the major cause of genetic mental retardation. The extra gene dosage effect associated with trisomy 21 results in abnormalities of the processing of amyloid precursor protein (APP) in patients with Down's syndrome. It is also well known that almost all patients over the age of 40 years with Down syndrome display Alzheimer's like neuropathology. Because of that, Down's syndrome is considered also a good model to study Alzheimer's disease. There are also some transgenic mice that can be used as a model for Down's syndrome, as mice with 16 cromosome parcial trisomy (Ts65DN), that express high amounts of APP in the hippocampus.

We studied the distribution of some elements of the Endogenous Cannabinoid System in patients with Down's syndrome at different ages. As expected there was an age-dependent expression of β-amyloid peptide, with an over-expression of CB_2 receptors in cells within β-amyloid plaques, and of FAAH enzyme in cells surrounding plaques.

In Ts65DN mice we found a reduction of CB_1 receptor mRNA levels and an increase in CB_2 receptor mRNA compared with controls. This increase in CB_2 mRNA was accompanied by protein expression in selected cell types in the hippocampus.

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CANNABIDIOL REDUCED THE STRIATAL ATROPHY CAUSED 3-NITROPROPIONIC ACID *IN VIVO* BY MECHANISMS INDEPENDENT OF THE ACTIVATION OF CANNABINOID RECEPTORS

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We recently demonstrated the occurrence of profound alterations of cannabinoid receptor signaling during the neurodegeneration of the striatum caused by the exposure to 3-nitropropionic acid (3NP) *in vivo*, and that the administration of the plant-derived cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) was neuroprotective, which indicated that the early loss of cannabinoid receptor signaling could be instrumental in 3NP toxicity (Lastres-Becker et al., *Neuroreport* 15, 2375-2379, 2004). In the present study, we wanted to further explore the potential mechanisms involved in the neuroprotective effect exerted by this plant-derived cannabinoid, by examining in the same animal model a series of cannabinoid-based compounds, with more selectivity for different elements of the cannabinoid signalling system. We used the CB_1 receptor agonist arachidonyl-2-chloroethylamide (ACEA), the CB_2 receptor agonist HU-308, and cannabidiol (CBD), a phytocannabinoid with negligible affinity for the two cannabinoid receptor subtypes but exhibiting, as Δ^9-THC, a notable antioxidant potential. As expected, the administration of 3NP caused a significant depletion of GABA contents in the striatum, accompanied by a parallel reduction in mRNA levels for several markers of striatal GABAergic projection neurons, such as proenkephalin (PENK), substance P (SP) and neuronal-specific enolase (NSE), which indicated that 3NP caused the preferential degeneration of these neurons. We also found reductions in mRNA levels for superoxide dismutase-1 (SOD-1) and -2 (SOD-2), which supported the notion of a 3NP-induced loss in endogenous defenses against oxidative stress. The application of CBD, but not of ACEA or HU-308, during the period of 3NP exposure completely recovered 3NP-induced reductions in GABA contents and mRNA levels for SP, NSE and SOD-2, and partially attenuated the reductions in mRNA levels for SOD-1 and, to a lesser extent, PENK, thus indicating that CBD may be neuroprotective but acted preferentially on striatal neurons that project to the substantia nigra. In summary, the present study demonstrates that CBD may act as a neuroprotective agent reducing the striatal atrophy generated by the exposure to 3NP, which may be relevant for Huntington's disease, a neurodegenerative disorder characterized by the preferential loss of striatal projection neurons. This capability seems to be based on the antioxidant potential of CBD since this cannabinoid has negligible activity at the CB_1 or CB_2 receptors, whereas the use of selective agonists for both receptor subtypes did not replicate CBD effects.

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THE ACTIVATION OF CANNABINOID CB_2 RECEPTOR IMPAIRS Aβ REMOVAL FROM ALZHEIMER´S DISEASE HUMAN TISSUES

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Alzheimer's disease (AD) brain is characterized by the presence of beta-amyloid (Aβ) plaques which are surrounded by activated astrocytes and microglial cells. Previous studies have demonstrated that glial cells become activated in response to these deposits that also trigger pro-inflammatory cytokines release as well as an increase in phagocytic activity. It has also been recently shown that adult mouse astrocytes are capable of removing Aβ precipitates from transgenic mice tissue sections (Wyss-Coray et al, Nature Medicine 9(4):453-7, 2003)

We have previously shown that activated glial cells express CB_2 receptors in areas of neuroinflammation in the human brain. Specifically, we reported the induction of CB_2 expression in microglial cells located in the vicinity of Aβ enriched-plaques (Benito et al, J Neurosci 23(35):11136-41, 2003). The objective of the present work was to explore the possible role of CB_2 receptors on the function glial cells exposed to Aβ peptide. To that end, several human glial cell lines were used and tested for their ability to remove Aβ plaques from frozen tissue sections from cortical regions of AD brains. Afterwards, we used several CB_2 ligands to check the possible consequences of CB_2 activation on this activity.

Our results indicate that U373 glioblastoma cell line as well as THP1-derived macrophages are able to remove Aβ deposits *in situ*. Additionally, we found that CB_2 activation leads to profound changes in this cell activity. Specifically, JWH-015 treated U373 cells showed a reduced capability to remove Aβ deposits (25% decrease, approx.) from human AD tissue sections. These preliminary results are indicative of a remarkable role of CB_2 receptors in glial function in AD.

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THE ENDOCANNABINOID SYSTEM CONTROLS A KEY EPILEPTOGENIC CIRCUIT IN THE HIPPOCAMPUS

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Complex behaviours, cognition requires intense neuronal activity. Excessive neuronal activity, however, can lead to neuronal damage, cell death and seizures. The endocannabinoid system tightly controls neuronal excitability as the dramatic increase of seizure susceptibility of the cannabinoid receptor 1 (CB1) knockout mice demonstrates. However, there is controversy concerning the nature of the neurons directly modulated by endocannabinoids.

In this study we aim to identify which neuronal population is responsible for the endocannabinoid mediated protection against kainic acid (KA) induced seizures, an animal model of temporal lobe epilepsy.

To be able to successfully dissect the exact target of endocannabinoid action in seizure protection, a battery of pharmacological, molecular, electrophysiological and genetic tools was applied. Namely, we have generated two new conditional CB1 mutant lines bearing a deletion of CB1 in either in cortical glutamatergic or in forebrain GABAergic neurons. In these animals, KA-induced seizure behaviour, CB1 mRNA co-expression with specific markers of glutamatergic or GABAergic neurons and endocannabinoid-mediated synaptic events were studied.

Here we show that endocannabinoids directly target hippocampal glutamatergic neurons. Furthermore, we show that functional CB1 cannabinoid receptors are present on the terminals of glutamatergic hilar mossy cells of the dentate gyrus, co-localizing with vesicular glutamate transporter 1 (VGluT1). Conditional deletion of the CB1 gene either in cortical glutamatergic neurons or in forebrain GABAergic neurons, as well as virally induced deletion of the CB1 gene in the hippocampus, demonstrate that the presence of CB1 in glutamatergic hippocampal neurons is both necessary and sufficient to provide substantial endogenous protection against kainate-induced seizures.

The direct endocannabinoid-mediated control of hippocampal glutamatergic neurotransmission may constitute a promising therapeutic target for the treatment of disorders associated with excessive excitatory neuronal activity.

HIPPOCAMPAL CANNABINOID CB_1 RECEPTOR REDISTRIBUTION IN EPILEPTIC RAT BRAIN IS DEPENDENT ON NMDA RECEPTOR ACTIVATION AND INDEPENDENT OF ONGOING SEIZURE ACTIVITY

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Earlier published work from this laboratory has shown that cannabinoids demonstrate cannabinoid receptor type 1 (CB_1R) dependent anticonvulsant effects in the rat pilocarpine model of acquired epilepsy, a chronic model of spontaneous recurrent seizures that is dependent on NMDA receptor activation during pilocarpine-induced status epilepticus (SE). We have presented previous findings showing that epileptic animals exhibit a long-term redistribution of CB_1R within the hippocampus using immunohistochemical and [^{35}S]GTPγS autoradiography studies (Falenski et al., 2004, *ICRS* 167). However, several questions regarding this redistribution have yet to be addressed. Specifically, the current study was designed to determine whether redistribution of CB_1R is dependent on NMDA receptor activation. Additionally, studies were carried out on chronically epileptic animals to determine if ongoing seizure activity is required to maintain the CB_1R redistribution.

To evaluate the NMDA receptor component, rats were administered the NMDA receptor antagonist MK801 prior to pilocarpine injection. Although MK801-treated animals still experienced status epilepticus (SE), they did not develop spontaneous recurrent seizures. Six weeks following treatment, control, pilocarpine (epileptic), and pilocarpine+MK801 (nonepileptic) animals were sacrificed and brains were processed for immunohistochemical analysis for CB_1-R expression using modifications of established techniques (Tsou et al., 1998, *Neuroscience* 83, 393-411). To evaluate the contribution of ongoing seizure activity, animals were treated with phenobarbital (60-mg/kg, i.p.) for 12 days to suppress epileptic seizures, after which time brains were processed for immunohistochemical analysis, [^{3}H]WIN55,212-2 autoradiography, and WIN55,212-2-stimulated [^{35}S]GTPγS autoradiography. Results of these studies indicate that MK801 pretreatment prevented the redistribution of CB_1R-immunoreactivity that occurs in epileptic animals, which indicates it is dependent on NMDA receptor activation. Additionally, seizure suppression in epileptic animals did not affect the already established redistribution of CB_1R in all parameters examined. Ultimately, these results indicate that the redistribution of CB_1R is not dependent on SE alone, nor does it occur specifically as a consequence of ongoing epileptic seizures, suggesting that it is a plasticity change that occurs in association with epileptogenesis.

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DIFFERENTIAL CONTROL BY G_Q/G_{11} PROTEINS OF IN VIVO ENDOCANNABINOID LEVELS AND ENDOCANNABINOID-MEDIATED REGULATION OF NEURONAL EXCITABILITY

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Metabotropic receptors coupled to G-proteins of the G_q/G_{11} family contribute to nervous system functions by modulating synaptic transmission. Studies carried out so far only *in vitro* have suggested that these G-proteins also play a role in endocannabinoid biosynthesis. Endocannabinoids are known to exert neuroprotective effects (van der Stelt & Di Marzo, 2005, for a recent review) and to affect neuronal excitability. We, therefore, analysed endocannabinoid levels in the hippocampi of mice lacking the α-subunits of G_q and G_{11}, $G\alpha_q$ and $G\alpha_{11}$, selectively in forebrain principal neurons. Furthermore, we investigated the consequences of potential changes in endocanabinoid levels in these mutant mice, by analysing: 1) two endocannabinoid-mediated forms of short-term synaptic plasticity in their hippocampus, i.e. DSE and DSI (Wilson & Nicoll, 2002); and 2) their response to an excitotoxic stimulus, kainic acid (KA), previously shown to trigger endocannabinoid-mediated neuroprotection (Marsicano et al., 2003).

We found that the hippocampi of forebrain-specific $G\alpha_q/G\alpha_{11}$-null mice contain lower basal anandamide, but not 2-arachidonoylglycerol (2-AG), levels. By contrast, the hippocampal concentration of 2-arachidonoylglycerol, but not anandamide, is reduced in response to excitotoxic challenge with KA. In addition, endocannabinoid-mediated retrograde inhibition of both excitatory and inhibitory neurotransmitter release is abrogated in forebrain-specific $G\alpha_q/G\alpha_{11}$-deficient mice. These mutant mice also exhibit two typical features of forebrain-specific CB_1-receptor knockouts, i.e. increased seizure susceptibility to KA and impaired activation of neuroprotective mechanisms previously shown to be activated by endocannabinoids via CB_1 receptors, such as immediate early gene synthesis (Marsicano et al., 2003). Increased susceptibility to KA is normalized through enhancement of endocannabinoid levels with the endocannabinoid reuptake inhibitor, OMDM-2, while the competitive CB_1 receptor antagonist SR141716A causes no further aggravation. By contrast, OMDM-2 is less effective in wild-type mice, where SR141716A causes worsening of KA-induced seizures.

These findings demonstrate that G_q/G_{11}-mediated signalling controls differentially both basal and KA-induced anandamide and 2-AG synthesis *in vivo,* and suggest that impaired endocannabinoid signalling is a mechanism, albeit not the only one, by which loss of G_q/G_{11} leads to increased excitability.

CONCENTRATIONS OF Δ^9-TETRAHYDROCANNABINOLIC ACID DURING GROWTH AND DEVELOPMENT OF CANNABIS PLANTS

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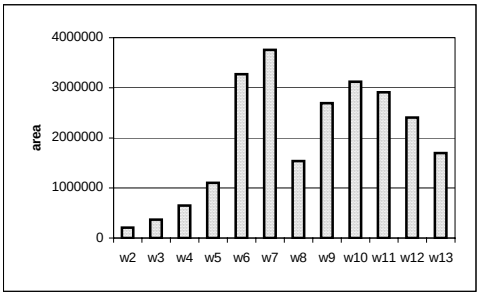
Introduction: Under the general conditions applied for growing Medicinal Grade Cannabis (MGC 1001), the point when about 50% of the stigmas on the inflorescences have turned brown marks the start to harvest. Normally this happens when plants are 9-10 weeks old, after the cuttings have been fully rooted and transferred to the greenhouse. To correlate this rule of thumb applied by the growers to the actual content of Δ^9-tetrahydrocannabinolic acid (THC-A) in the plants, the following investigation was undertaken.

Material and Methods: From the content of 1 production batch (total of 200 plants), 6 individual plants were randomly selected. At weekly intervals, from week 2 till week 12, one flowering top from each plant was obtained. The total of 6 tops were finely cut, extracted, and analyzed for their THC-A content by HPLC. Since at the time of this investigation, no certified THC-A standard, was available, the results are expressed as areas. However, by means of a in house standard the HPLC method used was shown linear over the range analyzed, with good precision (Rsd $\leq$ 1.5%).

Results and Conclusion



Although 7 weeks old plants contain the highest absolute amounts of THC-A, they only have a few branches with inflorescences, and small primary flowers. Therefore, as a whole, the relative THC-A concentration is still rather low. To stimulate branching and development of many more inflorescences, plants undergo a calculated water and fertilizer management scheme. The results of this procedure can be seen as a drop of THC-A concentration in week 8, followed by a gradual increase again up to week 10. The absolute content in the second optimum, at week 10 is a little lower than in week 7; however, due to excessive branching of 10 weeks old plants they contain relatively the most of Δ^9-tetrahydrocannabinolic acid. This furthermore correlates exactly with the point when about 50% of the stigmas on the inflorescences have turned into brown. Δ^9-Tetrahydrocannabinol analyzed concomitantly by HPLC is only present in very small quantities when compared to its acid form. Its absolute concentrations generally follow the pattern of THC-A.

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