18TH ANNUAL

SYMPOSIUM

OF THE

INTERNATIONAL CANNABINOID RESEARCH SOCIETY

AVIEMORE, SCOTLAND

JUNE 25-29, 2008

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MacDONALD AVIEMORE
HIGHLAND RESORT

Aviemore, Scotland

June 25-29, 2008

PROGRAM AND ABSTRACTS

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2008 Symposium on the Cannabinoids

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The 2008 ICRS Symposium on the Cannabinoids
is dedicated to the memory of
Drs. J. Michael Walker and Billy Ray Martin.

Registration: June 25, 2008 (16.00 – 20.00)

Day 1
Thursday, June 26th

8.30	Opening Remarks		
Topic A. SAR Studies and New Synthetic Molecules Moderators: Patty Reggio and Giulio Muccioli			Page #
8.45	Alessia Ligresti, Alberto Minassi, Luciano De Petrocellis, Orazio Taglialatela-Scafati, Marco Allarà, Giovanni Appendino and Vincenzo Di Marzo	STEREO- AND ENANTIO-SPECIFIC ACTIVITY OF FATTY ACID CYCLOPROPANOLAMIDES AT CANNABINOID AND VANILLOID RECEPTORS	1
9.00	Herbert H. Seltzman, Marcus F. Brackeen, Daniel J. Watkins, Jason P. Burgess, Anne F. Gilliam, Brian F. Thomas, Yanan Zhang, Tiffany Langston, and Hernan A. Navarro	LOCKEDNESS - CONFORMATIONALLY RESTRICTED ENANTIOMERIC PROBES OF THE hCB1 RECEPTOR	2
Topic B. Receptor Structure and Signal Transduction Moderators: Allyn Howlett and Gemma Baillie			
9.15	Michal Jonasz Urbanski, Anne Simon, Zsolt Lenkei, Jost Leemhuis, Dieter K. Meyer and Bela Szabo	THE LACK OF SOMATODENDRITIC EFFECTS OF CANNABINOIDS IS DUE TO CONSTITUTIVE ACTIVITY OF CB1 RECEPTORS AND SUBSEQUENT RECEPTOR INTERNALIZATION	3

9.30	Brian Hudson and Melanie Kelly	INTERACTION BETWEEN THE CB1 CANNABINOID RECEPTOR AND THE β2 ADRENERGIC RECEPTOR AFFECTS CB1 AND β2 RECEPTOR SIGNALING	4
9.45	John McPartland	ADAPTIVE EVOLUTION AND PURIFYING SELECTION AT INDIVIDUAL AMINO ACID RESIDUES IN THE CB1 SEQUENCE	5
10.00	Barbara Bosier, Emmanuel Hermans and Didier M. Lambert	AGONIST-SELECTIVE REGULATION OF AP-1 ELEMENT SUPPORTS THE COMPLEX REGULATION OF TYROSINE HYDROXYLASE EXPRESSION BY DISTINCT CANNABINOID AGONISTS	6
10.15 - 10.45	COFFEE BREAK		
TOPIC C. ENDOCANNABINOIDS AND RELATED LIPIDS: BIOSYNTHESIS AND INACTIVATION Moderators: Mauro Maccarrone and Lina Thors			
10.45	Patricia Reggio, Alan Grossfield, Dow Hurst, Klaus Gawrisch, Scott Feller and Mike Pitman	MICROSECOND TIME SCALE MOLECULAR DYNAMICS SIMULATIONS: 2-AG ENTRY INTO THE CB2 RECEPTOR VIA THE LIPID BILAYER	7
11.00	Maurice Elphick and Michaela Egertová	LOCALIZATION OF NAPE-PLD EXPRESSION IN THE BRAIN: EVIDENCE OF A NOVEL ROLE FOR N-ACYLETHANOLAMINES AS ANTEROGRADE SYNAPTIC SIGNALLING MOLECULES	8
11.15	Takayuki Sugiura, Atsushi Yamashita, Seishi Kishimoto and Saori Oka	ANALYSIS AND STRUCTURE-ACTIVITY RELATIONSHIP OF LYSOPHOSPHATIDYLINOSITOL, A POSSIBLE ENDOGENOUS LIGAND FOR GPR55	9

11.30	Stefania Petrosino, Meliha Karsak, Luigia Cristino, Evelyn Gaffal, Thomas Tüting, Natsuo Ueda, Andreas Zimmer, Tiziana Bisogno and Vincenzo Di Marzo	PROTECTIVE ROLE OF PALMITOYLETHANOLAMIDE IN KERATINOCYTES AND ITS INVOLVEMENT IN CONTACT ALLERGIC DERMATITIS	10
11.45	Joel Schlosburg and Aron Lichtman	ENDOCANNABINOID MODULATION OF PRURITUS: FURTHER INVESTIGATIONS INTO ITCH	11
12.00	Daniel Fraga, Cristiane I.S. Zanoni, Carlos A. Parada and Glória E.P. Souza	INVOLVEMENT OF ENDOGENOUS CANNABINOIDS ON FEVER: DEPENDENCE ON PROSTAGLANDINS AND OPIOIDS SYNTHESIS/RELEASE	12
12.15	Michelle Roche, John P Kelly, Maeve O'Driscoll and David P. Finn	INHIBITION OF ENDOCANNABINOID DEGRADATION OR TRANSPORT IN VIVO MODULATES LPS-INDUCED INCREASES IN CIRCULATING CYTOKINE LEVELS	13
12.30 – 14.15	LUNCH		
14.15 – 17.00	**POSTER SESSION 1**		
16.30 – 17.00	COFFEE BREAK / POSTER SESSION 1		
17.00 – 17.45	**PLENARY SESSION 1** **"ENDOCANNABINOID OXYGENATION BY CYCLOOXYGENASE-2: CHEMISTRY AND CONSEQUENCES"** **LARRY MARNETT, PH.D.** Vanderbilt University		PS1
17.45 – 18.00	BREAK		

TOPIC C. (CONT.) Moderators: Stephen Alexander and Maria-Grazia Cascio			
18.00	Mauro Maccarrone, Filomena Fezza, Sergio Oddi, Chiara De Simone, Cinzia Rapino, Nicoletta Pasquariello, Enrico Dainese and Alessandro Finazzi-Agrò	BIOCHEMICAL PROFILE OF BIOTIN-ANANDAMIDE, A NOVEL TOOL FOR THE VISUALIZATION OF ANANDAMIDE ACCUMULATION	14
18.15	Tiziana Bisogno, Enrico Morera, Francesca Guida, Maria Grazia Cascio, Stefania Petrosino, Sabatino Maione, Giorgio Ortar and Vincenzo Di Marzo	TETRAHYDROLIPSTATIN ANALOGUES: NEW SELECTIVE PHARMACOLOGICAL TOOLS TO INVESTIGATE 2-AG METABOLISM	15
18.30	Nephi Stella	IDENTIFICATION OF A NOVEL 2-AG-HYDROLYZING ENZYME EXPRESSED BY MICROGLIAL CELLS	16
18.45	Yan Sun, Stephen Alexander, David Kendall and Andrew Bennett	INVOLVEMENT OF FATTY ACID BINDING PROTEINS IN THE TRANSPORT OF ENDOCANNABINOIDS TO PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS	17
19.00	Raphael Mechoulam and Itai Bab	AN ENDOGENOUS LIPID WITH NOVEL BONE ANABOLIC ACTIVITY IN VIVO	18
19.15 – 19.30	FREE TIME		
19.30 – 22.00	DINNER / FREE TIME		

Day 2
Friday, June 27th

8.25	Plenary Session Introduction		
Topic D. Neural Plasticity, Neuroprotection and Neurodegenerative Disorders Moderators: Ken Mackie and Matt Hill			Page #
8.30 – 9.15	**Plenary Session 2** "Endocannabinoid Mobilization and Synaptic Plasticity in the Hippocampus" **Bradley E. Alger, Ph.D.** University of Maryland School of Medicine		PS2
9.15	Bela Szabo, Tim Knop, Flora Kovacs, Thomas Freiman, Thomas J. Feuerstein and Michal J. Urbanski	ENDOCANNABINOID-MEDIATED RETROGRADE SYNAPTIC SIGNALING IN THE HUMAN CEREBRAL CORTEX	19
9.30	Joana Lourenco, Astrid Cannich, Christophe Mulle and Giovanni Marsicano	ENDOGENOUS ACTIVATION OF KAINATE RECEPTORS IS NECESSARY FOR A NOVEL FORM OF ENDOCANNABINOID-MEDIATED INHIBITION OF SYNAPTIC TRANSMISSION	20
9.45	Giuliano Pillolla, Antonio Luchicchi, Anna Lisa Muntoni, Miriam Melis and Marco Pistis	ENDOCANNABINOIDS FINE TUNE SLOW OSCILLATORY ACTIVITY OF VENTRAL TEGMENTAL AREA DOPAMINE NEURONS	21
10.00	Alex Straiker and Ken Mackie	MUSCARINIC-CANNABINOID SUPPRESSION OF EXCITATION	22
10.15 – 10.45	Coffee Break		

TOPIC D. (CONT.) Moderators: Javier Fernández-Ruiz and Gareth Pryce			
10.45	Matthew Hill, Andrea Titterness, Anna Morrish, Erica Carrier, Tiffany Lee, Brian Christie, Boris Gorzalka and Cecilia Hillard	VOLUNTARY EXERCISE PROMOTES HIPPOCAMPAL NEURAL PROGENITOR CELL PROLIFERATION THROUGH AN ENHANCEMENT OF ENDOGENOUS CANNABINOID SIGNALING	23
11.00	Duncan Ryan, Alison Drysdale, Roger Pertwee and Bettina Platt	MITOCHONDRIA ARE NOVEL TARGETS FOR CANNABIDIOL'S NEUROPROTECTIVE ACTION	24
11.15	Gareth Pryce, Gavin Giovannoni and David Baker	INHIBITION OF SPASTICITY USING PERIPHERALIZED CB1 RECEPTOR AGONISTS	25
11.30	Hua Zhang, David Hilton, Oliver Hanemann, Mathieu Widmer, Richard Hosking and John Zajicek	HISTOPATHOLOGICAL STUDY OF CANNABINOID RECEPTORS EXPRESSION IN HUMAN MULTIPLE SCLEROSIS	26
11.45	Moisés García-Arencibia, Concepción García, Patricia Rodríguez-Valsero, José Antonio Ramos and Javier Fernández-Ruiz	EARLY AND LATE CHANGES IN CB1 AND CB2 RECEPTORS IN THE BASAL GANGLIA OF MICE WITH DELETION OR MUTATION OF SPECIFIC PARK GENES	27
12.00	Koen Van Laere, Cindy Casteels, Sophie Lunskens, Karolien Goffin, Nathalie Gerard, Guy Bormans, Igor Grachev and Wim Vandenberghe	IN VIVO PET BRAIN IMAGING OF THE TYPE 1 CANNABINOID RECEPTOR IN PARKINSON'S DISEASE	28
12.15	Jose Martinez-Orgado, Francisco J. Alvarez, Hector Lafuente, M.Carmen Rey-Santano, Victoria E. Mielgo, Elena Gastiasoro, Miguel Rueda, Ana I. Castillo, Enrique Hilario, Roger G. Pertwee and Julián Romero	CEREBRAL AND EXTRACEREBRAL BENEFITS OF CANNABIDIOL IN A PIGLET MODEL OF NEWBORN HYPOXIC-ISCHAEMIC ENCEPHALOPATHY	29

12.30 – 14.15	LUNCH
13.00 – 14.00	NIDA CAREER DEVELOPMENT INFOLUNCHEON **"I need a new medicine – How to develop phytocannabinoids"** Presenters: Stephen Wright, R&D Director, GW Pharmaceuticals Colin Stott, Director of R&D Operations, GW Pharmaceuticals
14.15 – 16.30	**POSTER SESSION 2**
16.00 – 16.30	COFFEE BREAK / POSTER SESSION 2
16.30 – 22.00	**TRIP TO CAIRNGORM MOUNTAIN** DINNER WILL BE PROVIDED

Notes:

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Day 3
Saturday, June 28th

8.25	Announcements		
Topic E1. Cognition, Stress, and Neuropsychiatric Disease Moderators: Cecilia Hillard and Anushka Goonawardena			
8.30	Sabrina F Lisboa, Leonardo BM Resstel, Sâmia RL Joca and Francisco S Guimarães	5HT1A-RECEPTORS ARE INVOLVED IN THE ATTENUATION OF BEHAVIORAL RESPONSE TO RESTRAINT STRESS INDUCED BY CANNABIDIOL (CBD) IN RATS	30
8.45	Daniela Viganò, Cinzia Guidali, Stefania Petrosino, Vincenzo Di Marzo and Daniela Parolaro	DIRECT AND INDIRECT MODULATION OF CANNABINOID SYSTEM IN A PHENCYCLIDINE MODEL OF SCHIZOPHRENIA	31
9.00	Jonathon C. Arnold, Aurelie A Boucher, Glenn E. Hunt, Iain S. McGregor, Jacques Micheau and Tim Karl	HETEROZYGOUS NEUREGULIN 1 MICE DISPLAY ALTERED NEUROBEHAVIOURAL RESPONSES TO REPEATED CANNABINOID EXPOSURE	32
9.15	James Burston, Dana Selley, Laura Sim-Selley and Jenny Wiley	ALTERATION OF CANNABINOID SIGNALING BY ANTIPSYCHOTICS	33
9.30	Tiziana Rubino, Natalia Realini, Daniela Viganò and Guidali Cinzia	ADULT FEMALE RATS PRE-EXPOSED TO THC IN ADOLESCENCE AS A MODEL TO TEST CANNABINOID SYSTEM INVOLVEMENT IN DEPRESSION	34
9.45	Allyn Howlett, Steven Franklin, Kofi-Kermit Horton, Laurence Miller and Linda Dykstra	CB1 RECEPTOR KNOCKOUT AFFECTS BRAIN ADAPTATIONS TO STRESS AND AGING	35

Time	Authors	Title	Page
10.00	Natalia Realini, Daniela Braida, Sandra Guidi, Valeria Capurro, Tiziana Rubino, Renata Bartesaghi and Daniela Parolaro	CHANGES IN HIPPOCAMPAL MORPHOLOGY AND SYNAPTIC PLASTICITY FOLLOWING CHRONIC Δ^9-THC TREATMENT IN ADOLESCENCE ARE ASSOCIATED WITH COGNITIVE IMPAIRMENT IN ADULTHOOD	36
10.15 – 10.45	COFFEE BREAK		
10.45	Michael J. Wesley, Colleen A. Hanlon, Mack Miller, and Linda J. Porrino	CHRONIC MARIJUANA USERS SHOW ALTERED NEURAL PROCESSING DURING DECISION MAKING AND FEEDBACK	37
11.00	Philip Robson and Tilden Etges	PSYCHIATRIC EFFECTS OF SATIVEX	38
TOPIC F1. APPETITE, ENERGY REGULATION, ADDICTION AND HOMEOSTASIS Moderators: Michael Cawthorne and Vincenzo Di Marzo			
11.15	Joshua Lile, Thomas Kelly, David Hudson and Lon Hays	SUBSTITUTION PROFILE OF VARIOUS DRUGS IN HUMANS DISCRIMINATING Δ^9-THC	39
11.30	Marco Pistis, Giuliano Pillolla, Antonio Luchicchi, Anna Lisa Muntoni and Miriam Melis	INHIBITION OF FAAH BLOCKS THE EXCITATORY EFFECTS OF NICOTINE ON MESOLIMBIC DOPAMINE NEURONS VIA CB1 AND PPAR-α RECEPTORS	40
11.45	Haiying Liu, Andrea Frassetto, Donald S. Williams, Richard J. Hargreaves, Richard Z. Chen and Tung M. Fong	EFFECT OF CB1R INVERSE AGONIST ON HEPATIC LIPID CONTENT QUANTIFIED WITH IN VIVO MR SPECTROSCOPY	41
12.00	Michael Cawthorne, Colin Stott, Stephen Wright, Geoffrey Guy, Mohamed Zaibi and Ed Wargent	THE METABOLIC EFFECTS OF TETRAHYDROCANNABIVARIN (THCV) AND CANNABIDIOL (CBD)	42

12.15	Cecilia Hillard, Christopher Roberts and Joseph Besharse	ENDOCANNABINOID SIGNALING REGULATES THE CIRCADIAN PATTERN OF WHEEL RUNNING BEHAVIOR	43
12.30 – 14.15	LUNCH		
13.30 – 14.15	FUNDING OPPORTUNITIES AT NIDA Presenter: Vishnudutt Purohit, D.V.M., Ph.D., Health Scientist Administrator, National Institute on Drug Abuse		
14.15 – 16.30	**POSTER SESSION 3**		
16.00 – 16.30	COFFEE BREAK / POSTER SESSION 3		
TOPIC G1. GASTROINTESTINAL, CARDIOVASCULAR AND OTHER PERIPHERAL FUNCTIONS Moderators: Keith Sharkey and Joanna Jamontt			
16.30	Keith Sharkey, Catherine Keenan, Hong Zhang, Nancy Buckley, Beat Lutz, Kamala Patel and Martin Storr	INHIBITORS OF ENDOCANNABINOID DEGRADATION REDUCE COLITIS BY ACTIVATION OF CB1 AND CB2 RECEPTORS	44
16.45	Mona R. El-Talatini, Anthony Taylor and Justin Konje	LONGITUDINAL STUDY SHOWING THE RELATIONSHIP BETWEEN ANANDAMIDE AND SEX STEROIDS AND GONADOTROPIN HORMONES IN WOMEN	45
17.00	Saoirse O'Sullivan	IN VITRO VASCULAR EFFECTS OF CANNABIDIOL (CBD) IN THE RAT ISOLATED AORTA	46

17.15	Sandor Batkai, Gregorz Godlewski, Shakiru O. Alapafuja, Spyros P. Nikas, Indu T. Bharatan, Alexandros Makriyannis, Benjamin F. Cravatt, Pal Pacher and George Kunos	THE NOVEL FAAH INHIBITOR AM-3506: CARDIOVASCULAR EFFECTS AND THERAPEUTIC IMPLICATIONS	47
17.30	Sabine Steffens, Fabienne Burger, Francois Mach and Fabrizio Montecucco	JWH-133 ADMINISTRATION DURING ISCHEMIA REDUCES INFARCT SIZE IN A MOUSE MODEL OF MYOCARDIAL ISCHEMIA/REPERFUSION	48
17.45 – 18.00	BREAK		
18.00 – 19.15	**BUSINESS MEETING**		
19.15 – 19.30	FREE TIME		
19.30 – 22.00	**DINNER / CEILIDH**		

Notes:

Day 4
Sunday, June 29th

8.25	Announcements		
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8.30	Wilma Steegenga, Caroline Lute, Jocelijn Meijerink, Mieke Poland, Michael Muller and Renger Witkamp	CANNABINOIDS CAN STIMULATE ARTEROSCLEROTIC PLAQUE FORMATION BY INDUCING DEDIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS INTO OSTEOBLASTS	49
8.45	Lauren Whyte, Erik Ryberg, Susan Ridge, Ken Mackie, Peter Greasley, Michael Rogers and Ruth Ross	GPR55 IS INVOLVED IN THE REGULATION OF OSTEOCLAST ACTIVITY IN VITRO AND BONE MASS IN VIVO	50
Topic H. Cancer Moderators: Manuel Guzmán and Aoife Gowran			
9.00	Christopher Fowler, Suichu Chung, Pär Stattin and Anders Bergh	THE ENDOCANNABINOID SYSTEM IN PROSTATE CANCER	51
9.15	Guillermo Velasco, Mar Lorenre, Arkaitz Carracedo, Sofía Torres, Francesco Natali, Ainara Egia, Cristina Blázquez, Sonia Hernández and Manuel Guzmán	AMPHIREGULIN RENDERS GLIOMA CELLS RESISTANT TO CANNABINOID-INDUCED APOPTOSIS	52
9.30	Somnath Mukhopadhyay, Inneke Johnson and Michael Schaller	CB2 CANNABINOID RECEPTOR-MEDIATED REGULATION OF PROSTATE CANCER GROWTH	53

9.45	María Salazar, Arkaitz Carracedo, Íñigo J. Salanueva, Sonia Hernández, Cristina Blázquez, Mar Lorente, Ainara Egia, Juan L. Iovanna, Manuel Guzmán, Patricia Boya and Guillermo Velasco	AUTOPHAGY MEDIATES CANNABINOID ANTI-TUMORAL ACTION	54
10.00	Marta Valenti, Paola Massi, Daniele Bolognini and Daniela Parolaro	INHIBITION OF HUMAN GLIOMA CELL MIGRATION AND INVASIVENESS INDUCED BY CANNABIDIOL, A NON-PSYCHOACTIVE CANNABINOID.	55
10.15 – 10.45	COFFEE BREAK		
TOPIC E2. COGNITION, STRESS, AND NEUROPSYCHIATRIC DISEASE			
10.45	Iddo Magen, Yosefa Avraham, Zvi Ackerman, Raphael Mechoulam and Elliot Berry	CANNABIDIOL (CBD) AMELIORATES COGNITIVE IMPAIRMENTS ASSOCIATED WITH A MODEL OF CHRONIC LIVER DISEASE IN MICE	56
TOPIC F2. APPETITE, ENERGY REGULATION, ADDICTION AND HOMEOSTASIS			
11.00	Ester Fride, Shimon Rabichev, Michal Schechter, Hodaya Dahan, Aron Weller, Shimon Ben-Shabat and David Branski	CANNABINOID CB1 RECEPTOR MANIPULATION AT BIRTH AFFECTS ADULT FUNCTIONS: IMPLICATIONS FOR 2-ARACHIDONYL GLYCEROL AS A FOOD SUPPLEMENT FOR INFANTS WITH 'FAILURE-TO-THRIVE'	57
11.15 – 12.15	**KANG TSOU MEMORIAL LECTURE** **"NEW INSIGHTS INTO ENDOCANNABINOID SIGNALLING AND FUNCTION BASED ON THE EXPRESSION OF DAGLα AND DAGLβ"** **PATRICK DOHERTY, PH.D.** *King's College London*		PS3

12.15 – 12.45	**MARTIN / WALKER MEMORIAL** Presenters: Andrea Hohmann and Richard Musty		
12.45 – 14.15	LUNCH		
14.15 – 17.00	**POSTER SESSION 4**		
16.30 – 17.00	COFFEE BREAK / POSTER SESSION 4		
TOPIC I. PAIN, SPASTICITY AND INFLAMMATION Moderators: Barbara Costa and Steven Kinsey			
17.00	Ildikó Rácz, Xavier Nadal, Judith Alferink, Josep E. Banos, Jennifer Rehnelt, Miquel Martìn, Alfonso Gutierrez-Adan, Elena Sanguino, Jorge Manzanares, Andreas Zimmer and Rafael Maldonado	MODULATION OF NEUROPATHIC PAIN BY ENDOCANNABINOIDS	58
17.15	Maulik Jhaveri, Clare Spicer, Stacey Knapp, David Kendall and Victoria Chapman	FUNCTIONAL SUPRA-SPINAL CB2 RECEPTORS IN NEUROPATHIC MODEL OF PAIN	59
17.30	Michael J. Dart, Megan E. Gallagher, Arturo Perez-Medrano, Sridhar Peddi, Tongmei Li, Derek W. Nelson, Jennifer M. Frost, Karin R. Tietje, Bo Liu, Xueqing Wang, Keith B. Ryther, William A. Carroll, Anthony V. Daza, George K. Grayson, Yihong Fan, Tiffany R. Garrison, Odile F. El-Kouhen, Betty B. Yao, Chang Z. Zhu, Madhavi Pai, Prasant Chandran, Anita K. Salyers, Gin C. Hsieh, Prisca Honore, Jill M. Wetter, Kennan C. Marsh and Michael D. Meyer	IDENTIFICATION OF THE THIAZOLYLIDENE AMIDE A-836339 AS A POTENT AND SELECTIVE CB2 RECEPTOR AGONIST FOR PAIN MANAGEMENT	60

17.45	István Katona, Rita Nyilas, Gabriella M. Urbán, Ken Mackie, Masahiko Watanabe and Tamás F. Freund	MOLECULAR ARCHITECTURE OF ENDOCANNABINOID SIGNALING AT NOCICEPTIVE SYNAPSES	61
18.00	Francesca Comelli, Isabella Bettoni, Mariapia Colleoni, Gabriella Giagnoni and Barbara Costa	ACTIONS OF THE MAGL INHIBITOR URB602 IN CHRONIC PAIN MODELS	62
18.15	Steven Kinsey and Aron Lichtman	FAAH MODULATION OF NEUROPATHIC PAIN: A DISSOCIATION BETWEEN PHARMACOLOGICAL AND KNOCKOUT APPROACHES	63
18.30	Ryan Butler, Gemma Ford, Michelle Hogan, Michelle Roche, Ian Robinson, David Barrett, David Kendall, Victoria Chapman and David Finn	EFFECTS OF THE FAAH INHIBITOR URB597 ON FEAR-CONDITIONED ANALGESIA AND ASSOCIATED ALTERATIONS IN SIGNAL TRANSDUCTION PROTEINS, LEVELS OF 2-AG AND FATTY ACID AMIDES	64
18.45	Sepideh Pooyania, Karen Ethans, Tony Szturm, Alan Casey, and Daryl Perry	A RANDOMIZED DOUBLE-BLINDED CROSSOVER STUDY ASSESSING THE EFFECT OF CANNABINOIDS ON SPASTICITY IN SPINAL CORD INJURED PERSONS: A PILOT STUDY	65
19.00 – 19.30	FREE TIME		
19.30 – 22.00	**ICRS BANQUET**		

Poster Session 1 - Topics A - C

DAY 1, THURSDAY JUNE 26TH: 14.15 – 17.00

Topic A. Chemistry, SAR Studies and New Synthetic Molecules		
Giuseppe Peddio, Barbara Pittau, Sabrina Piras, Paolo Lazzari and Luca Pani	DETERMINATION OF RIMONABANT THIOPHENE ANALOGUES IN RAT PLASMA AND BRAIN BY LIQUID CHROMATOGRAPHY– TANDEM MASS SPECTROMETRY.	P1
Yanan Zhang, Jason Burgess, Marcus Brackeen, Ann Gilliam, Kevin Page, S. Wayne Mascarella, Herbert H. Seltzman and Brian F. Thomas	DEVELOPMENT OF CONFORMATIONAL CONSTRAINED ANALOGS OF SR141716: SYNTHESIS, COMPUTATIONAL ANALYSIS AND BIOLOGICAL EVALUATIONS	P2
Raimo Saari, Jonna-Carita Törmä and Tapio Nevalainen	QUINOLYL, ISOQUINOLYL, AND QUINOXALINYL PHENYL AMINES AS CB2 RECEPTOR AGONISTS	P3
Janet Ralbovsky, Paul Beckett, Conrad Cowan, Larissa Rakhilina, Shirley Louise-May, Carla Gauss and Frederique Menzaghi	SYNTHESIS, SAR EVALUATION AND MOLECULAR MODELING OF MODIFIED PHENANTHRIDINES: NOVEL AND SELECTIVE CB2 AGONISTS	P4
Topic B. Receptor Structure and Signal Transduction		
Evangelia Kotsikorou, Judy Norris, Diane Lynch, Dow Hurst and Patricia Reggio	IDENTIFICATION OF THE HEMOPRESSIN BINDING SITE AT CB1	P5
Joong-Youn Shim	COMPARATIVE MODELING OF THE TRANSMEMBRANE HELICAL BUNDLE OF THE CB1 RECEPTOR	P6

John Tyrrell, Dow Hurst and Patricia Reggio	MODELING STUDIES OF THE CB1/DOPAMINE D2 SHORT HETERODIMER USING CORRELATED MUTATION ANALYSIS	P7
Tricia Hardt, Hengjun He, Jerry Hernandez, Maurice Elphick, Deborah Lewis and Dana Selley	CANNABINOID RECEPTOR INTERACTING PROTEIN (CRIP1A) AFFECTS CB1 SIGNALLING	P8
Pietro Marini, Luciano De Petrocellis, Aniello Schiano Moriello, Luigìa Cristino, Maura Palmery and Vincenzo Di Marzo	SYNERGISTIC EFFECTS OF CB1, MUSCARINIC AND δ-OPIOID RECEPTOR AGONISTS ON INTRACELLULAR CA2+ MOBILIZATION IN SH-SY5Y CELLS	P9
Frauke Steindel, Martin Häring, Giovanni Marsicano, Beat Lutz and Krisztina Monory	DIFFERENTIAL COUPLING OF G PROTEINS TO CB1 RECEPTORS IN HIPPOCAMPAL GLUTAMATERGIC AND GABAERGIC NEURONS	P10
Gemma Baillie, Teresa Barber, Dow Hurst, Jeremy Presland, Roger Pertwee, Mary Abood, Patricia Reggio and Ruth Ross	ALLOSTERIC MODULATION OF THE CANNABINOID CB1 RECEPTOR	P11
Carolyn Tanner, Roger G. Pertwee and Ruth A. Ross	INVESTIGATION OF THE EFFECTS OF CANNABINOID LIGANDS IN BRAIN MEMBRANES DERIVED FROM $CB1^{-/-}$ AND $CB2^{-/-}$ MICE	P12
Lesley Ford, Jeremy Presland, Angela King, Andrew Irving, Roger Pertwee and Ruth Ross	STUDIES IN TISSUES FROM $CB1^{-/-}$ AND $CB2^{-/-}$ MICE REVEAL JWH-015 IS AN AGONIST AT A NOVEL CANNABINOID RECEPTOR	P13
Christopher Henstridge, Nariman Balenga, Gillian Campbell, Maria Waldhoer and Andrew Irving	The GPR55 ligand, L-α-lysophosphatidylinositol, promotes Rho-dependent Ca2+ signaling	P14
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Notes:

Plenary Lecture 1

17.00 – 17.45

Thursday, June 26th, 2008

SELECTIVE OXYGENATION OF ENDOCANNABINOIDS BY CYCLOOXYGENASE-2

Larry Marnett, Ph.D.

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The two cyclooxygenase enzymes, COX-1 and COX-2 catalyze the oxygenation of arachidonic acid to prostaglandin endoperoxides, which are the common intermediates in the biosynthesis of prostaglandins and thromboxane. COX-2 efficiently utilizes neutral derivatives (esters and amides) of arachidonic acid as substrates. Foremost among these are the endocannabinoids, 2-arachidonoylglycerol and arachidonoylethanolamide. This raises the possibility that COX-2 oxygenation plays a role in a novel signaling pathway dependent on agonist-induced release of endocannabinoids and their selective oxygenation by COX-2. The products of COX-2 oxygenation of endocannabinoids are glyceryl prostaglandins and ethanolamide prostaglandins. The glyceryl prostaglandins, PGE_2-G PGI_2-G, exhibit interesting biological activities in inflammatory, neurological, and vascular systems. These compounds are produced in intact cells stimulated with physiological agonists and have been isolated from in vivo sources. This supports the hypothesis that endocannabinoids are substrates for a COX-2-selective signal transduction pathway.

PLENARY LECTURE 2

8.30 – 9.15

Friday, June 27th, 2008

ENDOCANNABINOID MOBILIZATION AND SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS

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University of Maryland School of Medicine
Baltimore, MD, 21201, U.S.A.

Endocannabinoids are important signaling molecules that modulate synaptic strength throughout the central nervous system. They are implicated in many animal behaviors, and they control both short- and long-term forms of synaptic plasticity in neurophysiological studies. Understanding the linkage between regulation of synaptic properties and behavioral functions will require a thorough understanding the cellular regulation of endocannabinoids. The main candidate endocannabinoids, anandamide and 2-arachidonyl glycerol, are derivatives of fatty acids, and are synthesized by enzymatic action on cellular plasma membrane phospholipids. Because they are not stored prior to use, endocannabinoids are said to become available 'on demand'. Synthesis and release could be coupled, such that synthesis leads directly to release, or synthesis and release could be dissociable phenomena representing different molecular processes. At the cellular physiological level it is difficult to distinguish between the two, and the term 'mobilization' has come to subsume both factors. In general, the regulation of endocannabinoid mobilization is not understood, and yet the degree and duration of mobilization are critical variables in determining the type of synaptic plasticity that is induced. Importantly, differences in our understanding of endocannabinoid mobilization may be emerging as neurochemical and cellular approaches are compared. One trigger for mobilization is an increase in intracellular calcium concentration, a second is activation of certain G-protein coupled receptors (e.g., muscarinic cholinergic, metabotropic glutamate, and dopamine D2), and a third is a synergistic interaction between G-protein products and calcium. This talk will present new data relating to the mechanism(s) of endocannabinoid mobilization in hippocampal pyramidal cells. The use of electrophysiological and novel optical approaches in this investigation will be emphasized.

PLENARY LECTURE 3

11.15 – 12.15

Sunday, June 29th, 2008

KANG TSOU MEMORIAL LECTURE

NEW INSIGHTS INTO ENDOCANNABINOID SIGNALLING AND FUNCTION BASED ON THE EXPRESSION OF DAGLα and DAGLβ

Patrick Doherty, Ph.D.

Wolfson Centre for Age-Related Diseases, King's College London, UK.

Determining where ligands for the CB1 and CB2 cannabinoid receptors are made is important for understanding the function of the endocannabinoid signalling system. 2-arachidonylglycerol (2-AG) is a ligand for both receptors and two closely related enzymes (DAGLα and DAGLβ) that synthesise 2-AG have now been cloned. We have previously reported that during development DAGL and CB1 transcripts can be found in newly born neurons with their protein products expressed in growing axons. I will discuss recent data that shows that DAGL and CB1 receptor function is required for the development of fasciculated axonal tracts, a phenotype that is consistent with adhesion molecules promoting axonal growth by activating an FGF receptor/DAGL/CB1 signalling cascade. In the adult brain there is a requirement for the post-synaptic synthesis of an endocannabinoid that can function as a retrograde synaptic messenger. Here, DAGLα and DAGLβ expression in neurons is restricted to dendrites, suggesting that they contribute to this endocannabinoid function. We now report that in the adult CNS, DAGLα and DAGLβ are also expressed by ependymal and proliferating cells in the adult subventricular zone (SVZ). These cells generate rapidly dividing progenitors that migrate along the rostral migratory stream (RMS) and populate the olfactory bulb (OB) with new neurons. We now show that in the young adult mouse, DAGL and CB2 antagonists (but not CB1 antagonists) inhibit cell proliferation in the SVZ, and that this is associated with a reduction in the appearance of new neurons in the OB. Furthermore, CB2 agonists and a FAAH inhibitor (but not CB1 agonists) stimulate cell proliferation in the SVZ and/or the appearance of new neurons in the OB of older animals. These data suggest that a rundown in endocannabinoid tone might be responsible for the reduced SVZ neurogenesis that is seen in older animals, and identify the CB2 receptors and FAAH as potential therapeutic targets to counteract this age-related phenomenon. Overall the data suggest that DAGLα and DAGLβ play a major role in endocannabinoid signalling in the developing and adult CNS, and thereby substantiate a physiological role for 2-AG as an endocannabinoid involved in a wide range of functions mediated by the CB1 and CB2 cannabinoid receptors.

STEREO- AND ENANTIO-SPECIFIC ACTIVITY OF FATTY ACID CYCLOPROPANOLAMIDES AT CANNABINOID AND VANILLOID RECEPTORS

Alessia Ligresti[1#], Alberto Minassi[2#], Luciano De Petrocellis[3#], Orazio Tagliatatela-Scafati[4#], Marco Allarà[1#], Giovanni Appendino[2#] and Vincenzo Di Marzo[1#]

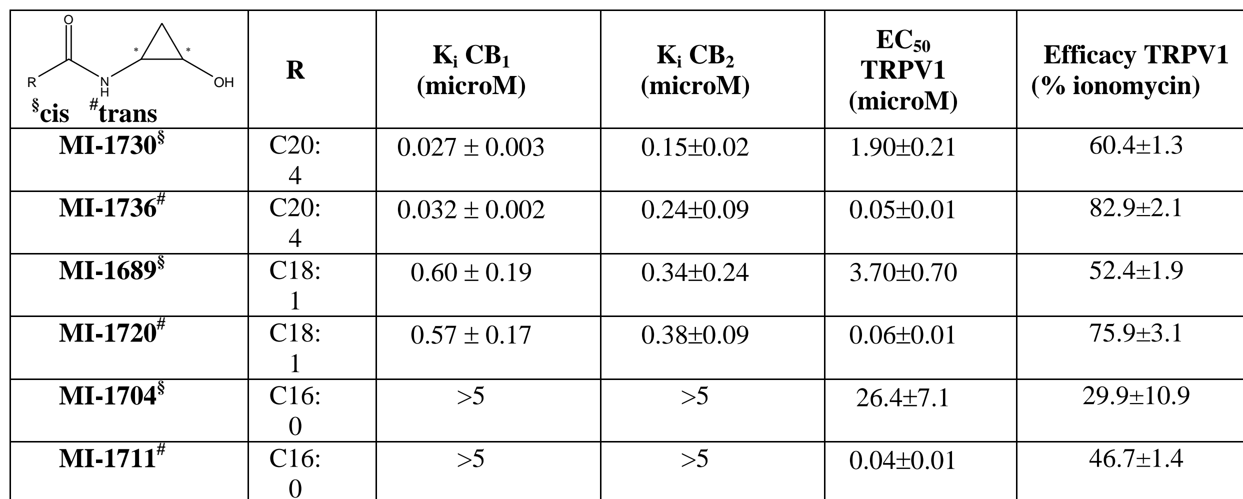
[#]Endocannabinoid Research Group; [1]Istituto di Chimica Biomolecolare, CNR Pozzuoli, Italy; [2#] Dipartimento di Chimica, Università di Novara, Italy; [3]Istituto di Cibernetica, CNR Pozzuoli, Italy; [3]Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", Italy

All the endocannabinoids identified so far contain no chiral centers, and this is true also for most of the endovanilloids (i.e. the endogenous agonists of TRPV1 vanilloid channels). Furthermore, no natural or synthetic potent TRPV1 agonist has been identified so far whose high potency was not mostly due to non-chiral vanilloid or catecholamine moieties. We investigated fatty acid ethanolamide structural requirements for the binding to CB_1 and CB_2 receptors and activation of TRPV1 receptors. In particular, we locked the ethanolamine moiety of various amides (differing in the acyl chain) using a cyclopropanol group with two chiral centers and affording four diastereoisomers.

Details on the non-enantioselective synthesis of the compounds will be provided at the meeting. Racemic mixtures were separated by chiral-phase HPLC. Affinity for human CB_1 and CB_2 receptors was assessed by binding assays, and the capability to activate the human TRPV1 receptor was investigated by means of intracellular Ca^{2+} assays (see Table).

The two racemic mixtures with the arachidonic moiety (MI-1730 and MI-1731) retained a similar affinity to anandamide for both cannabinoid receptors (CB_1>CB_2), and, for the TRANS mixture, exhibited potency at TRPV1 receptors comparable to capsaicin, thus behaving as true CB_1/TRPV1 "hybrids". Unlike oleoylethanolamide, the racemic mixtures with oleic acid (MI-1689 and MI-1720) were able to bind both cannabinoid receptors (with no selectivity) and, again in the case of the TRANS enantiomers, they still potently activated TRPV1. The racemic mixtures with palmitic acid (MI-1704 and MI-1711) exhibited no affinity for cannabinoid receptors, but, in the case of TRANS mixture, showed remarkable activity at TRPV1, unlike palmitoyl-vanillamide and -ethanolamide. When we separated the four arachidonoylcyclopropanolamides diastereoisomers in MI-1730 and MI-1731, we found little enantioselectivity at cannabinoid receptors. However, enantioselective TRPV1 activity was observed for both the TRANS and CIS couples (not shown in Table).

In conclusion, we reported for the first time a potent activity at TRPV1 for molecules lacking the vanillic moiety, and found that a cyclopropanol moiety can introduce strong activity at this receptor even in saturated fatty acids like palmitic acid. Furthermore, we found that the cyclopropanol function introduces appreciable affinity for cannabinoid receptors in oleic acid. Finally, we showed for the first time that activity at TRPV1 channels can be strongly sensitive to chiral centers. The new "hybrid" CB_1/TRPV1 ligands MI-1736 and MI-1720 might provide new attractive therapeutic tools for the treatment of pain and other disorders in which both receptor types are involved. Further studies are ongoing to assign the absolute stereochemistry of all enantiomers and to fully evaluate their function.



§cis #trans	R	K_i CB_1 (microM)	K_i CB_2 (microM)	EC_{50} TRPV1 (microM)	Efficacy TRPV1 (% ionomycin)
MI-1730§	C20:4	0.027 ± 0.003	0.15±0.02	1.90±0.21	60.4±1.3
MI-1736#	C20:4	0.032 ± 0.002	0.24±0.09	0.05±0.01	82.9±2.1
MI-1689§	C18:1	0.60 ± 0.19	0.34±0.24	3.70±0.70	52.4±1.9
MI-1720#	C18:1	0.57 ± 0.17	0.38±0.09	0.06±0.01	75.9±3.1
MI-1704§	C16:0	>5	>5	26.4±7.1	29.9±10.9
MI-1711#	C16:0	>5	>5	0.04±0.01	46.7±1.4

This work is dedicated to the memory of Prof. J. Michael Walker

LOCKEDNESS - CONFORMATIONALLY RESTRICTED ENANTIOMERIC PROBES OF THE hCB1 RECEPTOR

Herbert H. Seltzman, Marcus F. Brackeen, Daniel J. Watkins, Jason P. Burgess, Anne F. Gilliam, Brian F. Thomas, Yanan Zhang, Tiffany Langston, Hernan A. Navarro
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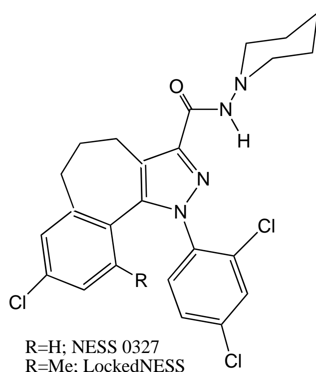
The aim of this study was to design and synthesize non-interconvertable, conformationally distinct rotamers known as atropisomers to probe conformational requirements of the cannabinoid antagonist Rimonabant in the cannabinoid CB1 receptor active site. Understanding these requirements could lead to a more rational design of potentially specific and higher activity drugs.

The methods involved NMR, chromatographic and computational examination of candidate compounds that looked for evidence of non-interconverting enantiomers and separation of these isomers for separate pharmacological study. The first compound, reported as NESS 0327, potentially limited the rotational conformations of the 4-chlorophenyl ring in the 5-position with a three-carbon bridge connecting it to the pyrazole 4-position. In this case the above evidence showed that the energy barrier between the enantiomers of NESS 0327 is too low to isolate the two conformers. We then synthesized a further sterically constrained methyl-substituted analog with the same bridge where the energy barrier was calculated and chromatographically demonstrated to be sufficient to isolate the enantiomeric atropisomers A & B. Two distinct isomers were pharmacologically characterized by binding affinity (hCB1), GTPγS and calcium flux assay.

The binding results (Ki, nM) at the human hCB1 receptor versus tritiated SR141716 and CP 55,940 were 15.9 & 59.3 (A+B); 13.3 & 32.7 (A); 10.5 & 18.1 (B); 4.77 & 11.2 (NESS 0327) respectively. The GTPγS assay showed all the analogs to be antagonists while in the calcium flux CA3 assay they behaved as partial agonists.

We conclude that suitably substituted analogs of Rimonabant can be locked into separate conformers for pharmacological study. Further, the current pair of atropisomers have comparable affinities for hCB1 (vs SR141716) indicating that the incident difference (orientation of the 4-chlorophenyl ring) is tolerated by the receptor and does not alter binding or function.

Supported by commercial funds to HHS, Grant R01 DA19217 to BFT and internal R&D funds to HAN.



THE LACK OF SOMATODENDRITIC EFFECTS OF CANNABINOIDS IS DUE TO CONSTITUTIVE ACTIVITY OF CB_1 RECEPTORS AND SUBSEQUENT RECEPTOR INTERNALIZATION

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The $G\alpha_{i/o}$ protein-coupled CB_1 cannabinoid receptor is widely distributed in the nervous system. Activation of CB_1 receptors on axon terminals leads to inhibition of voltage-gated calcium channels and subsequent inhibition of neurotransmitter release and synaptic transmission (Szabo & Schlicker, Handb Exp Pharmacol 168: 318–56, 2005). Surprisingly, cannabinoid agonists applied to the somatodendritic region of neurons do not elicit effects expected from a $G\alpha_{i/o}$ protein-coupled receptor, like activation of potassium currents and inhibition of voltage-gated calcium channels (Freiman et al., J Physiol 575: 789-806, 2006). The hypothesis of the present work was that somatodendritic cannabinoid effects are missing, because the constitutively active somatodendritic CB_1 receptors are removed from the cell surface by internalization (Lenkei et al., J Neurosci 22; 3141-3153).

We used CB_1 receptor mutants for testing our hypothesis. Like in other G protein-coupled receptors, a tyrosine residue located in the conserved DRY motive (position 3.46, T210) plays an important role in the constitutive activity and localization of CB_1 receptors. Compared with the wild-type receptor (CB_1R-WT), the mutant CB_1R-T210I exhibits enhanced agonist and diminished inverse agonist affinity, consistent with a shift toward the constitutively active form and increased internalization. On the contrary, the mutant CB_1R-T210A exhibits diminished agonist and enhanced inverse agonist affinity and localizes on the cell membrane (D'Antona et al., Biochem 45: 5606-5617, 2006). Primary hippocampal neurons were transfected with CB_1R-WT or the mutants CB_1R-T210I and CB_1R-T210A. Neurons were superfused and depolarization-evoked calcium transients were determined by fluorometric imaging (using the calcium sensitive dye fura-4F, AM). The cannabinoid receptor agonist WIN55212-2 (5×10^{-7} M) did not affect the calcium transients in neurons transfected with CB_1R-WT or CB_1R-T210I. In contrast, in neurons transfected with CB_1R-T210A, WIN55212-2 significantly inhibited the calcium transients (by 45 ± 8 %; $P < 0.05$). The functional results were compatible with the results of anatomical localization studies. In cultured hippocampal neurons, the mutant CB_1R-T210A was observed on the somatodendritic cell membrane of neurons. In contrast, only very few CB_1R-WT or CB_1R-T210I were found on the somatodendritic membrane.

The results support the hypothesis that the lack of somatodendritic effect of cannabinoids is due to the constitutive activity and subsequent internalization of CB_1 receptors.

INTERACTION BETWEEN THE CB_1 CANNABINOID RECEPTOR AND THE β_2 ADRENERGIC RECEPTOR AFFECTS CB_1 AND β_2 RECEPTOR SIGNALING

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Many family A G-protein coupled receptors (GPCRs) have been found to form dimers or higher order oligomers with themselves or with other GPCRs. These interactions have been shown to influence many aspects of receptor function including trafficking, pharmacology and signaling. The CB_1 receptor has been shown to undergo these interactions with itself as well as with other GPCRs including the D_2 dopamine, the orexin-1, and the opioid receptors. We have recently demonstrated using bioluminescence resonance energy transfer (BRET) that the CB_1 receptor also dimerizes with the β_2 adrenergic receptor and that this interaction influences the trafficking of these receptors (Hudson and Kelly, 2007; 17th Annual Symposium on the Cannabinoids). The present study describes interactions between CB_1 and β_2 receptor signaling pathways that may also result from dimerization.

In order to study CB_1 and β_2 receptor signaling, an infrared fluorescence based assay was employed to assess phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK) and cyclic AMP response element binding protein (CREB) in 293H cells. When cells expressed only the CB_1 receptor the cannabinoid agonist WIN 55,212-2 (WIN) stimulated an increase in phospho-ERK through a PTX-sensitive pathway and an increase in phospho-CREB (pCREB) through a PTX-insensitive pathway. In contrast, when cells expressed only the β_2 receptor the β agonist isoproterenol (ISO) increased both pERK and pCREB through PTX-insensitive pathways. If CB_1 and β_2 were co-expressed the efficacies of both WIN and ISO to increase pERK were enhanced, while the efficacy of WIN to stimulate pCREB was reduced. Co-application of WIN and ISO resulted in an additive effect on pERK and an inhibitory effect on pCREB. The CB_1 inverse agonist AM251 enhanced the ISO pERK response in cells expressing both receptors, while the β_2 antagonist timolol had no effect on the WIN responses. The PTX sensitivity of all responses was not altered by co-expression of both receptors.

Together, these results demonstrate complex signaling interactions between the CB_1 and β_2 receptors. Co-expression of these receptors appears to shift CB_1 signaling towards its more traditional PTX-sensitive Gi pathway, while generally enhancing β_2 signaling. The results of the present study, in combination with the previous report of dimerization between the CB_1 and β_2 receptors, suggests a need to reevaluate cannabinoid and adrenergic interactions in tissues that co-express these receptors.

Acknowledgements: CIHR (MEMK); NSERC and Killam Trust studentships (BDH)

ADAPTIVE EVOLUTION AND PURIFYING SELECTION AT INDIVIDUAL AMINO ACID RESIDUES IN THE CB1 SEQUENCE

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Introduction: Positive selection on a gene confers adaptive evolution upon its carrier. The identification of genes shaping adaptive evolution in humans has captured the imagination of bioinformatics researchers. For example *MRGX2*, a nociception-specific gene, shows signs of positive selection. Codons corresponding to the *MRGX2* ligand binding region were under strongest positive selection. On the other hand, *CNR1* (the gene encoding CB1) as a whole is under strong purifying selection, where mutations in its sequence reduce carrier fitness and they are usually removed from the population. This study examined *CNR1* on a codon-by-codon basis, to identify individual amino acids under positive selection or purifying selection.

Methods: Selection pressure was calculated as Ka/Ks, a ratio normalized as the number of nonsynonymous nucleotide substitutions per potential nonsynonymous sites divided by the number of synonymous nucleotide substitutions per potential synonymous sites. Ka/Ks was calculated with the CodeML package in PAML, performed upon a multiple sequence alignment constructed from 19 *CNR1* orthologs. Codons corresponding to the domains within CB1 were examined, as were specific amino acid residues involved in ligand binding and G-protein interactions (identified via a literature search of site-directed mutagenesis studies).

Results: The seven α-helix domains in *CNR1* were under strong purifying selection (mean Ka/Ks of 197 codons = 0.0526 ±0.0033), followed by the three IC loops (0.0570 ±0.0072), three EC loops (0.0603 ±0.0071), C-terminal region (0.0640 ±0.0055), and N-terminal region (0.0782 ±0.0043), differences were significant (Kruskal-Wallis $p = 0.0001$). The set of residues involved in ligand binding (0.0518 ± 0.0118), G-protein coupling (0.0516 ± 0.0126), and receptor desensitization (0.0514 ± 0.0211) were also under strong purifying selection. Nevertheless these biologically significant residues did not count among the codons under strongest purifying selection, the 13 codons in *CNR1* under strongest purifying selection have not undergone site-directed mutagenesis studies.

Conclusion: Although positive selection captures the imagination, it is equally important to identify genes undergoing purifying selection. Ka/Ks analysis may identify heretofore unrecognized amino acid residues that confer strongly selected biological functions.

AGONIST-SELECTIVE REGULATION OF AP-1 ELEMENT SUPPORTS THE COMPLEX REGULATION OF TYROSINE HYDROXYLASE EXPRESSION BY DISTINCT CANNABINOID AGONISTS

Barbara Bosier, Emmanuel Hermans and Didier M. Lambert

Unité de Chimie Pharmaceutique et de Radiopharmacie & Unité de Pharmacologie expérimentale, Université catholique de Louvain, B-1200 Brussels, Belgium

Δ^9-THC-mediated alteration of motor and emotional behaviours frequently correlates with modification of catecholamine transmission systems. Beside acute alteration of neurotransmitter release, modulations of complex intracellular signalling pathways by cannabinoid also lead to long-term adaptation of cell functions. In this respect, we previously reported CB_1-mediated opposite regulation of tyrosine hydroxylase (TH) gene expression when comparing the responses to the unrelated agonists HU 210 and CP 55,940.
Given their crucial regulatory role within the TH promoter, we now further investigated the influence of these agonists on CRE and AP-1 *cis*-enhancer elements using reporter gene assays. Consistent with CB_1-mediated reduction of cAMP accumulation, both ligands decreased CRE-driven luciferase activity. In contrast, in cell engineered to examine AP-1 activity, HU 210 caused a concentration-dependent reduction of luciferase activity whereas CP 55,940 failed to regulated AP-1. We next investigate the role of these response elements in cannabinoid-mediated regulation of TH expression. Site directed mutagenesis of CRE consensus within the TH promoter strongly reduced efficacies of both agonists. Besides, mutation of the AP-1 site totally impaired both HU 210 and CP 55,940-mediated regulation of TH transcription. These data suggest that CRE activity is required to control the efficacy of cannabinoid responses while AP-1 element is crucial for determining the agonist-selective responses.
In addition, responses observed both on AP-1 or on TH promoter-driven constructs were sensitive to pertussis toxin, suggesting that agonist-selective responses are mediated through Gi/o-dependent mechanism. Finally, different kinase inhibitors were used to discriminate between signalling pathways involved in cannabinoid-mediated gene transcription. HU 210 and CP 55,940 were found to differentially modulate TH promoter activity through activation of different subsets of intracellular signalling cascades. Surprisingly, PKC inhibitors efficiently inhibited HU 210 effects on both AP-1 and TH promoter, suggesting an agonist-selective regulation of PKC-dependent responses.
Taken together, our results demonstrate that commonly used cannabinoid agonists with similar pharmacodynamic properties display differential effects regarding AP-1 *cis*-enhancer element. This could explain the complex regulations of TH gene expression, leading to totally distinct delayed responses. Our data suggest that complexity may arise from the activation of distinct subsets of Gi/o proteins and their related effectors, supporting the concept of functional selectivity.

MICROSECOND TIME SCALE MOLECULAR DYNAMICS SIMULATIONS: 2-AG ENTRY INTO THE CB2 RECEPTOR VIA THE LIPID BILAYER

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It is commonly assumed that Class A GPCR ligands enter and exit the receptor via extracellular space. While this assumption makes sense for charged, hydrophilic ligands such as the cationic neurotransmitters, a similar entrance/exit point is difficult to rationalize for hydrophobic ligands such as 2-arachidonoylglycerol (2-AG), the endogenous ligand of the Class A cannabinoid CB2 receptor. In work reported here, we tested the hypothesis that 2-AG may enter CB2 via the lipid bilayer. Microsecond time scale molecular dynamics simulations of 2-AG (NVT ensemble, T=310K, with velocity resampling occurring every nanosecond) were conducted in a system composed of the CB2 receptor in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer. The system contained 124 POPC and 38 2-AG molecules. Simulations revealed that 2-AG can enter CB2 from the POPC bilayer by inserting between transmembrane helix 6 (TMH6) and TMH7 extracellular to the highly conserved W6.48(258). The initial interaction site for the 2-AG head group is S7.39(285), however, the ligand quickly establishes a long-standing interaction with D275 in the EC-3 loop of CB2. The entry of 2-AG into the CB2 binding pocket produces rearrangements in the intracellular domains of CB2 that are commonly associated with GPCR activation. These include a transient (150 ns) break in a key salt bridge between D6.30(240) and R3.55(136) and movement of the IC-3 loop away from the TMH bundle towards lipid. [Support: NIH RO1 DA03934 and KO5 DA021358 (PHR)]

LOCALIZATION OF NAPE-PLD EXPRESSION IN THE BRAIN: EVIDENCE OF A NOVEL ROLE FOR *N*-ACYLETHANOLAMINES AS ANTEROGRADE SYNAPTIC SIGNALLING MOLECULES

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N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) catalyses formation of N-acylethanolamines (NAEs) and was recently identified as a member of the zinc metallohydrolase family of enzymes (Okomoto et al. 2004; J. Biol. Chem., 279, 5298). Analysis of brain levels of NAEs in NAPE-PLD$^{-/-}$ mice indicates that this enzyme is primarily involved in biosynthesis of long-chain saturated NAEs, with little or no in vivo contribution to formation of the endocannabinoid anandamide and other polyunsaturated NAEs (Leung et al. 2006; Biochem., 45, 4720). However, nothing is known about the physiological roles of long-chain saturated NAEs in the nervous system. To facilitate further investigation of this issue, we have analysed expression of NAPE-PLD in mouse brain (Egertová et al. 2008; J. Comp. Neurol. 506, 604) and rat brain.

NAPE-PLD mRNA is detected in neurons in many regions of the mouse brain, but with particularly high levels of expression in dentate gyrus granule cells. Furthermore, using novel NAPE-PLD antibodies validated for specificity by analysis of NAPE-PLD$^{-/-}$ mice, we have discovered that this enzyme appears to be primarily targeted to the axons and axon terminals of neurons in several regions of the brain, but most strikingly in axons of dentate gyrus granule cells (mossy fibres) and axons of the vomeronasal nerve. Recently, we have extended our analysis of NAPE-PLD expression to the rat brain. Our NAPE-PLD antibodies specifically label a band with the expected molecular mass for NAPE-PLD (~ 46 kDa), which is concentrated in the membrane fraction of rat brain homogenates. The general distribution of NAPE-PLD in rat brain appears to be broadly similar to mouse brain. However, there are differences in the relative abundance of NAPE-PLD in brain regions. For example, the intensity of NAPE-PLD-immunoreactivity in the thalamus is much higher in rat brain than in mouse brain. These observations are consistent with the relative abundance of NAPE-PLD activity in rat brain regions, with the highest levels of enzyme activity detected in the thalamus (Morishita et al. 2005; J. Neurochem. 94, 753).

Collectively, our data lead us to postulate that long-chain saturated NAEs generated by NAPE-PLD in axon terminals may act as anterograde synaptic signalling molecules, exerting effects on postsynaptic neurons via as yet unknown molecular mechanisms. Consistent with this hypothesis, in many regions of the brain the enzyme that inactivates NAEs (fatty acid amide hydrolase, FAAH) is located in neuronal somata/dendrites that are postsynaptic to NAPE-PLD expressing axons.

ANALYSIS AND STRUCTURE-ACTIVITY RELATIONSHIP OF LYSOPHOSPHATIDYLINOSITOL, A POSSIBLE ENDOGENOUS LIGAND FOR GPR55

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GPR55 is a seven transmembrane, G protein-coupled receptor. Previously, several investigators reported that GPR55 may be a novel receptor for cannabinoids, yet the details remain to be determined. Recently, we explored a possible endogenous ligand for GPR55 using HEK293 cells which expressed GPR55. We found that lysophosphatidylinositol, an acidic lysophospholipid, induced rapid phosphorylation of ERK and a Ca^{2+} transient in GPR55-expressing cells. Notably, lysophosphatidylinositol failed to stimulate mock-transfected cells. Anandamide, virodhamine and abnormal cannabidiol did not induce the phosphorylation of ERK and Ca^{2+} transients in GPR55-expressing cells. These results strongly suggest that GPR55 is a specific and functional receptor for lysophosphatidylinositol (*Biochem. Biophys. Res. Commun*., 362, 928-934, 2007). In this study, we first examined the tissue levels of lysophosphatidylinositol using 17:0 lysophosphatidylinositol as an internal standard. We found that rat brain contains 20 nmol/g tissue of lysophosphatidylinositol. The predominant fatty acyl moiety of brain-derived lysophosphatidylinositol is stearic acid followed by palmitic acid. We then compared in detail the biological activities of various molecular species of lysophosphatidylinositol, various species of lysophospholipids, cannabinoids and related molecules using HEK293 cells expressing GPR55. Various types of lysophospholipids, such as lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylserine, did not exhibit appreciable agonistic activities. Interestingly, lysophosphatidylglycerol possessed weak agonistic activity, although its activity was far less potent compared with that of lysophosphatidylinositol. The agonistic activities of 2-arachidonoylglycerol, free arachidonic acid and CP55940 were negligible. Various types of detergents, such as sucrose monolaurate and sodium dodecylbenzenesulfonate, also did not induce the phosphorylation of ERK or Ca^{2+} transients, indicating that the effects of lysophosphatidylinositol are not attributed to non-specific physico-chemical effects. These results provided further evidence that GPR55 is a specific receptor for lysophosphatidylinositol.

PROTECTIVE ROLE OF PALMITOYLETHANOLAMIDE IN KERATINOCYTES AND ITS INVOLVEMENT IN CONTACT ALLERGIC DERMATITIS

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Palmitoylethanolamide (PEA) is produced by mammalian cells together with anandamide. It potentiates the effects of anandamide at both cannabinoid and TRPV1 (transient receptor potential vanilloid type-1) receptors, and it directly activates peroxisome proliferator-activated receptor-α (PPAR-α) nuclear receptors. Controversial data exist as to its possible agonist activity at the orphan G-protein-coupled receptor, GPR55. Pharmacological studies have shown analgesic and anti-inflammatory properties of PEA. In particular, several of its anti-inflammatory actions were suggested to occur at the level of mast cells and keratinocytes. Furthermore, PEA was shown to be effective against contact dermatitis in humans, whereas in animal models of contact allergic dermatitis, it has been suggested that CB_1, CB_2, TRPV1 and PPAR-α receptors might play a protective role. In particular, double CB_1/CB_2 null mice were found to be less protected against 2,4-dinitrofluorobenzene (DNFB)-induced contact allergic dermatitis, which is accompanied by elevation of anandamide and 2-AG levels in inflamed ears (Karsak et al., *Science*, 2007). Therefore, the aim of this study was to examine whether PEA is produced during DNFB-induced contact allergic dermatitis in mice, and to investigate if it has any direct protective action on keratinocytes.
Wild-type (C57BL/6J) or double CB_1/CB_2 null mice were sensitized by DNFB on the shaved abdomen on two consecutive days, and on day 5 their ears were spread with DNFB. Subsequently, mice were sacrificed, and ear skin was used to determine the amounts of PEA by LC-APCI-MS and to carry out immunohistochemical studies on the expression of CB_2, TRPV1, PPAR-α and NAAA (*N*-acylethanolamine-hydrolyzing acid amidase), the enzyme mostly responsible for PEA degradation. PEA levels were found to be elevated in the ear skin of DNFB-sensitized mice after challenge with DNFB, particularly in double CB_1/CB_2 null mice, which exhibited a much higher inflammatory phenotype than wild-type mice. DNFB treatment induced an up-regulation of TRPV1 and PPAR-α receptors in ear skin keratinocytes of double CB_1/CB_2 null mice as compared to wild-type mice, and a down regulation of NAAA.
We next investigated the protective effect of PEA on human keratinocytes (HaCaT cells), plated into six-well culture plates for 1 day and then stimulated for 24h with the toll-like receptor 3 (TLR3) ligand poly-(I:C) (polyinosinic acid-polycitidilic acid) (100 microg/ml). After stimulation, the expression of the MCP-2/CCL8 chemokine, previously shown to be down-regulated by CB_1/CB_2 agonists in DNFB-induced contact allergic dermatitis in mice (Karsak et al., *Science*, 2007), was quantified by immunocytochemistry. A strong increase of MCP-2/CCL8 staining was found in poly-(I:C)-stimulated cells, confirmed by real-time quantitative PCR analysis. PEA (1-10 microM) counteracted this effect of poly-(I:C).
These findings suggest that PEA is an endogenous protective agent against inflammation induced by contact allergic dermatitis and that it might act at the level of keratinocytes, which express high levels of its putative direct or indirect molecular targets. Experiments are ongoing to identify the mechanism of action of this effect of PEA, and to determine if it can be observed also in vivo in DNFB-treated mice.

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This work is dedicated to the memory of Prof. J. Michael Walker

ENDOCANNABINOID MODULATION OF PRURITUS: FURTHER INVESTIGATIONS INTO ITCH

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Pruritus, an unpleasant nocifensive sensation that results in the desire to scratch the affected area, greatly reduces quality of life. Due to the variety of pathophysiological conditions underlying pruritus symptomology, common treatments are not always effective. Initial results from small clinical studies indicate that oral or topical administration of cannabinoid receptor agonists reduce both acute and chronic itch. Using a mouse model of allergenic pruritus, in which local subcutaneous administration of the mast cell degranulator compound 48/80 elicits scratching behavior, we have demonstrated that systemic Δ^9-THC reduces scratching (ED50 = 0.66 mg/kg, 95% CI 0.52 to 0.82 mg/kg, ip), accompanied with approximately equipotent suppression of overall activity. However, local subcutaneous administration of low doses of Δ^9-THC (0.3-0.75 mg/kg) reduced scratching while minimally affecting locomotor activity. Conversely, high doses of the CB_1 receptor antagonist rimonabant elicited scratching behavior and increased the magnitude of compound 48/80-induced scratching.

In the next series of experiments, we used complementary genetic and pharmacological approaches to target fatty acid amide hydrolase (FAAH), the primary enzyme responsible for the degradation of the endocannabinoid anandamide. FAAH (-/-) mice displayed a phenotypic decrease in compound 48/80-induced scratching. In addition, the irreversible FAAH inhibitor URB597 dose-dependently reduced the response to compound 48/80 scratching, but without the increased hypomotility associated with CB_1 receptor activity. In fact, a 10 mg/kg dose of URB597 produced comparable reductions in scratching (48% reduction from vehicle) to those of traditional allergenic pruritus treatments (loratadine: 52%, dexamethasone: 48% reduction). Neither URB597 nor Δ^9-THC reduced scratching responses in CB_1 (-/-) mice. Furthermore, a non-pruritogenic dose of rimonabant reversed the phenotypic reduction in compound 48/80 scratching in FAAH (-/-) mice. On the other hand, URB597 continued to reduce scratching behavior in CB_2 (-/-) mice, and wild type mice treated with a selective CB_2 receptor agonist. These data suggest that FAAH suppression reduces the scratching response through a CB_1 receptor mechanism of action. These lines of evidence suggest that cannabinoid receptor activation/inhibition modulate scratching behavior. In conclusion, FAAH represents a promising target to treat pruritus without cannabimimetic side effects.

INVOLVEMENT OF ENDOGENOUS CANNABINOIDS ON FEVER: DEPENDENCE ON PROSTAGLANDINS AND OPIOIDS SYNTHESIS/RELEASE

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AIM: The involvement of endocannabinoids in the control of body temperature and in the fever response is not yet well understood. For this reason, the present study aimed to investigate the contribution of the endogenous cannabinoids in the febrile response.

METHODS: Changes in rectal temperature were measured in a 30 min interval up to 6h by inserting a thermistor probe in the rectum of male Wistar rats. All data are reported as mean ± standard error mean in the peak of temperature response or percentage of reduction from the stimuli. All pre-treatments were injected 30 min before the stimuli.

RESULTS: The i.c.v. administration of anandamide (AEA) induced a dose dependent increase on body temperature (4.0h: Vehicle= -0.0±0.1; AEA 0.01μg= 0.4±0.1; AEA 0.1μg= 0.9±0.1; AEA 1.0μg= 1.5±0.0; AEA 10.0μg= 1.5±0.1°C). The increase on body temperature induced by the i.c.v administration of AEA 1.0μg was followed by a decrease in the tail skin temperature (4.0h Rectal: Vehicle= -0.1±0.1; AEA= 1.2±0.1; Tail: Vehicle= 0.0± -0.1; AEA= -0.7±0.1°C). The selective CB_1 agonist ACEA induced a bell shaped increase on body temperature (5.0h: Saline= 0.1±0.0; ACEA 0.001μg= 0.6±0.2; ACEA 0.01μg= 1.4±0.1; ACEA 0.1μg= 0.8±0.2; ACEA 1.0μg= 0.6±0.1°C). The i.c.v injection of the selective CB_2 agonist AM1241 did not induce change on body temperature. The i.c.v. pre-treatment with the selective CB_1 antagonist AM251 reduced in a dose dependent fashion the fever induced by AEA 1.0μg i.c.v (5.0h: Vehicle/Vehicle = 0.0±0.1; AM251 10.0μg/Vehicle= 0.2±0.1; Vehicle/AEA 1.0μg= 1.8±0.1; AM251 10.0μg/AEA 1.0μg= 0.6±0.2; AM251 5.0μg/AEA 1.0μg= 0.4±0.1; AM251 1.0μg/AEA 1.0μg= 1.6±0.2°C). The pre-treatment with AM251 5.0 μg, i.c.v. reduced the fever induced by ACEA 0.01μg (5.0h: AM251/Saline= 0.1±0.1; Saline/ACEA= 1.5±0.1; AM251/ACEA= 0.5±0.1°C). The non-selective cyclooxygenase (COX) inhibitor, indomethacin (2.0mg/kg, i.p.) and the selective COX-2 inhibitor celecoxib (5.0mg/kg, p.o.,) reduced by 62% and 40%, respectively, the fever induced by AEA (1.0μg, i.c.v.). The non-selective opioid antagonist naloxone (1.0mg/kg, s.c.) abolished the fever induced by AEA (1.0μg, i.c.v.). At this dose AEA increased β-endorphin concentration in the cerebrospinal fluid (CSF). AM251 (5.0μg, i.c.v.) reduced this β-endorphin increase in the CSF (Veh/Veh= 49.6 ± 27.3; AM251/Veh= 99.1 ± 41.2; Veh/AEA= 436.7 ± 62.8; AM251/AEA= 249.2 ± 43.4 pg/ml). At this dose AM251 also reduced the fever induced by LPS/50 μg/kg, i.p. (62%), IL-1β/3 ng (51%), TNF-α/250 ng (60%), IL-6/300 ng (73%) and endothelin (ET)-1/1 pmol (50%).

CONCLUSIONS: All together these results suggest that endogenous cannabinoids, through the activation of the CB_1 receptors, are involved in the development of febrile response induced by LPS and several mediators that orchestrate this response. This involvement seems to be dependent on prostaglandin and opioids synthesis/release.

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INHIBITION OF ENDOCANNABINOID DEGRADATION OR TRANSPORT *IN VIVO* MODULATES LPS-INDUCED INCREASES IN CIRCULATING CYTOKINE LEVELS

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The endogenous cannabinoid system plays an important role in regulating the immune system. Modulation of endogenous cannabinoids represents an attractive alternative for the treatment of inflammatory disorders. This study investigated the effects of URB597, a selective inhibitor of fatty acid amide hydrolase (FAAH), the enzyme catalysing degradation of the endogenous cannabinoid anandamide, and AM404, an inhibitor of anandamide transport, on lipopolysaccharide (LPS)-induced increases in plasma cytokine levels in rats. URB597 (0.6mg/kg) or AM404 (20mg/kg) were administered 30 or 60 min respectively, prior to LPS (100µg/kg) administration. Antagonists were administered just prior to endocannabinoid modulators. Blood was collected by cardiac puncture 2 hours post LPS, plasma removed and cytokine levels determined using R&D system cytokine ELISA kits. Both URB597 and AM404 potentiated the LPS-induced increase in plasma TNFα levels. The peroxisome proliferator-activated receptor (PPAR)γ antagonist, GW9662, attenuated the AM404-induced augmentation of TNFα levels. Furthermore, the selective cannabinoid CB_1 and CB_2 receptor antagonists, AM251 and AM630 respectively, and the transient receptor potential vanilloid receptor-1 (TRPV1) antagonist, SB366791, reduced LPS-induced TNFα plasma levels both alone and in combination with AM404. In contrast, AM404 inhibited LPS-induced increases in circulating IL-1β and IL-6. AM251 attenuated the immunosuppressive effect of AM404 on IL-1β. None of the antagonists altered the effect of AM404 on LPS-induced IL-6. Moreover, AM251, AM630 and SB366791, administered alone, inhibited LPS-induced increases in plasma IL-1β and IL-6 levels. In conclusion, inhibition of endocannabinoid degradation or transport *in vivo* potentiates LPS-induced increases in circulating TNFα levels, an effect which may be mediated by PPARγ and is also reduced by pharmacological blockade of CB_1, CB_2 and TRPV1. The immunosuppressive effect of AM404 on IL-1β levels is mediated by the cannabinoid CB_1 receptor. Improved understanding of endocannabinoid-mediated regulation of immune function has fundamental physiological and potential therapeutic significance.

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BIOCHEMICAL PROFILE OF BIOTIN-ANANDAMIDE, A NOVEL TOOL FOR THE VISUALIZATION OF ANANDAMIDE ACCUMULATION

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Anandamide (arachidonoylethanolamide, AEA) acts as endogenous agonist of both cannabinoid and vanilloid receptors. During the last two decades, its metabolic pathways and biological activity have been extensively investigated and relatively well-characterized. In contrast, the effective nature and mechanism of AEA transport remain at present a controversial and still unsolved issue. Here we report the characterization of a biotinylated analogue of AEA (b-AEA), that has the same lipophilicity of the parent compound. In addition, by means of biochemical assays and fluorescence microscopy, we show that b-AEA is accumulated inside the cells in a way superimposable on that of AEA. Conversely, b-AEA does not interact nor interfere with the other components of the endocannabinoid system, *i.e.* type-1 and type-2 cannabinoid receptors, vanilloid receptor, AEA synthetase (NAPE-PLD) or AEA hydrolase (FAAH). Taken together, our data suggest that b-AEA could be a very useful probe for visualizing the accumulation and intracellular distribution of this endocannabinoid.

TETRAHYDROLIPSTATIN ANALOGUES: NEW SELECTIVE PHARMACOLOGICAL TOOLS TO INVESTIGATE 2-AG METABOLISM

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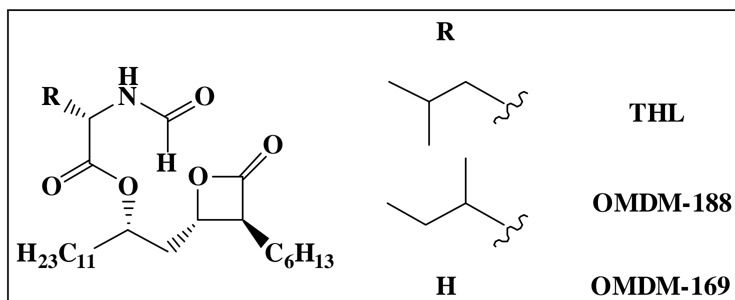
Enzymes for the biosynthesis and degradation of the endocannabinoid 2-arachidonoyl glycerol (2-AG) have been cloned and are the sn-1-selective-diacylglycerol lipases α and β (DAGLα and β) and the monoacylglycerol lipase (MAGL), respectively. Although several new compounds were screened as possible DAGL and/or MAGL inhibitors, the only DAGL inhibitors developed so far, except for the lipase inhibitor tetrahydrolipstatin (THL, IC_{50}~100 nM), are not suitable for systemic use in vivo, whereas of the two MAGL inhibitors developed so far, URB602 is quite weak (IC_{50}~30-200 μM), and *N*-arachidonoyl-maleimide, although relatively potent in vitro (IC_{50}<1 μM), has not been tested yet in vivo.
In the present study we synthesized 15 structural analogues of THL aiming at developing potent and selective inhibitors of 2-AG biosynthesis. For this purpose, the alcoholic portion of THL containing the characteristic substituted beta-lactone moiety was left unchanged, whereas the *N*-formyl-L-leucine moiety was modified by replacing the formyl group and/or the amino acid side chain. Moreover, the influence of the absolute configuration at the stereogenic centre bearing the formylamino group in THL was also evaluated. All new THL-derivatives were tested for their inhibitory effect on human recombinant DAGLα and their potential selectivity was evaluated against COS-7 cell MAGL and other proteins of the endogenous cannabinoid system (FAAH, CB_1 and CB_2).
All THL-derivatives whose formylamino group was chemically modified or lacking were inactive as DAGLα inhibitors (IC_{50}>25μM). The chemical modification of the pentenoic skeleton allowed, instead, the identification of 3 new THL-derivatives with an IC_{50} against DAGLα in the nM range. In particular, OMDM-188 was a potent DAGLα inhibitor (IC_{50}=16±1nM) and almost inactive on all the examined proteins of the endocannabinoid system. In addition, and surprisingly, we identified OMDM-169 as the first THL-analogue able to target MAGL more efficaciously than DAGLα.. OMDM-169 inhibited MAGL form both the membrane (IC_{50}=176±23nM) and cytosolic (IC_{50}=407±30nM) fractions of COS-7 cells, and its selectivity over DAGLα (IC_{50}=2.55±0.31μM) ranged from 7- to 14-fold. We also investigated the potential anti-hyperalgesic effect of OMDM-169. The compound (1.25-5 mg/kg, i.p.) dose-dependently inhibited the 2nd phase of formalin-induced nocifensive behaviour, this effect being attenuated by pre-treatment with the selective CB_2 and CB_1 receptors antagonists, AM251 and AM630 (1mg/kg, i.p.), respectively. Experiments are ongoing to establish if these effects are accompanied by elevation of cellular or tissue levels of 2-AG.



In conclusion, we have reported here the synthesis of potent THL-analogues able to inhibit 2-AG metabolism with high potential pharmacological and therapeutic utility.

This work is dedicated to the memory of Prof. J. Michael Walker

IDENTIFICATION OF A NOVEL 2-AG-HYDROLYZING ENZYME EXPRESSED BY MICROGLIAL CELLS

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Microglial cells, the macrophages of the brain, play a key role in the control and propagation of neuroinflammation. It is known that microglia produce endocannabinoids and express CB2 receptors, and that 2-AG stimulates microglial cell migration through CB2 receptors. Although endocannabinoid-hydrolyzing enzymes constitute a promising therapeutic target and their inhibition in microglia is likely to control neuroinflammation response and propagation, little is known about their expression in these immune cells. We have previously shown that the microglial cell line BV-2 expresses a novel, uncharacterized 2-AG-hydrolyzing enzyme. Here, in collaboration with the Cravatt laboratory, we performed an activity-dependent protein profiling of the serine hydrolase activities expressed by BV-2 cells and identified three novel 2-AG-hydrolyzing enzymes: ABHD6, ABHD12 and NTE. Using both a pharmacological approach and shRNA knockdown, we show that ABHD6 constitutes a major player in the control of 2-AG hydrolysis and bioactivity in BV-2 cells. These results suggest that ABHD6 constitutes a novel component of the endocannabinoid signaling system and represents a promising target for the control of neuroinflammation.

INVOLVEMENT OF FATTY ACID BINDING PROTEINS IN THE TRANSPORT OF ENDOCANNABINOIDS TO PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS

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Cannabinoids have several potential targets in the cell and the mechanisms by which cannabinoids are transported within cells to their specific site(s) of action remains unclear. Recent data have demonstrated that certain cannabinoids and related compounds such as fatty acid ethanolamides are ligands for the nuclear receptor sub-family of peroxisome proliferator-activated receptors (PPARs). Members of the intracellular fatty acid binding protein family (FABPs) serve as acceptors of fatty acids and related compounds and have been suggested to selectively deliver these to PPARs. Two members of the FABP family, FABP3 and FABP7, are expressed at relatively high levels in the brain, a major site of synthesis and physiological action of cannabinoids.

To assess the binding affinity of cannabinoids for FABPs, the ability of cannabinoid–like molecules to displace a BODIPY 558/568-labelled 12-carbon probe from the ligand binding pocket of either purified FABP3- or FABP7–GST fusion proteins was assessed by fluorescence polarization assay. Binding of the fluorescent probe to FABP3 and 7 was inhibited in a concentration dependent manner by arachidonic acid (AA) (FABP3 IC_{50} ~ 18 μM; FABP7 IC_{50} ~ 8 μM). Anandamide (AEA) (FABP3 IC_{50} ~ 15 μM; FABP7 IC_{50} ~ 5 μM), palmitoylethanolamide (PEA) (FABP3 IC_{50} ~ 23 μM; FABP7 IC_{50} ~ 11 μM) and oleoylethanolamide (OEA) (FABP3 IC_{50} ~ 12 μM; FABP7 IC_{50} ~ 4 μM) showed similar or higher binding affinity for both FABP3 and FABP7 compared to AA, whereas 2-arachidonoylglycerol (2-AG) bound with less potency (FABP3 IC_{50} ~ 4700 μM; FABP7 IC_{50} ~ 1600 μM). In contrast, Δ^9-tetrahydrocannabinol and WIN55212-2 showed no obvious displacement of fluorescent ligand from either FABP. To assess the effects of FABP3 and FABP7 on PPAR-mediated transcriptional activation, CHO cells were transiently transfected with a luciferase reporter construct containing three PPRE (Peroxisome Proliferator Response Element) in combination with FABP3 or 7 and PPARα, β or γ. FABP3 was found to selectively increase (2.2-fold, $P < 0.001$) PPARα-mediated transcriptional activation while FABP7 selectively increased (3.2-fold, $P < 0.001$) the transcriptional activation of PPARγ. No effects of FABP3 or 7 were observed upon the basal transcription activation of PPARβ. Treatment of cells transfected with PPARα and FABP3 with 10μM OEA caused a significant elevation of transcription over and above that obtained with either FABP3 or OEA alone. A similar result was obtained with PPARγ and FABP7 treated with 10μM AEA.
Furthermore, when cells transfected with FABP3- or FABP7-GFP fusions were treated with 20μM OEA or AEA, respectively, there was an increase in the nuclear localization of the fluorescent constructs as determined by confocal microscopy. These studies suggest FABP3 and FABP7 may play important roles in both the transport of endocannabinoids and the resulting activation of signal transduction pathways within the cell.

LATE BREAKING TALK

AN ENDOGENOUS LIPID WITH NOVEL BONE ANABOLIC ACTIVITY IN VIVO

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ENDOCANNABINOID-MEDIATED RETROGRADE SYNAPTIC SIGNALING IN THE HUMAN CEREBRAL CORTEX

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The CB_1 cannabinoid receptor is typically localized on axon terminals and its activation leads to presynaptic inhibition of neurotransmission (Szabo & Schlicker, Handb Exp Pharmacol 168: 318–56, 2005). During the process of retrograde signaling, the presynaptic CB_1 receptor is activated by endogenous cannabinoids (endocannabinoids) synthesized by postsynaptic neurons (Chevaleyre et al., Ann Rev Neurosci 29:37-75, 2006). Although this latter physiological phenomenon has been frequently demonstrated in the brain of rodents, it is not known, whether it also occurs in the human brain. The aim of the present experiments was to study endocannabinoid-mediated retrograde signaling in the human brain.

Cortical tissue mostly removed during tumor and epilepsy surgery was used. 250 µm-thick slices were cut with a vibratom and superfused. The electrical activity of pyramidal neurons was recorded with patch-clamp techniques. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of ionotropic glutamate receptor antagonists; as expected, sIPSCs were blocked by the $GABA_A$ receptor antagonist bicuculline. In order to enhance the activity of cannabinoid-sensitive presynaptic axons, muscarinic acetylcholine receptors were continuously stimulated by carbachol (2.5-5 x 10^{-6} M). Under these conditions, depolarization of postsynaptic pyramidal neurons (by 9 depolarizing pulses at a frequency of 1 Hz [from -70 mV to 0 mV for 100 ms]) led to suppression of GABAergic sIPSCs. This depolarization-induced suppression of inhibition (DSI) was 18 ± 3 % during the 10 s following the depolarization. DSI was abolished by the CB_1 receptor antagonist rimonabant (10^{-6} M), verifying involvement of endocannabinoids and CB_1 receptors. The cannabinoid receptor agonist WIN55212-2 (5 x 10^{-6} M) also inhibited sIPSCs (maximally by 32 ± 5 %). In the presence of WIN55212-2, the depolarization no longer elicited suppression of sIPSCs - an observation compatible with the action of endocannabinoids on CB_1 receptors. Interestingly, DSI was greater in those neurons, in which carbachol elicited a greater than average increase in sIPSCs and in those neurons in which WIN55212-2 (applied after the DSI) elicited a greater than average inhibition of sIPSCs.

This is the first demonstration of endocannabinoid-mediated retrograde synaptic signaling in the human brain. This kind of short-term synaptic plasticity, together with long-term synaptic plasticity, is thought to be important for memory and learning. The interference of exogenous cannabinoid agonists and antagonists with retrograde synaptic signaling in human brain tissue suggests that these compounds will interfere with memory and learning in man.

ENDOGENOUS ACTIVATION OF KAINATE RECEPTORS IS NECESSARY FOR A NOVEL FORM OF ENDOCANNABINOID-MEDIATED INHIBITION OF SYNAPTIC TRANSMISSION

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The endocannabinoid system (ECS) mediates many forms of transient and long-lasting synaptic plasticity in the brain. In the hippocampus, endocannabinoid-mediated inhibition of GABAergic transmission depends mainly on the depolarization of the postsynaptic cell. Here, we show that activity-dependent activation of kainate receptors on GABAergic interneurons is necessary for a novel form of ECS mediated depression of inhibitory transmission. Using whole-cell patch-clamp recordings of mouse hippocampal CA1 pyramidal cells (P15-20), we monitored evoked inhibitory post-synaptic currents (IPSCs) in basal conditions and after stimulation of glutamatergic afferent fibers. A decrease of GABAergic transmission was observed in these conditions in control slices (15.18±1.6%; Min et al., PNAS 1999, 96:9932). This effect (called KDi, for Kainate-dependent Depression of inhibitory transmission) was absent in mutant mice lacking the GluR5 subunit of kainate receptors (KARs, 1.2±1%), which is expressed exclusively in GABAergic interneurons at this age. Post-synaptic activation of group I metabotropic glutamate receptors (mGluRs) and intracellular Ca^{2+} rise were also necessary steps of KDi. Consistently, this form of short-term plasticity was fully blocked by bath applications of the CB1 receptor antagonist SR141716A (SR, 5 μM) in WT slices, and was absent in slices from constitutive and GABAergic-specific conditional CB1-KO mice. Interestingly, KDi was fully restored upon elevated levels of K^+ in GluR5 KO mice (-11.4 ± 4), suggesting that GluR5-containing KARs mediate depolarization of GABAergic interneurons in our conditions. Ongoing double patch clamp experiments on identified interconnected pairs of GABAergic interneurons and pyramidal cells seem to confirm that endogenous release of glutamate induces a KA-dependent depolarization of GABAergic interneurons. These observations strongly suggest that glutamate release has a double effect on GABAergic transmission: First, by direct activation of GluR5-containing KARs, it depolarizes GABAergic neurons; Second, through activation of post-synaptic mGluRs, it stimulates retrograde endocannabinoid-dependent inhibition of GABA release to induce KDi.
These data show that endogenous glutamatergic activity on both GABAergic and glutamatergic neurons regulates ECS-dependent synaptic plasticity.

ENDOCANNABINOIDS FINE TUNE SLOW OSCILLATORY ACTIVITY OF VENTRAL TEGMENTAL AREA DOPAMINE NEURONS

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Endogenous cannabinoids (eCBs) play a role in the regulation of neuronal circuitry dynamics underpinning high-order brain functions, including reward, motivation, learning, and memory. Among the neural structures involved in this complex neuronal network, ventral tegmental area (VTA) dopamine (DA) neurons are particularly susceptible to eCBs influence.
Short and long term eCBs actions finely shape DA synaptic inputs to generate pattern of electrical activity that control DA release and functional effects on target cells. Along with single spiking and bursting pattern, DA cells exhibit robust oscillatory activities characterized by repetition of clusters of action potentials at low frequencies (slow oscillation, SO 0.5-1.5 Hz).
Administration of the exogenous CB1 agonist WIN 55212-2 in anesthetized rats enhances DA neuronal activity and SO. To ascertain the physiological relevance of CB1-mediated increase of SO in DA neurons, eCBs levels were enhanced by the administration of URB597 (URB), a selective inhibitor of the enzyme fatty-acid amide hydrolase. DA neurons recorded from URB pre-treated rats (0.1 mg/kg i.v.) showed slightly, but not significant, differences in electrophysiological parameters such as frequency and percent of burst firing. On the other hand, spectral analysis revealed an enhanced expression of SO activity in URB pre-treated rats *versus* naïve rats (URB $P_{0,5\text{-}1,5}$ 0.5 ±0.09 n=15; controls $P_{0,5\text{-}1,5}$ 0.5 ±0.09 n=15 P<0.001 Anova and Tukey *post hoc* test). The CB1 receptor antagonist SR141716A (0.5 mg/kg i.v.), ineffective *per se*, was able to prevent URB-induced actions (URB $P_{0,5\text{-}1,5}$ 0.5 ±0.09 n=15; SR $P_{0,5\text{-}1,5}$ 0.3 ±0.06 n=9 P<0.001 Anova and Tukey *post hoc* test 0.3). These results point towards an involvement of eCB and CB1 receptors in SO of VTA DA neurons.
Recent experimental evidence ascribes to prefrontal cortex (PFC) a pivotal role in DA cells SO. In particular, oscillation of DA neurons reflects excitatory and inhibitory PFC-VTA pathway activity, providing both temporal and spatial codes relevant in information processing. Tuning of DA neuron SO by the eCBs system may bear relevance in several neuropsychiatric disorders (such as schizophrenia and ADHD) in which dysfunctions of cortical and sub-cortical DA transmission are characterized by alterations in oscillatory activity.

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MUSCARINIC-CANNABINOID SUPPRESSION OF EXCITATION

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Marijuana and hashish are major drugs of abuse. Δ^9-THC, their chief psychoactive ingredient, acts in the brain via cannabinoid CB1 receptors. These receptors have been implicated in several forms of synaptic plasticity – depolarization-induced suppression of excitation (DSE) and metabotropic suppression of excitation (MSE) as well as activation-dependent desensitization. When cultured as solitary neurons on a limited substrate, hippocampal neurons form synapses (or autapses) onto themselves. These 'autaptic' hippocampal neurons express all of the above cannabinoid-related forms of plasticity, indicating that CB1 exhibits considerable functional and temporal heterogeneity at a single set of synapses and making them an accessible model for fine dissection of the underpinnings of cannabinoid signaling.

Here we report that coincident activation of muscarinic acetylcholine receptors with oxotremorine-M (oxo-M) and CB1-dependent DSE results in a substantial presynaptic inhibition of excitatory transmission (40-45%) lasting approximately 10 minutes. The induction is blocked by CB1 antagonist SR141716 and muscarinic M3/M5 receptor antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) and is absent in CB1 receptor knockout cultures. Notably, after induction, this inhibition is reversed by SR141716, but not by non-specific muscarinic antagonist atropine, indicating that the inhibition is expressed via activation of CB1 receptors. Consistent with this, analysis of paired pulse ratios of excitatory postsynaptic currents and of spontaneous miniature currents indicates a presynaptic site of expression. This inhibition is mimicked by coapplication of candidate endocannabinoid 2-AG and oxo-M indicating that depolarization is not required for induction. We refer to this inhibition as muscarinic cannabinoid suppression of excitation (MCSE).

MCSE is not mimicked by co-activation of M3/M5 receptors with other presynaptic receptors, e.g. $G_{i/o}$ GABAB or adenosine A1 receptors, indicating a degree of specificity in MCSE.

MCSE may represent a novel form of coincidence detection resulting in a medium-term depression of synaptic signaling. Coincidence detection is important for learning processes. If MCSE is found to occur outside the autaptic preparation, it may have major implications for the study of cannabinoids, addiction, learning, and neuronal signaling in general.

VOLUNTARY EXERCISE PROMOTES HIPPOCAMPAL NEURAL PROGENITOR CELL PROLIFERATION THROUGH AN ENHANCEMENT OF ENDOGENOUS CANNABINOID SIGNALING

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Voluntary exercise and endogenous cannabinoid activity have independently been shown to regulate hippocampal plasticity in a common fashion. The aim of the current study was to determine if the endocannabinoid system is regulated by voluntary exercise, and in turn, if these changes contribute to the exercise-induced enhancement of cytogenesis in the hippocampus. In the initial experiment, we examined the effects of eight days of free access to a running wheel on the binding and signaling parameters of the CB_1 receptor and tissue content of the endocannabinoid ligands anandamide and 2-AG in the hippocampus and prefrontal cortex. It was found that engagement in voluntary exercise increased the maximal binding capacity of the cannabinoid CB_1 receptor, CB_1 receptor-mediated GTPγS binding and the tissue content of the endocannabinoid anandamide (and to some degree 2-AG) in the hippocampus but not in the prefrontal cortex. In the subsequent experiment, the cannabinoid CB_1 receptor antagonist AM251 (1 mg/kg) was administered daily to animals given free access to a running wheel for 8 days, after which cell proliferation in the hippocampus was examined through immunohistochemical analysis of the cell cycle protein Ki-67. Voluntary exercise increased proliferation of progenitor cells, as evidenced by the increase in Ki-67 positive cells in the granule cell layer of the dentate gyrus in the hippocampus. However, this effect was abrogated by concurrent treatment with AM251, indicating that the increase in endocannabinoid signaling in the hippocampus is driving the exercise-induced increase in cell proliferation. These data demonstrate that the endocannabinoid system in the hippocampus is sensitive to environmental change and is a critical mediator of experience-induced plasticity.

MITOCHONDRIA ARE NOVEL TARGETS FOR CANNABIDIOL'S NEUROPROTECTIVE ACTION

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Cannabinoids and the endocannabinoid system have attracted considerable interest for therapeutic applications such as analgesia. Nevertheless, the mechanism of action of one of the main non-psychoactive phytocannabinoids, cannabidiol (CBD), has so far remained elusive. This is despite its well recognised, and potentially beneficial, properties as an anti-convulsant and neuroprotectant. Here, we characterised the mechanisms by which CBD regulates Ca^{2+} homeostasis and mediates neuroprotection in neuronal preparations.

In human neuroblastoma preparations (SH-SY5Y) treated with mitochondrion-acting toxins (using the cell viability marker, Alamar Blue), CBD (0.1 & 1 μM) was neuroprotective against the uncouplers FCCP (53% protection) and tetraphenylphosphonium (TPP^+; 64%), and modestly protective against hydrogen peroxide- (16%) and oligomycin- (15%) mediated cell death. The psychoactive phytocannabinoid, THC, was also shown to be a potent neuroprotectant (42-50% protection) in a CB_1 receptor independent manner.

Studies in hippocampal cultures using Fura-2 AM Ca^{2+} imaging suggested that CBD-mediated Ca^{2+} regulation is bi-directional, depending on the excitability of cells. Under physiological K^+/Ca^{2+} levels, CBD caused a subtle rise in $[Ca^{2+}]_i$, whereas elevated levels of extracellular K^+, or exposure to the K^+ channel antagonist 4AP, caused CBD to reduce $[Ca^{2+}]_i$ and prevent seizure-like activity. Regulation of $[Ca^{2+}]_i$ was not primarily mediated by interactions with ryanodine or IP_3 receptors of the endoplasmic reticulum, established by co-application of the antagonists dantrolene and 2-APB. Instead, dual-imaging experiments with a cytosolic (Fura-2 AM) and a mitochondrial (Rhodamine-FF) fluorophore suggested that mitochondria act as the compartment for Ca^{2+} regulation, with mitochondrial Ca^{2+} responses preceding cytosolic changes. Additionally, application of FCCP and the mitochondrial Na^+/Ca^{2+} exchange inhibitor, CGP 37157, but not the mitochondrial permeability transition pore inhibitor cyclosporin A, abolished subsequent CBD responses.

Thus, it appears that CBD has the capacity to affect cellular Ca^{2+} homeostasis bi-directionally dependent upon baseline excitability. Under pathological conditions involving mitochondrial dysfunction and neuronal Ca^{2+} dysregulation, CBD may prove beneficial in preventing apoptotic signalling, via a restoration of Ca^{2+} homeostasis.

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INHIBITION OF SPASTICITY USING PERIPHERALIZED CB_1 RECEPTOR AGONISTS

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Cannabis contains hydrophobic compounds that rapidly penetrate the central nervous system (CNS). Thus, it has no means of avoiding stimulation of CB_1 receptors (CB_1R) in the cognitive centres of the brain that mediate psychoactive effects, whilst activating CB_1R in the motor control circuits within: the brain, spinal cord and peripheral neuromuscular compartment that may limit spasticity or other symptoms of multiple sclerosis. Exclusion of CB_1R agonists from the CNS can be achieved by utilizing properties of the blood:brain barrier. Thus one can target CB_1R within the periphery by generating hydrophilic compounds or by targeting hydrophobic compounds to CNS-exclusion pumps within the blood:brain barrier. We have identified compounds such as naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl) methanone (Dziadulewicz EK. et al. 2007 J Med Chem. 50:3851) and 1′,1′-dimethylheptyl-Δ^8-tetrahydro-cannabinol -11-oic acid (CT3, ajulemic acid Burstein SH et al. 2004. Life Sci. 75:1513), which fit these profiles. We have shown that they can inhibit spasticity in experimental allergic encephalomyelitis, which is a model of multiple sclerosis. These were active after intravenous and oral administration at doses that did not induce adverse cannabimimetic effects, unless CNS-exclusion pumps were inhibited by pretreatment with cyclosporin A. Although low doses of CT3 have been reported to exhibit cannabimimetic effects in mice (Vann RE et al. 2007. J Pharmacol Exp Ther. 320:678), these are probably explained by the finding that some ICR/CD-1 (outbred) mice have a non-functional CT3-CNS-exclusion pump, which is active in inbred mouse strains. Although high doses of compounds reached the CNS, therapy was evident using between 10-400 fold lower doses. Exclusion of CB_1R agonists from the brain centres controlling unwanted cannabimimetic effects may increase the therapeutic window and harness the benefit that the cannabinoid system has to offer, whilst limiting unwanted side-effects.

HISTOPATHOLOGICAL STUDY OF CANNABINOID RECEPTORS EXPRESSION IN HUMAN MULTIPLE SCLEROSIS

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A number of *in vitro* and *in vivo* studies performed in animals suggest that cannabinoids, via their binding to CB1 and/or CB2 receptors, have some neuroprotective effects that might be exploited in the treatment of neurodegenerative diseases, including multiple sclerosis (MS). In this regard, our group has been studying the clinical applications of cannabinoids in MS for a number of years, our current hypothesis being that cannabinoids may prevent disease progression in addition to simply relieve symptoms. While a number of possible mechanisms have been explored using animal models of MS, few data have been published using human tissue. We thus examined the distribution of cannabinoid receptors (CB1 and CB2) in different pathological stages of MS using paraffin-embedded brain sections (from MS patients and control individuals) and detection of the cannabinoid receptors and various markers of CNS cells by immunohistochemistry and immunofluorescence.

MS lesions were classified using the BÖ/Trapp staging system into active, chronic active and chronic inactive plaques. Both white matter and grey matter plaques (including shadow plaques) were examined in addition to normal appearing white matter (NAWM). Our results confirmed that CB1 was expressed in neurons, macrophages, oligodendrocytes and T-lymphocytes; CB2 was expressed in neurons, microglia, macrophages, astrocytes and T-lymphocytes. In contrast to some previous reports, CB1 was expressed in reactive/inactive astrocytes and injured axons. Quite unexpectedly, CB1 and CB2 immunoreactivity could be detected in cerebral blood vessels (endothelial cells and smooth muscle cells) in both MS patients and control individuals. In MS group, 14 out of total 15 cases (93.3%) showed CB2 immunoreactivity on blood vessels, mainly in chronic inactive plaques; in contrast, only 2 patients (13.3%) showed CB1 expression on blood vessels. In the control group (16 cases in total), 11 cases (68.8%) showed CB2 expression on blood vessels, while CB1 was expressed on blood vessels in 6 cases (37.5%). We also noted that CB2 was expressed on astrocytes and adjacent blood vessels in NAWM.

Further work needs to be performed, particularly regarding the level of CB2 expression in blood vessels and its possible role in modulating the blood-brain barrier; and reduced neuroinflammation via endothelial CB2 receptors, which might in turn influence MS disease course.

EARLY AND LATE CHANGES IN CB_1 AND CB_2 RECEPTORS IN THE BASAL GANGLIA OF MICE WITH DELETION OR MUTATION OF SPECIFIC *PARK* GENES

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There is evidence that losses and/or malfunctioning of the cannabinoid signaling system, in particular at the level of CB_1 receptors, may be an early event in the pathogenesis of different chronic neurodegenerative disorders. These losses might trigger excitotoxicity, calcium influx, oxidative stress or other cytotoxic events that are normally under the control of CB_1 receptors. By contrast, induction/up-regulation of CB_2 receptors seems to be a later event possibly linked to the occurrence of astrocytosis and microgliosis that are triggered as a consequence of brain injury. We are presently studying both phenomena, the early loss of CB_1 receptors and the late up-regulation of CB_2 receptors, in the basal ganglia in different mouse models of parkinsonism generated by deletion or mutation of specific genes associated with Parkinson's disease in humans [*PARK-1* (α-synuclein), *PARK-2* (parkin) or *PARK-6* (PINK-1)]. Our data reveal a biphasic response for CB_1 receptors, with losses at early ages in these mice, when dopaminergic dysfunction rather than neuronal death is the major event that takes place, followed by up-regulatory responses at the late ages characterized by a profound nigrostriatal pathology. At these late ages, it is possible that CB_2 receptors may be induced in glial cells and represent, together with CB_1 receptors, a promising target for the control of disease progression.

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IN VIVO PET BRAIN IMAGING OF THE TYPE 1 CANNABINOID RECEPTOR IN PARKINSON'S DISEASE

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Introduction : The endocannabinoid system (ECS) has been implied in the pathogenesis of PD and/or LID. Ex vivo studies in animal models of PD have yielded conflicting data. We have characterized the availability of the type 1 cannabinoid (CB1) receptor *in vivo* in early and advanced Parkinson's disease (PD) patients with and without levodopa-induced dyskinesia (LID) using PET imaging and the high-affinity highly selective radioligand [^{18}F]-MK9470.

Methods: 29 PD patients (20M/9F) in different stages of disease severity were included : 9 medication-free patients with early PD (Hoehn-Yahr stage I; disease duration (dd) 2.1±1.4y; age 56.8±14.0y), 10 with advanced PD without LID (LATE-D; age 71.2±5.1y; dd 11.2±3.5y) and 10 with advanced PD and LID (LATE+D; age 61.9±7.6y; dd 12.2±4.3y), as well as twelve healthy controls (CON; age 57.5±10.8; 6M/6F). All underwent dynamic PET scanning with 310±56 MBq [^{18}F]-MK9470. Parametric standardized uptake value (SUV) images reflecting global receptor availability were calculated and corrected for partial volume effects. For regional analysis, SUV values were normalized on the individual total grey matter SUV. Statistical parametric mapping (p_{height} <0.001 uncorrected) and subcortical volume-of-interest (based on individual T1 MPRAGE MRI) statistical analysis were performed.

Results: Compared to CON, early PD patients showed no differences in global SUV values.; decreases were found in both LATE+D and LATE-D groups in the neocortex and cerebellum (mean SUV decrease = -23 %, p<0.03), but not in subcortical areas.
As for regional changes compared to CON, the early PD group showed relative increases in motor and mesolimbic dopaminergic projection areas (putamen, nucleus accumbens and anterior cingulate; all p<0.001). In LATE-D and LATE+D, similar changes were found at more stringent significance. In LATE-D but not in LATE+D, significant increases were found in the premotor cortex (p<0.001). Direct regional comparison of late-stage patients showed decreased relative CB1 receptor availability in the anterior putamen and premotor cortex in the LATE+D group (p<0.005).

Conclusion: This study provides the first *in vivo* imaging evidence for changes in the ECS in PD patients with and without L-DOPA induced dyskinesias. The relationship of these changes to the underlying pathophysiological neurodegenerative processes in the dopaminergic pathways and duration of drug therapy needs further imaging investigations.

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CEREBRAL AND EXTRACEREBRAL BENEFITS OF CANNABIDIOL IN A PIGLET MODEL OF NEWBORN HYPOXIC-ISCHAEMIC ENCEPHALOPATHY

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Aims: to explore neuroprotective and possible adverse effects of the non-psychoactive plant cannabinoid, cannabidiol (CBD), and to compare CBD with a CBD + Δ^9-tetrahydrocannabinol mixture (Sativex^R, SVX) in an animal model of newborn hypoxic-ischaemic encephalopathy.

Methods: 3 to 5-day-old piglets underwent hypoxia-ischaemia (HI: temporary occlusion of both carotid arteries + hypoxia). At 15 and 240 min post-insult, they received iv CBD at 0.1 mg/kg (HI+CBD, n=8), SVX at 0.1 mg/kg (HI+SVX, n=4) or vehicle (HI+VEH, n=8). Non-HI sham-operated piglets served as additional controls (SHAM, n=3). Brain damage was studied both by near-infrared spectroscopy (NIRS) and amplitude-integrated EEG (aEEG) monitoring during the experiment (6 h) and by histological assessment (Nissl and FluoroJadeB staining) at the end. Some piglets were allowed to recover 1 h post-insult to allow performance of a neuroconduction assay until 72 h post-insult.

Results: in HI+VEH total haemoglobin index (THI) increased and fractional tissue oxygen extraction (FTOE) decreased, suggesting severe cerebral haemodynamic and metabolic impairment; in HI+CBD THI and FTOE were similar to SHAM. In HI+VEH EEG showed a sustained and profound decrease in amplitude, indicating cerebral hypofunction; and seizures were observed in all piglets. In HI+CBD EEG amplitude recovered to 46.4±7.8% baseline and seizures appeared only in 4/8 piglets (both $p<0.05$). EEG impedance increased in HI+VEH, indicating brain oedema; this increase was blunted by CBD. The number of viable neurons decreased and that of degenerating neurons increased in HI+VEH; CBD reduced both effects by more than 50%. Preliminary data pointed to a normalization of neuroconduction from 24 h postinsult in HI+CBD. CBD administration was free from adverse effects; it was, moreover, associated with systemic beneficial effects: blunting of cardiac troponine increase, modulation of hypotension, preservation of lung compliance, and reduction of oxygenation index. SVX did not provide more benefits than pure CBD in NIRS or aEEG studies, but did induce an increase in cerebral blood flow and volume, as well as a decrease in blood pressure over the first 3 h post-insult.

Conclusion: administration of CBD alone after HI reduced brain damage and was associated with extracerebral benefits. SVX did not provide any additional cerebral benefits but, unlike pure CBD, was associated with some potentially undesirable vascular effects.

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$5HT_{1A}$-RECEPTORS ARE INVOLVED IN THE ATTENUATION OF BEHAVIORAL RESPONSE TO RESTRAINT STRESS INDUCED BY CANNABIDIOL (CBD) IN RATS

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Introduction: CBD is a non-psychotomimetic compound from *Cannabis sativa* related to anxiolytic- and antipsychotic-like effects in rodents. Although the mechanisms of CBD effects are not completely understood, it is proposed to facilitate the endocannabinoid system and to activate serotonin 5-HT1A receptors. Since either facilitation of endocannabinoid-mediated neurotransmission or 5-HT1A receptor activation may promote adaptation to stress, the aim of this work was to test the hypothesis that CBD would attenuate behavioral consequences of restraint stress (RS). We also investigated if these responses depend on activation of 5-HT1A receptors.

Methods: Male Wistar rats received an ip injection of vehicle or CBD (1, 10 or 20 mg/kg) and, 30 min after, they were submitted to 60 min restraint. 24h later, animals were tested on the elevated plus-maze (EPM), an animal model to detect anxiogenic and anxiolytic-like effects. In a second experiment, we test the effect of an antagonist of 5HT1A receptors (WAY100635) on CBD-induced effects.

Results: RS induce anxiogenic-like behavior in the EPM that where reduced by CBD (% open arm entries: $F_{3,25}=7.72$, $p<0.001$). Pretreatment with WAY blocked CBD effects on % open arms (post-hoc analysis, $p>0.05$ compared to controls).

Conclusion: Our results corroborate the hypothesis that CBD is able to attenuate behavioral consequences of restraint stress. Moreover, it can be suggested that CBD effects are mediated by the activation of 5-HT1A receptors.

DIRECT AND INDIRECT MODULATION OF CANNABINOID SYSTEM IN A PHENCYCLIDINE MODEL OF SCHIZOPHRENIA

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Recent advances in the neurobiology of cannabinoids have renewed interest in the association between cannabis and schizophrenia. Our studies suggested that chronic phencyclidine (PCP) treatment, a model of cognitive and negative symptoms of schizophrenia, altered cannabinoid system in term of CB1 receptor functionality, FAAH and endocannabinoid levels mainly in the prefrontal cortex. In fact, in this region we observed a significant reduction in GTPγS binding (-41%) accompanied by an increase in 2AG levels (+39%) with a trend to decrease in anandamide levels (-27%). Moreover, we demonstrated that prolonged cannabis use worsened behavioural and biochemical effects induced by PCP suggesting that endocannabinoid-based therapeutic interventions could represent a useful framework for schizophrenic disorders.
On this basis, in the present study we first evaluated behavioural and biochemical responses induced by chronic PCP exposure by modulating endocannabic tone using direct (CB1 receptor antagonist AM251) and indirect (anandamide uptake inhibitor AM404) cannabinoid compounds.
Chronic AM251 co-treatment improved the PCP-altered recognition memory as presented by a significant increase in the recognition index respect to PCP treated rats. This compound was able to counteract the reduction in CB1 receptor functionality produced by PCP in the prefrontal cortex.
To better understand the role of endocannabinoid ligands and the specific contribute of these compounds in the PCP behavioural aspects, we studied the effects of indirect cannabinoid compounds in different symptoms of schizophrenia in rats. We co-treated rats with chronic PCP and AM404, the prototypical anandamide uptake inhibitor, in order to evaluate the responses induced by an increased endogenous cannabinoid tone.
Chronic AM404 was able to modulate cognitive deficit induced by PCP.
In parallel, we evaluated the effects of acute administration of AM251 and AM404 on hyperlocomotion and stereotypies produced by acute PCP administration. These cannabinoid modulators differently affected PCP-induced positive-like symptoms.
Taken together these results suggest that pharmacological modulation of the cannabinoid system can represent a new perspective in the treatment of schizophrenia.

HETEROZYGOUS NEUREGULIN 1 MICE DISPLAY ALTERED NEUROBEHAVIOURAL RESPONSES TO REPEATED CANNABINOID EXPOSURE

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Cannabis use may increase the risk of developing schizophrenia by precipitating the disorder in genetically vulnerable individuals. Neuregulin 1 (*NRG1*) is a schizophrenia susceptibility gene and mutant mice heterozygous for this gene (*Nrg1* HET mice) exhibit a schizophrenia-relevant phenotype. We have recently shown that *Nrg1* HET mice are more sensitive to the acute neurobehavioural effects of Δ^9-tetrahydrocannabinol (THC) - THC selectively enhanced c-Fos expression in the ventrolateral septum (LSV) and prepulse inhibition (PPI) in *Nrg1* HET mice with no such effects being observed in their wild type-like (WT) littermates. As chronic exposure to high doses of cannabinoids is more reliably associated with precipitating psychotic reactions, the present study examined the effects of repeated administration (15 days) of CP 55,940 (400 μg/kg i.p.) on *Nrg1* HET and WT mice. *Nrg1* HET mice showed accelerated tolerance to the locomotor suppressant and hypothermic actions of CP 55,940 compared to WT mice. In the light-dark emergence test, similar anxiogenic effects of CP 55,940 were observed in both genotypes on the first day of exposure. However, while WT mice became tolerant to these effects with repeated injections of CP 55,940, *Nrg1* HET maintained their avoidance of the light zone up to day 15 of exposure. In the PPI model, CP 55,940 differentially affected *Nrg1* HET and WT mice on day 1, but both genotypes were completely tolerant to the effects of the cannabinoid by day 7. We also examined the neuronal correlates of chronic CP 55,940 exposure by measuring FosB expression using immunohistochemistry. Following 15 days of CP 55,940 injections, *Nrg1* HET mice showed a significant increase in FosB expression in the LSV compared to vehicle controls, which was not observed in WT mice. Taken together, these data reinforce the existence of cannabinoid-neuregulin 1 interactions in the CNS. This research may enhance our understanding of how genetic factors increase individual vulnerability to schizophrenia and cannabis-induced psychosis.

ALTERATION OF CANNABINOID SIGNALING BY ANTIPSYCHOTICS

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Previous research has suggested that there is an interaction between antipsychotic compounds and the cannabinoid system. Firstly, (Sundram et al, 2005 and Andersson et al, 2005) have shown that rodents that were chronically treated with the typical antipsychotic haloperidol or the atypical antipsychotic clozapine showed a alteration in cannabinoid CB1 receptor levels. Another study showed that administration of the dopamine antagonist eticlopride, significant increased the level of 2-AG in the limbic forebrain (Patel et al, 2003). Additionally, (Marchese et al, 2003) showed that when doses of haloperidol and Δ^9-THC that produced no tetrad effects by themselves were administered together, they produced significant tetrad effects.

Despite these converging lines of research, as yet, there is no direct explanation for this interaction. To this end, we assessed the effects of haloperidol and clozapine alone and in combination with 2-AG and/or the MAGL inhibitor N-arachidonyl maleimide (NAM) in behavioral tests (including the THC tetrad) as well tests of CB1 receptor mediated G-protein activation (including endocannabinoid mediated [^{35}S]GTPγS binding assay).

Our initial findings show that neither haloperidol nor clozapine stimulated [^{35}S]GTPγS binding, and they to not bind to CB1 receptors at concentrations up to 30 μM. However, when 2-AG concentration-effect curves were generated in the presence or absence of haloperidol (1 μM) or clozapine (10 μM), the E_{max} value for 2-AG-stimulated [^{35}S]GTPγS binding was increased by approximately 30 % in the presence of either antipsychotic, in both cerebellar and striatal membrane homogenates. Interestingly, our initial behavioral work with the MGL inhibitor NAM and haloperidol showed that the combination of NAM and haloperidol produced significant tetrad effects at a haloperidol dose of 0.03 mg/kg, a dose that did not produce significant locomotor suppression or catalepsy by itself.

These results suggest that both antipsychotics may enhance endocannabinoid mediated G-protein activation, which may contribute to either the therapeutic effect of these compounds or may in part be responsible for the side effect profile (especially with haloperidol). Future work will focus on examining whether this biochemical effect has a behavioral correlate, as well as examining whether this effect is mediated through a receptor distinct from CB1.

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ADULT FEMALE RATS PRE-EXPOSED TO THC IN ADOLESCENCE AS A MODEL TO TEST CANNABINOID SYSTEM INVOLVEMENT IN DEPRESSION

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We recently demonstrated that adult female rats exposed during adolescence to the psychoactive ingredient of marijuana Δ^9-tetrahydrocannabinol (THC), display a depressive-like behaviour measured in the forced swim test. Moreover, in the sucrose preference test these rats showed a significant reduction in preference, that might be interpreted as anhedonia, a loss of interest in rewarding activities seen in many people with depression. At the biochemical level, these animals have significant reduction in CB1 receptor density and functionality in brain areas belonging to a circuit involved in emotional processing and reward (ventral tegmental area, nucleus accumbens and amygdala). These results prompted us to speculate that activation of CB1 receptors by endocannabinoids in these brain areas is fundamental for normal emotional behaviour and stress response, and that the adolescent period represents a time window where the endocannabinoid system is specially vulnerable to exogenous modulation.

To test these hypotheses, adult female rats were subjected to the same paradigm of THC treatment as adolescents, that is with increasing doses of THC for 11 days and left undisturbed for thirty days. They were then tested in the forced swim and sucrose preference assays, and showed no significant difference from control rats in these behavioural parameters. At the biochemical level, no significant reduction in CB1 receptor density and functionality were evident in the ventral tegmental area, nucleus accumbens and amygdala, as conversely observed in adolescent-treated rats. These results suggest that adult female rats are less sensitive to chronic THC exposure than adolescent ones, and strengthen the hypothesis that decreased CB1 receptor functionality in the ventral tegmental area, nucleus accumbens and amygdala play a fundamental role in triggering depressive-like behavior.

Finally, to further test this, adult female rats pre-exposed to THC in adolescence were injected with URB597 (1mg/kg ip) and then tested in the forced swim procedure. These animals spent more time in active behaviours (swimming) and less time immobile than rats treated with vehicle. This result suggests that the increase in the endocannabinoid tone produced by URB597 was able to reduce the depressive-like behaviour, thus confirming the importance of the endocannabinoid system in the regulation of emotionality.

CB_1 RECEPTOR KNOCKOUT AFFECTS BRAIN ADAPTATIONS TO STRESS AND AGING

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CB_1 cannabinoid receptors in the brain have been implicated in the response to stress. One approach to determine the association between the endocannabinoid system and CNS mechanisms is to investigate phenotypic differences between transgenic CB_1(-/-) (KO) versus C57Bl/6 wild-type (WT) mice. In order to determine the response to stress, mice were subjected to a chronic unpredictable stress (CUS) paradigm, which consisted of random exposure to 2-3 stressors per day over a 2 week period. The influence of CUS on the ability of the mice to acquire a Conditioned Place Preference (CPP) to cocaine was assessed over the next six days. On alternating days, mice were injected with saline or cocaine (10.0 mg/kg) paired with placement in one of the conditioning compartments. When mice were allowed access to all compartments, CPP had been acquired in non-stressed or stressed WT mice (49 ±11% , 27 ±9% increase in time in the cocaine-paired compartment, respectively), but not CB_1KO mice (3.8 ±7% increase). However, CB_1KO mice that had been exposed to CUS displayed a preference for the cocaine-paired compartment (52.4 ±19% increase). In the stressed compared with unstressed mice, striatal gene expression of pre-prodynorphin was greater, and D4 dopamine receptor was lower, in both WT and CB_1KO, indicative of stress-associated differences unrelated to CB_1 receptor ablation. However, in the stressed mice, pre-proenkephalin gene expression was significantly greater in the WT than in the CB_1KO mice. It is hypothesized that increased enkephalin neuronal activity serves as a compensatory mechanism in achieving homeostasis in WT mice, whereas the failure to compensate with an increased enkephalin in CB_1KO may be a factor in the apparent increased reinforcement value of substances of abuse. Examination of hippocampal gene expression revealed a trend toward decreased neuronal growth factors BDNF and NT3 in young adult CB_1KO compared with WT mice. Because expression of these neuronal growth factors is further reduced in old adult (WT as well as CB_1KO) mice, the CB_1KO phenotype appears to be consistent with accelerated depletion of neuronal growth factors. These data suggest that CB_1 receptors in can impact multiple brain coping mechanisms.

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CHANGES IN HIPPOCAMPAL MORPHOLOGY AND SYNAPTIC PLASTICITY FOLLOWING CHRONIC Δ9-THC TREATMENT IN ADOLESCENCE ARE ASSOCIATED WITH COGNITIVE IMPAIRMENT IN ADULTHOOD

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Marijuana and hashish, psychoactive products of the hemp plant, are the illicit drugs most frequently used by human adolescents. The peri-adolescent period represents a critical phase for cerebral development that is characterized by strong neuronal plasticity, with sprouting and pruning of synapses, changes in dendritic spine density, maturation and rearrangement of major neurotransmitter pathways.

This remodeling process is thought to support the emerging adult cognitive style. Since the endocannabinoid system plays an important role during the brain development exposure to cannabinoids during this developmental phase may conceivably lead to subtle but lasting changes in the brain and behavior.

Literature regarding long-term consequences of adolescent cannabinoid exposure on cognitive behavior is scarce and not always in accordance: the current study therefore aimed to assess whether an experimental model of adolescent chronic exposure to ^{9}-THC, may induce lasting effects on learning and memory in male rats. The behavioral aspects was completed with neurochemical and morphological analysis aimed to show the presence of alterations in synaptic plasticity.

Adolescent male rats have been treated with THC or its vehicle from 35 to 45 postnatal days (PND). Once animals reached the adult age (75 PND) the following tests have been performed: the 8 arm radial maze task was used to investigate specific aspects of spatial memory. Passive avoidance test was then performed to evaluate emotional learning. No alteration was found in emotional memory, but in the 8 arm radial maze vehicle group exhibited a greater performance, in comparison with THC-pre-treated animals, suggesting a deficit in spatial memory.

To correlate memory impairment to altered neuroplasticity, level of proteins mainly involved in synaptic plasticity was investigated in the most relevant areas for learning and memory, hippocampus and prefrontal cortex. We found significant alteration in pre- and post- synaptic proteins expression (↓23%VAMP2, ↓41%PSD95) and in the astroglial marker GFAP (↓21%) in the hippocampus of pretreated rats, indicating a possible alteration in the mechanisms underlying synaptic plasticity.

Finally, using the Golgi-Cox staining we performed a detailed morphological analysis of dendritic structure in the dentate gyrus of the hippocampus, that is thought to be part of a circuit essential for the establishment of memory. Male pretreated rats had a significantly lower total dendritic length than vehicle group. This difference was accompanied by parallel differences in total dendritic number with THC pretreated rats having less dendrites than controls. Spine density analysis showed that THC induced a decrease in spine density both on the proximal and distal dendritic tree.

The biochemical results suggest that THC pretreated rats may establish less synaptic contacts and/or less efficient synaptic connections throughout the hippocampus. Taken together these data suggest that cannabis consumption during adolescence may induce long-term effects ending in alteration of learning, memory and synaptic plasticity.

CHRONIC MARIJUANA USERS SHOW ALTERED NEURAL PROCESSING DURING DECISION MAKING AND FEEDBACK

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Chronic marijuana (MJ) users have an inability to develop and implement successful decision making strategies while performing the Iowa Gambling Task (IGT; Whitlow et al., 2004). We previously used block design fMRI to characterize differences in overall brain activity between MJ users and controls while performing the task. The IGT, however, is a complex task and a block design did not allow us to examine activity specific to various phases of the task. The purpose of this study was to use event-related fMRI to isolate neural responses to specific portions of the IGT (e.g. decision making and feedback) that may contribute the observed deficit in MJ Users.

Demographically matched controls (12) and MJ users (14) performed three runs of the IGT each consisting of 135 gambling trials. Each trial contained two distinct events, a decision making event and a feedback event. Using standard linear regression techniques, activity associated with decision making and feedback events was isolated for both controls and MJ users and compared to control events. Furthermore, activity for both events was directly compared between controls and MJ users.

During decision making, both controls and MJ users used a similar network of activity including the thalamus, cingulate, striatum and insula (BA 14). The spatial distribution of activity differed between groups however, with controls relying more on the anterior cingulate (ACC, BA 24) and striatum while MJ users relied on activity in more posterior cingulate and insula. Comparing activity for decision making between controls and MJ users revealed that controls had significantly greater thalamic, striatal and ACC activity than MJ users while MJ users had greater activity in the posterior cingulate and prefrontal cortex (PFC). During feedback both controls and MJ users had increased activity in the thalamus, ACC, striatum, and PFC. Directly comparing activity between controls and MJ users, however, showed that activity in controls was much greater than that of MJ users.

These data suggest that the inability to develop and implement successful decision making strategies in MJ users involves inadequate neural responses to, and processing of, the decision making and feedback portions of the IGT. That MJ users show less activity than controls to the feedback portion of the task suggests that MJ users are less responsive to this portion of the task. Results will be discussed with respect to aspects of emotional processing in MJ users.

PSYCHIATRIC EFFECTS OF SATIVEX

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Introduction: Cannabis smoking may cause transient psychiatric symptoms as part of the intoxication syndrome, including euphoria, anxiety, depression, paranoid ideas, illusions, hallucinations and delusional beliefs. All of these effects are thought to be primarily related to the Δ-9-THC content of cannabis. Evidence has accumulated that cannabis smoking in adolescence may increase the risk of a psychotic illness in later life, although it must be noted that studies upon which this finding is based all have methodological shortcomings. Interest in the therapeutic potential of cannabinoid medicines (CM) in a wide range of medical conditions has increased significantly over the past decade, and many of the beneficial effects appear to be mediated through the partial agonist action of THC at the CB_1 receptor. There are many differences between recreational cannabis smokers and medicinal recipients of CM including the motivation of the user, constituents, purity, dose, and pharmacokinetics. However, it is important to consider what degree of psychiatric risk THC-containing medicines may pose to patients.

Method: Sativex, a CM delivered by an oromucosal spray, is currently the subject of an extensive international clinical trial programme in various indications. Each activation of the spray contains 2.7mg THC and 2.5mg cannabidiol (CBD). Neuropathic pain and spasticity in multiple sclerosis (MS) have been the focus of several placebo-controlled clinical trials. Data from 496 MS patients who received Sativex and 434 who received placebo have been pooled in order to examine the timing, severity, reversibility, and impact upon the patient of psychiatric adverse events (PAE).

Results: PAE occurred more commonly following Sativex than placebo, particularly disorientation (5.4% vs 1.2%), depression (3% vs 1.8%), dissociation (2.8% vs 0.2%), hallucinations (1.8% vs 0.2%), confusional state (1% vs 0), and paranoia (0.8% vs 0.2%). Suicidal ideation was uncommon (0.4%). Anxiety and insomnia occurred more frequently following placebo. A large majority of patients experiencing PAE did so early in treatment (88% within first 28 days). Incidence of PAE after one month was 3.2% for Sativex and 3.8% for placebo. PAE were usually either mild (38%) or moderate (44%) in intensity, and only a minority (14%) led to withdrawal from Sativex. More Sativex-related PAE (81%) resolved spontaneously than placebo-related events (53%). Importantly, when withdrawal from Sativex was indicated because of PAE, this resulted in complete resolution of the PAE in all but one event (of depression).

Conclusion: The timing of PAE and beneficial effects in Sativex clinical trials indicates that a risk/benefit analysis can be formulated reliably within a one-month treatment trial. There was no evidence from these studies that Sativex poses any long-term psychiatric risks to patients. The presence of CBD may inhibit some unwanted effects of THC.

SUBSTITUTION PROFILE OF VARIOUS DRUGS IN HUMANS DISCRIMINATING Δ^9-THC

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There is evidence that non-cannabinoid neurotransmitter systems (i.e., opioid and GABA) are involved in some of the central effects of Δ^9-THC. The purpose of this study was to examine the involvement of these neurotransmitters systems in the discriminative-stimulus effects of Δ^9-THC in humans. Healthy subjects who reported moderate cannabis use, but did not meet criteria for cannabis dependence, were enrolled as outpatients at the University of Kentucky General Clinical Research Center. The primary outcome measure for this study was the discriminative-stimulus effects of oral Δ^9-THC. To this end, drug-discrimination procedures were used, which consisted of three phases. During the sampling phase, subjects received 25 mg oral Δ^9-THC, which was identified as Drug X. Drug X is used as an example; a unique letter code was used for each subject. During the acquisition phase, subjects were required to correctly identify when they receive placebo (i.e., Not Drug X) or 25 mg Δ^9-THC. Finally, a test phase was conducted to determine if doses of Δ^9-THC, triazolam and hydromorphone shared discriminative-stimulus effects with the training dose of Δ^9-THC. Methylphenidate was also included as a negative control. In addition to drug-discrimination, several other measures were collected to determine a profile of Δ^9-THC effects, including physiological indices, self-report questionnaires, a time estimation procedure, as well as memory and psychomotor performance tasks. Eight subjects learned to discriminate Δ^9-THC and completed the entire experimental protocol. The training dose of Δ^9-THC functioned as a discriminative-stimulus and produced prototypical cannabimimetic effects. For example, Δ^9-THC decreased skin temperature, elevated heart rate and increased subject ratings typically associated with cannabis and drugs of abuse. Significant effects of Δ^9-THC generally emerged at 2 h, and peaked at 3-4 h. All of the drugs tested increased subject ratings on the self-report questionnaires and had effects on performance and/or physiological measures, but only Δ^9-THC substituted for the training dose. These results suggest that the discriminative-stimulus effects of Δ^9-THC are not directly mediated through central opioid or GABA systems. Preliminary data from an ongoing follow up study in which the cannabinoid agonist nabilone is being tested in subjects discriminating Δ^9-THC will also be presented, and indicate that the discriminative-stimulus effects of Δ^9-THC are primarily mediated through cannabinoid receptors in humans.

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INHIBITION OF FAAH BLOCKS THE EXCITATORY EFFECTS OF NICOTINE ON MESOLIMBIC DOPAMINE NEURONS VIA CB1 AND PPAR-α RECEPTORS

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The endogenous cannabinoid system has been implicated in the modulation of addictive behaviours and in the mechanisms of action of diverse drugs of abuse, including nicotine. For example, blockade of CB1 receptors by the antagonist SR141716A (rimonabant) reduces nicotine self-administration (Cohen et al., Behav Pharmacol, 13:451; 2002) and nicotine-induced increase in dopamine (DA) release in the nucleus accumbens (Cheer et al., J Neurosci, 27:791; 2007).

The mesolimbic DA system processes many responses evoked by drugs of abuse, such as their rewarding properties, affective motor behaviour and emotional responses. Both nicotine and cannabinoids stimulate DA neurons, whereas endocannabinoids have been shown to regulate DA neuron synaptic functions and mediate short-term forms of synaptic plasticity. Thus, we studied the contribution of the endocannabinoid system in the electrophysiological effects of nicotine on DA neurons. To this aim, we carried out extracellular single cell recordings in anesthetized rats and whole-cell patch-clamp experiments in brain slices from ventral tegmental area (VTA) DA neurons.

Nicotine (0.2 mg/kg, i.v.) stimulated the firing rate and the percent of burst firing of VTA DA neurons both *in vivo* (154±36% of baseline firing rate and +18±4% burst firing, n=11, $p<0.05$) and in slices. Rimonabant (0.5 mg/kg, i.v., 4 min before nicotine; n=11) did not antagonize nicotine excitatory effects on DA neurons (175±50% of baseline firing rate and +17±6% of burst firing; n=11; $p>0.05$ vs controls). However, blockade of FAAH with URB597 (0.1 mg/kg, i.v. 1-2 hours before recordings) completely prevented the stimulatory actions of nicotine, which became inhibitory instead (74±6% and -17±4% of baseline firing and percent of burst firing, respectively; n=7). The effect of URB597 was incompletely prevented by rimonabant (0.5 mg/kg, i.v.), suggesting that activation of CB1 receptors by enhanced anandamide levels contributed only partially to antagonize nicotine-induced excitation. Besides anandamide, it is known that oleoylethanolamide (OEA) brain levels are also increased by URB597. Thus, we tested whether OEA was involved in the blockade of nicotine effects on DA neurons. Interestingly, OEA (1 μM) blocked the excitatory actions of nicotine in brain slices through the activation of nuclear peroxisome proliferator-activated receptors-alpha (PPAR-α).

We conclude that inhibition of fatty acid ethanolamides' hydrolysis completely abolished nicotine-induced excitation of DA neurons probably inducing a functional negative modulation of nicotinic acetylcholine receptors, cooperatively mediated by both CB1 and PPAR-α receptors.

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EFFECT OF CB1R INVERSE AGONIST ON HEPATIC LIPID CONTENT QUANTIFIED WITH IN VIVO MR SPECTROSCOPY

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Although CB1R inverse agonists reduce body weight reduction, inhibit food intake, and increase energy expenditure, their effect on hepatic lipid was yet to be determined. Because of the sensitivities of MR spectroscopy to various biochemical contents in tissue, such as hepatic lipid, we used a MR spectroscopy (MRS) technique to evaluate the effect of CB1R inverse agonist on hepatic lipid content in living mice.

We used a spatially-localized MRS method to obtain hydrogen spectra from mouse liver. From the MR spectrum, water and fat signals were quantified numerically from their corresponding peaks. The method was based on point resolved spectroscopy (PRESS), implemented on a Bruker 9.4T NMR system (TR/TE=7sec/7ms, 1 1 voxel, NSA=16). Two groups of DIO mice (n=6/ group) were given vehicle and AM251 at 3mg/kg/day respectively for 10 days. Hepatic lipid content was measured with MRS immediately prior to and post the treatment under anesthesia (pentobarbital).

Typical localized MR spectrum obtained from mouse liver reveals both water and fat components in the tissue as two distinct peaks. The hepatic lipid percentage is defined as: 100 x lipid / (lipid +water). Both lipid and water contents were quantified by integrating spectral area under their corresponding peaks. The MRS result showed a statistically significant reduction in intra-hepatic lipid after 10 days of treatment with AM251. In terms of normalized liver fat change from that of pre-treatment, the treated group showed -28.2%+/-5.0% reduction in liver fat content compared with -8.0%+/-4.4% in the control group ($p < 0.007$).

Treatment with a CB1R inverse agonist was found to be effective in reducing hepatic lipid content in DIO mice compared with that of the control group. The study showed the sensitivity and feasibility of the method in revealing the change in hepatic lipid content in a routine in vivo study involving mice.

THE METABOLIC EFFECTS OF TETRAHYDROCANNABIVARIN (THCV) AND CANNABIDIOL (CBD)

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Introduction: The potential benefits of CB-1 receptor antagonism in the area of obesity and metabolic syndrome have been widely explored. Cannabidiol (CBD) has been shown to have anti-inflammatory effects and Mechoulam *et al* (2006, 2008) have explored its protective effects on islet cell destruction in a model of type 1 diabetes. However, the potential that combinations of CBD with a CB-1 antagonist may provide additional metabolic benefit has not been explored. GW Pharma Ltd have bred proprietary strains of *Cannabis sativa L.* containing high levels of the neutral CB-1 antagonist, delta-9-tetrahydrocannabivarin (THCV) and other strains containing high levels of CBD.

Methods: The metabolic effects of purified THCV (0.3 & 3.0mg/kg), purified CBD (3.0mg/kg), and combinations of THCV + CBD Botanical Drug Substance (BDS) (0.3mg/kg + 0.3mg/kg & 3.0mg/kg + 3.0mg/kg) were evaluated in genetically obese (ob/ob) mice and were compared with vehicle controls and a positive control (AM251, 10mg/kg/day). Mice were treated daily for a total of 36 days. Food and water intake were measured daily and body weight was assessed twice weekly. Other metabolic endpoints included: 24h energy expenditure, thermic response to a mixed meal, oral glucose tolerance, body fat composition, plasma levels of glucose, lactate, insulin, triglycerides, cholesterol, HDL-cholesterol and free fatty acids, insulin and adiponectin levels. Liver weight, liver triglyceride concentration and liver glycogen concentration were also measured.

Results: Pure THCV acutely decreased food intake but over the 5 week dosing period there was no overall effect on food intake. However, 24h energy expenditure and the thermic response to food was increased. The low dose of THCV improved insulin sensitivity but this was not seen with the high dose. THCV increased the liver triglyceride concentration, as did AM251.

Pure CBD had no effect on food intake or energy expenditure or insulin sensitivity. It reduced the plasma total cholesterol concentration significantly whilst increasing the HDL-cholesterol concentration. It markedly reduced liver triglyceride levels whilst increasing liver glycogen content.

In the 1:1 mix of THCV and CBD BDS, CBD did not ameliorate the thermogenic effect of THCV whilst THCV did not modulate the effect of CBD in raising the plasma HDL-cholesterol concentration nor in reducing fatty liver.

Conclusion: This is the first demonstration of potential beneficial effects of CBD in hypercholesterolaemia and non-alcoholic fatty liver disease. In addition, in combination with THCV it potentially addresses a number of components of the metabolic syndrome.

ENDOCANNABINOID SIGNALING REGULATES THE CIRCADIAN PATTERN OF WHEEL RUNNING BEHAVIOR

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Circadian rhythms are regular changes in mental and physical characteristics that occur with a period of roughly one day and are driven by a molecular clock. The sleep-wake cycle, feeding, body temperature, and activity patterns are examples of processes with circadian rhythms. The mechanisms by which the molecular clock regulates behavior are not well understood. Given the role of endocannabinoid (eCB) signaling in many circadian processes, we hypothesize that eCB signaling is a link between the molecular clock and behavioral patterns. There is evidence in the literature that support this hypothesis. For example, Δ^9-tetrahydrocannabinol (THC) exposure results in changes in the amount of time that animals spend in each phase of the sleep-wake cycle (Barratt and Adams, Biol Psychiatry, 6**,** 207-14, 1973.) and treatment of rats with 0.1 mg/kg THC for 1 week results in inversion of the circadian rhythm of brain temperature (Perron et al., Neuroreport, 12**,** 3791-4, 2001). In addition, eCB signaling is circadian; *N*-arachidonylethanolamine (AEA) increases in the hypothalamus during the light and falls in the dark (Murillo-Rodriguez et al., Life Sci, 79**,** 30-7, 2006.). CB1 receptor null mice have higher circulating glucocorticoid concentrations at the light-dark transition, but not at other times of the day (Cota et al., Endocrinology, 148**,** 1574-81, 2007.).

We examined the circadian expression of three proteins involved in eCB function using real time PCR. Male mice were held at a light cycle of 6 am-6 pm. CB1 receptor expression is not altered over the time course of the day; however, the expression of both fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) are significantly circadian. The mRNA for FAAH is highest at the onset of light (6 am) while the mRNA for MGL is highest in the dark phase (10 pm). Since protein expression is delayed by several hours, these data suggest that FAAH activity is highest in the light phase while MGL activity is highest in the middle of the dark phase. These data suggest that AEA and 2-arachidonoylglycerol (2-AG) concentrations should be out of phase with each other; as has been demonstrated previously (Valenti et al., Cell Mol Life Sci, 61**,** 945-50, 2004).

To further explore this hypothesis, we examined activity (wheel running) patterns in mice with genetic deletions of either the CB1 receptor or FAAH. In male mice null for FAAH, wheel running activity during the last few hours of the light phase (anticipatory running) was significantly increased compared to wild-type controls. On the other hand, wheel running in the first few hours of the light phase (residual running) was decreased. In CB1 receptor null mice, the anticipatory running was significantly reduced. These data support the hypothesis that eCB signaling modulates a circadian behavior, wheel running, in a subtle but significant manner. In particular, intact eCB signaling is required for the onset and offset of behavior that occurs in concert with the light cycle.

INHIBITORS OF ENDOCANNABINOID DEGRADATION REDUCE COLITIS BY ACTIVATION OF CB_1 AND CB_2 RECEPTORS

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The endocannabinoid system mediates protection against intestinal inflammation. Pharmacological activation of the CB_1 and the CB_2 receptors, as well as the use of blockers of endocannabinoid degradation, have been shown to exert protective effects against colitis. The mechanisms involved remain unclear. Here we investigated the beneficial effects of blocking endocannabinoid degradation and/or cellular reuptake in experimental colitis in mice. To further characterize the mechanisms involved, we performed experiments in CB_1 gene-deficient ($^{-/-}$) and $CB_2^{-/-}$ mice.

Colitis was induced in wild type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice (C57Bl6/N background) by treatment with trinitrobenzene-sulphonic acid (TNBS). Animals were treated with the FAAH blocker URB597 (5mg/kg), the endocannabinoid membrane transport inhibitor VDM11 (5mg/kg) or combinations of both drugs. Drugs were injected either 30 min before the induction of colitis or 30 min before and then twice daily for 3 days. Macroscopic scoring, histology and myeloperoxidase levels were evaluated three days after the induction of colitis. Quantitative PCR was performed on the colon to investigate whether FAAH mRNA expression is altered during experimental colitis.

Inflammation was significantly reduced in wild type mice in the presence of URB597, VDM11 or both as evaluated by macroscopic damage score, myeloperoxidase levels and colon length. Both drugs added together produced no greater benefit than either alone and the beneficial effects were not enhanced by multiple doses of the drugs. The protective effects of either URB597 or VDM11 were abolished in $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. Quantitative RT-PCR following induction of experimental colitis showed that expression of FAAH mRNA was significantly reduced in wild type mice in the absence of drugs early in the expression of colitis (1 day), but these levels returned to baseline at 3 days after the induction of colitis in the inflamed region of distal colon. Interestingly FAAH mRNA levels were upregulated in the proximal colon 3 days after the induction of colitis.

In conclusion, drugs targeting endocannabinoid degradation have the potential to relieve experimental colitis and thus might be developed in the future as tools for the treatment of human inflammatory bowel disease. Activation of CB_1 and CB_2 receptors by endogenously released cannabinoids ameliorates experimental colitis. In addition, we show that alterations in FAAH mRNA expression occur in the pathophysiological response to experimental colitis.

LONGITUDINAL STUDY SHOWING THE RELATIONSHIP BETWEEN ANANDAMIDE AND SEX STEROIDS AND GONADOTROPIN HORMONES IN WOMEN

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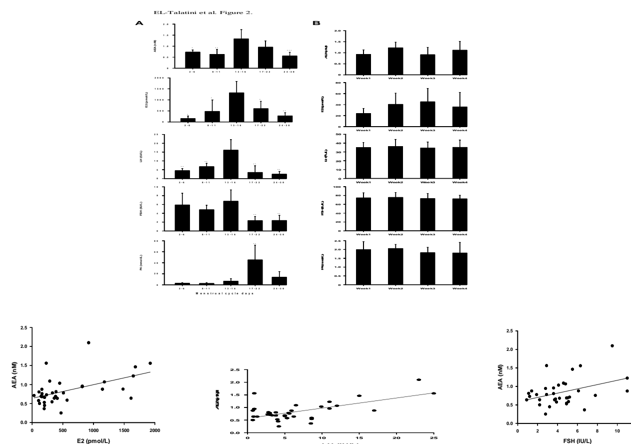
Introduction: The levels of the endocannabinoids, anandamide (AEA) are crucial for early pregnancy success; reduced levels at implantation favour success and the reverse with high levels. Plasma AEA levels are thought to be modulated partly by circulating progesterone. The aims of our study were to investigate the changes in plasma AEA levels throughout the menstrual cycle in menstruating women with reference to post –menopausal. Additionally, we investigated relationship between plasma AEA levels and serum ovarian steroids and gonadotropin involved in human fertility.

Methods: A total 12 healthy volunteers (7-pre-menopausal aged 21-40years, and 5 postmenopausal aged 54-68years) with BMI of 20-24kg/m^2 were recruited into the longitudinal study. The Premenopausal women had regular cycles. All women in the study had no medical problems and not on any hormonal therapy for last 2years. Plasma AEA measured by using UPLC-MS/MS and serum FSH, LH, E2 and progesterone measured by using automated ADVIA Centaur Assay System. The menstrual cycle was divided to 5 phases; early follicular (d2-6), mid follicular (d8-d11), ovulation (d12-16), early luteal (D17-22), late luteal phase (D24-31).Ovulation identified by using urine LH surge Kit, and confirmed by peak serum FSH, LH, and E2 levels. Also, blood samples taken from menopausal women (>2years post-menopause) weekly over 4 week period.

Results: Fig1 Shows Mean (SD) plasma AEA levels were0.73 (0.09) nM in early follicular, 0.63(0.22) nM in mid follicular phase, peak level of plasma AEA at ovulation (1.33±0.4n M), and 0.96(0.27) nM in the early luteal, and lowest level in the late luteal phase 0.55(0.16) nM. All were significantly different to the levels in post –menopausal women (1.05±0.15n M) except those in early luteal phase.
Fig2 shows statistically positive correlation between plasma AEA levels and serum estradiol (P<0.0015), LH (P<0.0001), and FSH (P, 0.022) but not progesterone (P<0.841) in Premenopausal women.



Conclusion: Peak plasma AEA occurs at ovulation and is positively related to estradiol, FSH, LH levels. These findings suggest these hormones are involved in the regulation of AEA levels.

IN VITRO VASCULAR EFFECTS OF CANNABIDIOL (CBD) IN THE RAT ISOLATED AORTA

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Cannabidiol (CBD) is a major component of *Cannabis sativa* that lacks the psychotropic effects of Δ^9-tetrahydrocannabinol (THC). CBD is anti-inflammatory, anti-oxidant, neuroprotective and beneficial in diabetes (see Pertwee, 2008, *Br J Pharmacol.* 153:199-215). However, the receptor sites of action for CBD remain largely unknown due to its low affinity for cannabinoid receptors. We have shown that THC causes time-dependent vasorelaxation of the rat isolated aorta, sensitive to peroxisome proliferator-activated receptor gamma (PPARγ) antagonism (O'Sullivan *et al*., 2005, *Biochem Biophys Res Commun.* 337:824-31). The aim of the present study was to establish whether CBD activates PPARγ and whether similar vascular responses are observed.

Male Wistar rats (200-300 g) were killed by cervical dislocation. The thoracic aorta was isolated, cut into 3-5 mm lengths and mounted on a Mulvany-Halpern myograph. Vessels were bathed in oxygenated Krebs' solution at 37°C and set to a baseline tension of 10 mN. U46619 and methoxamine were added to increase tone by at least 10 mN. When stable contraction was maintained, the vasorelaxant effects of a single concentration of CBD or vehicle control on induced tone was assessed.

CBD (10 μM) caused significant relaxation of the rat aorta compared to vehicle-treated aortae at all time-points over 2 h (2 h, vehicle 20 ± 2 cf CBD 70 ± 4 % relaxation, mean ± SEM, n=13, P<0.001, Student's t test). After 2 h, the residual relaxation (the vasorelaxant effect of CBD minus the vasorelaxant effect of vehicle/time) was 50 ± 3 % relaxation. In the presence of the PPARγ receptor antagonist GW9662 (1 μM), the residual vasorelaxant effect of CBD was reduced (2 h & GW9662, 33.0 ± 6.1 % relaxation, P<0.05, ANOVA). The vasorelaxant effect of CBD was <u>not</u> affected by removing the endothelium, nitric oxide synthase inhibition, PTX pre-treatment (200 ng ml^{-1}, 2 h), capsaicin pre-treatment (10 μM, 1 h) or the CB_1 receptor antagonist AM251 (1 μM). When arteries were contracted with a high K^+ buffer, there was no difference in the vasorelaxant effect of CBD compared with control. By contrast, when tone was induced with U46619 in Ca^{2+}-free Krebs-Hensleit solution, the vasorelaxant effect of CBD was blunted (2 h, 9.3 ± 2.5 % relaxation, P<0.01). The contractile response to the re-introduction of Ca^{2+} in Ca^{2+}-free, high K^+ buffer was also reduced in a concentration-dependent manner in the presence of CBD (R_{max} vehicle 2.49 ± 0.06 g tension; & 1 μM CBD 2.10 ± 0.13, P<0.05; & 10 μM CBD 1.86 ± 0.11, P<0.01; & 30 μM CBD 1.38 ± 0.11, P<0.01).

The results of the present study demonstrate that CBD causes significant vasorelaxation over time in the rat isolated aorta. This does not appear to be due to activation of cannabinoid receptors, TRPV1, the endothelium or potassium channels. In common with THC, this response could be partially inhibited by a PPARγ antagonist, however, the majority of the vasorelaxant effects of CBD appear to be through calcium channel inhibition.

THE NOVEL FAAH INHIBITOR AM-3506: CARDIOVASCULAR EFFECTS AND THERAPEUTIC IMPLICATIONS

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We have earlier reported that in experimental models of hypertension an anandamide-mediated counterregulatory system is activated as the body's attempt to work against the elevation of blood pressure. Treatment of anesthetized hypertensive rats with the fatty acid amide hydrolase (FAAH) inhibitor URB597, which effectively prevents anandamide breakdown, transiently lowered blood pressure and the inappropriately high cardiac contractility to normotensive levels, whereas the CB_1 receptor antagonist rimonabant produced the opposite effects. Neither drug affected the blood pressure and cardiac contractility of normotensive animals. As an attempt to further explore the therapeutic potential of FAAH inhibition in hypertension, we have developed a novel, *in vivo* effective FAAH inhibitor, AM-3506, and tested its effects on blood pressure in both anesthetized and conscious, chronically instrumented animals.

In in vitro enzyme activity and ligand binding assays, AM-3506 had a K_i of 31 nM for FAAH and 28.8 μM for MGL. Additionally, it had low affinity for CB_1 (K_i: 191 nM) and CB_2 receptors (K_i: 577 nM), and did not inhibit anandamide transport at concentrations up to 20 μM. In an activity-based enzyme-profiling assay using brain tissue from mice treated in vivo with AM-3506, a dose of 0.1 mg/kg AM-3506 i.p. caused >85% inhibition of FAAH with no appreciable inhibition of a host of other targets, including MGL, ABHD4 and ABHD6. Using the same profiling paradigm, URB597 was ~ 10 times less potent than AM-3506 in inhibiting FAAH.

In anesthetized spontaneously hypertensive rats (SHR), the basal mean arterial pressure (MAP) of 179.8±7.6 mmHg was reduced to 113.7±20.4 mmHg by an i.v. bolus injection of 1 mg/kg AM-3506 with no sign of recovery for over an hour. Comparable, but shorter lasting effects were obtained with 5 mg/kg URB597, in agreement with the difference in *in vitro* potency. The hypotensive effect of AM-3506 is dose-dependent; 0.1 mg/kg dose is already causing a statistically significant, 36.7±8.3 mmHg decrease in MAP. Since anesthesia may influence cardiovascular variables, we also tested AM3506 in conscious, freely moving, chronically cannulated SHR. As in the anesthetized animals, AM-3506 caused a dose-dependent and long lasting reduction in MAP to near normotensive levels (173.0±3.8 to 126.0±2.2 mmHg). Heart rate was unaffected by AM3506 in either anesthetized or conscious SHR. In additional experiments in conscious SHR pretreated with rimonabant (3 mg/kg), 1 mg/kg AM3506 failed to reduce MAP, which indicates that its hypotensive effect is mediated by CB_1 receptors. Since AM-3506 does not bind to CB_1 receptors, the most likely mechanism to explain these findings is that by blocking FAAH, AM-3506 prevents the breakdown of endogenous anandamide, which then activates CB_1 receptors to lower blood pressure. This explanation is further supported by the finding that an analog of AM-3506 devoid of FAAH inhibitory activity was unable to reduce blood pressure in SHR.

Because inhibition of FAAH does not elicit behavioral effects predictive of addictive potential, FAAH inhibitors such as AM-3506 may be considered for the treatment of hypertension.

JWH-133 ADMINISTRATION DURING ISCHEMIA REDUCES INFARCT SIZE IN A MOUSE MODEL OF MYOCARDIAL ISCHEMIA/REPERFUSION

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Acute myocardial infarction is the leading cause of morbidity and mortality in developed and developing nations. Although the prompt restoration of antegrade flow in the infarct-related coronary artery is the mean therapy for improving survival, reperfusion itself may cause damage to ischemic myocardial tissue, which is well-known as “reperfusion injury”. A previous study has shown that pretreatment with cannabinoid agonists before ischemia reduced myocardial ischemia reperfusion injury in mice in a CB_2 dependent manner. In the present study, we investigated if acute administration of the CB_2 agonist JWH-133 during myocardial ischemia could reduce infarct size in a mouse model. Myocardial ischemia in 8 to 12 week old C57Bl6 mice was induced by left coronary artery occlusion for 30 minutes, followed by 24 h of reperfusion. 5 minutes before reperfusion, mice received one intraperitoneal injection of 20 mg/kg JWH-133 or vehicle. The infarct size (n=12 per group) was assessed by 1 % triphenyltetrazolium chloride staining and quantification of area of infarction (I), area of risk (AAR) as well as total left ventricle (T). Histological analysis was performed on frozen heart sections (n=4 per group). Acute treatment with JWH-133 significantly reduced the infarct size as compared to control mice (I/AAR 19.27 % ± 1.91 vs. 29.98 % ± 3.13 in controls, p: <0.01; I/T 11.39 % ± 1.33 vs. 16.75 % ± 1.63, p: <0.05; mean ± SEM). This effect was associated with reduced numbers of neutrophils in the myocardium of JWH-133-treated mice. Our data demonstrate that systemic administration of JWH-133, when administered after myocardial ischemia onset, reduces infarct size in mice. These findings suggest a potential therapeutic use for the treatment of myocardial ischemia and reperfusion injury. We are currently investigating the molecular mechanism underlying this beneficial effect.

CANNABINOIDS CAN STIMULATE ARTEROSCLEROTIC PLAQUE FORMATION BY INDUCING DEDIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS INTO OSTEOBLASTS

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Introduction: Vascular smooth muscle cells (VSMCs) are one of the players involved in the process of arterosclerotic plaque formation. Upon receipt of local triggers, mainly received from activated macrophages, these cells can differentiate from a contractile into a synthetic, osteoblast-like phenotype. In this study we examined whether this phenotypical switch is affected by phyto, synthetic or endocannabinoids.

Methods: VSMCs were cultured in normal or osteogenic medium in the presence or absence of a variety of cannabinoids. Osteoblast differentiation was monitored by measuring the activity of the well established bone marker alkaline phosphatase (ALP) and by measuring calcification of the extracellular matrix, a typical feature of mature osteoblasts. Pertussis toxin and receptor specific antagonists were used in the same type of experiments to examine the involvement of the cannabinoid receptor type 1 and 2 (CB1 and CB2) in dedifferentiation of the VSMCs under influence of the cannabinoids. In addition, we exposed the VSMCs to conditioned medium obtained from monocytes and macrophages in the presence or absence of cannabinoids.

Results: Strong induction of the osteoblast markers was observed in VSMCs in response to treatment with the highly potent synthetic cannabinoid HU210 or the endocannabinoid noladin ether (NE). No phenotypical switch was observed with other phyto, synthetic or endocannabinoids. We found that HU210 and NE can activate osteoblast differentiation of VSMCs on their own but can also work synergistically with factors excreted by macrophages. The results obtained with receptor specific agonists or antagonists indicated that the osteoinductive effect of HU210 and NE was not mediated via the CB1 or CB2 receptor.

Conclusion: Our results show that HU210 and NE can contribute to arterosclerotic plaque formation both alone as well as in concert with factors excreted by macrophages via a CB1 and CB2-independent mechanism.

GPR55 IS INVOLVED IN THE REGULATION OF OSTEOCLAST ACTIVITY IN VITRO AND BONE MASS IN VIVO

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Both CB_1 and CB_2 receptor knockout mice have been previously shown to have abnormal bone phenotypes and cannabinoid receptor ligands shown to affect bone cell function. GPR55 is a G protein coupled receptor that is structurally distinct from CB_1 and CB_2 but is activated by various endogenous, synthetic and plant cannabinoids. The aim of this study was to investigate whether GPR55 has a role in bone physiology.

Human osteoclasts were generated by culturing peripheral blood-derived monocytes from healthy donors with M-CSF and RANKL. Using immunostaining and quantitative PCR we found that GPR55 is expressed in human osteoclasts and that the level of GPR55 receptor mRNA increases during osteoclast differentiation. To study the functional effect of GPR55 in human osteoclasts, cells were treated with O1602, a candidate ligand for GPR55. In the presence of 1nM-1μM O1602 there was no effect on the formation of $\alpha v\beta 3$-positive osteoclasts. However, O1602 increased the proportion of actively-resorbing osteoclasts (i.e. cells with F-actin rings) and increased resorption pit area; at 50nM, cells with actin rings were 236 % ± 31 of control ($P<0.01$) and resorption area was 217 % ± 28 of control ($P<0.01$). This demonstrates for the first time that this GRP55 agonist stimulates the activity of human osteoclasts *in vitro*. Furthermore, the putative GPR55 antagonist, cannabidiol (CBD, 500nM), significantly inhibited the increase in resorption and F-actin ring number seen after treatment with 50nM O1602 alone ($P<0.0001$, n = 4). We also found that treatment of human osteoclasts with 1μM O1602 resulted in an increase in GTP-bound Rho, as demonstrated in a pull down assay. This is consistent with the $G\alpha_{12/13}$ coupling that has been previously described and may also relate to the stimulatory effects of O1602 on osteoclast polarisation and resorption. Micro CT analysis of tibiae and femorae from $GPR55^{-/-}$ and WT age-matched littermate control revealed a significant increase in bone mass in both young and adult (12 week and 8 month respectively) *male* $GPR55^{-/-}$ mice. Thus, trabecular bone volume was significantly increased (12 wk old male; tibia +36% ($P<0.01$) femur +71% ($P<0.05$); 8 month male tibia +33% ($P<0.05$), femur +28% ($P<0.05$)) and consistent with this bone surface (8 month male tibia +46% ($P= 0.0377$), femur +55% ($P=0.0409$) and trabecular number (8 month male tibia +46% ($P=0.0332$), femur +55% ($P=0.0327$)) were also increased. This was not seen in female mice of the same age.

Taken together, these data suggests that, in addition to CB_1 and CB_2, GPR55 plays a key role in bone physiology. Furthermore, small molecules that interact with this receptor may be useful therapeutics in diseases such as osteoporosis that are caused by excessive osteoclast activity.

THE ENDOCANNABINOID SYSTEM IN PROSTATE CANCER

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Introduction: Prostate cancer is the commonest form of cancer affecting men, with >180000 cases being reported in the US annually. There is evidence that endocannabinoids can affect the invasive behaviour of prostate cancer cells *in vitro* (Nithipatikom *et al.*, *Cancer Res* 64 [2004] 8826-30), but there is no data available concerning the expression of CB receptors in prostate cancer tissue samples, or whether such expression is correlated to differentiation and cancer progression.

Method: We used formalin-fixed, paraffin-embedded specimens of normal and tumour tissue from patients who were diagnosed with cancer following transurethral resection surgery for prostatic enlargement. At the time of sample collection (between 1975 and 1990), the standard treatment strategy was watchful waiting until evidence of metastatic progression was clinically observed. This means that the expression of biomarkers can be correlated with disease outcome in an essentially untreated population. The samples (up to 4 for each normal tissue sample and up to 5 for each tumour sample) from >400 patients were investigated immunochemically using a CB_1 receptor antibody (AbCam ab23703, lot no. 280229). The samples were scored individually for intensity and distribution of the staining by an evaluator who was not given access to the clinical or outcome data. Median values were then calculated. The antibody was also tested upon a formalin-fixed, paraffin-embedded sample of human cerebellum.

Results: In the normal prostate tissue, immunoreactivity primarily was found on the surfaces of the glandular epithelial cells and none in the stroma. In the cerebellum, the immunoreactivity was consistent with the distribution of the CB_1 receptor. An interim analysis has been conducted for the first 290 patients, for whom median scores could be calculated for 263 (normal tissue) and 265 (tumour tissue) cases. The scale used (here termed abIS) allows for scores between 0 (absent) and 3 (intense, wide distribution), and the majority of the cases had abIS scores between 1.5 and 2.4. For the normal tissue, a total of six samples (2%) had abIS >2.5, and all six were from patients whose corresponding tumour tissue had a Gleason score of 8-10. For the tumour tissue, a total of 26 cases (10%) had abIS values >2.5, of who 3 (5%), 1 (1%), 4 (8%) and 18 (22%) were for Gleason scores of 4-5, 6, 7 and 8-10 respectively. This high abIS value was only found among 8% (14/183) of patients who had not metastasised during the long follow up period, whereas it was found in 23% (5/22) cases where metastases had been observed. Median abIS values of exactly 2.5 were also found more often in the tumour tissue (40/265) than in the normal tissue (20/263), but the test-retest reliability of this score was less robust than for the >2.5 values, and there was no obvious relation to the Gleason score.

Conclusion: High levels of ab23703 immunoreactivity are more often found in prostate cancer tissue with a poor prognosis than in normal tissue. Given that the endocannabinoid system can affect the invasivity of prostate cancer tumour cells *in vitro*, its modulation may be a possible therapeutic approach for prostate cancer.

AMPHIREGULIN RENDERS GLIOMA CELLS RESISTANT TO CANNABINOID-INDUCED APOPTOSIS

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Gliomas, one of the most malignant forms of cancer, exhibit a high resistance to conventional therapies. Identification of the molecular mechanisms responsible for this resistance is therefore of great interest to improve the efficacy of the treatments against these tumors. Δ^9-Tetrahydrocannabinol (THC), the major active ingredient of marijuana, and other cannabinoids inhibit tumor growth in animal models of cancer, including glioma, an effect that relies, at least in part, on the ability of these compounds to induce apoptosis of tumor cells. By analyzing the gene expression profile of two sub-clones of glioma cells with different sensitivity to cannabinoid-induced apoptosis, here we identified the epidermal growth factor receptor ligand amphiregulin as a candidate factor to mediate the resistance of glioma cells to cannabinoid treatment. Amphiregulin was highly overexpressed in the cannabinoid-resistant cell line C6.4, both in culture and in tumor xenografts, which was associated with increased extracellular signal-regulated kinase (ERK) activation. Silencing of amphiregulin expression or amphiregulin neutralization decreased ERK phosphorylation and sensitized C6.4 cells to cannabinoid treatment. Amphiregulin-induced ERK activation mediated the resistance of C6.4 cells to THC, at least in part, by blunting the expression of p8 and TRB3 - two genes involved in cannabinoid-induced apoptosis of glioma cells. These observations were also evident in two human glioma cell lines with different sensitivity to cannabinoid-induced cell death. Our findings therefore contribute to unraveling the molecular bases undelying the emerging notion that targeted inhibition of the EGFR pathway can improve the efficacy of antitumoral therapies.

CB_2 CANNABINOID RECEPTOR-MEDIATED REGULATION OF PROSTATE CANCER GROWTH

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Cannabinoids (including endocannabinoids) have been shown to regulate cell death or cell growth, depending on the cell type and concentration of the cannabinoid compounds (Guzmán et al., Pharmacol Ther. 95:175-84, 2002). Recent studies showed that non-specific CB_1/CB_2 receptor agonist WIN-55,212-2 treatment produced cell cycle arrest and induction of apoptosis in LNCaP human prostate cancer cells in a CB_1 cannabinoid receptor-dependent manner Sarfaraz et al., J Biol. Chem 281:39480–91, 2006; Sarfaraz et al., Am Assoc Cancer Res 2007;48:521, 2007). In the present study, we have found that in low passage (when they maintained their androgen-sensitivity) LNCaP cells, CB_2 cannabinoid receptor expression is higher compared to that of non-malignant prostate epithelial cells (PrEC). However, under similar cell culture condition no significant difference was observed for CB_1 receptor expression between LNCaP and PrEc. We further showed that CB_2 receptor agonist JWH015 treatment significantly reduced LNCaP cell proliferation, viability and FBS-induced motility and these responses were blocked by CB_2 receptor antagonist SR144528. Interestingly, we found that activation of CB_2 receptors inhibited LNCaP cell proliferation and viability without affecting the same in non-malignant PrEc cells. Taken together these results suggest that CB_2 receptor activation produced less adverse effects on normal prostate epithelial cells compared to LNCaP prostate cancer cells.

We have further identified that differential phosphorylation of focal adhesion kinase (FAK) at Tyr 397 and Tyr 576 along with a decrease in vascular endothelial growth factor (VEGF) signaling as possible molecular mechanisms that potentially link CB_2 receptor stimulation to these effects. Since toxicity in non-cancerous tissue limits efficacy of current cancer treatments, elucidating the mechanisms underlying this selective cannabinoid efficacy in human tissues is of clinical importance. To date most of the anti-tumor effects of cannabinoids have been correlated with the CB_1 receptors rather than CB_2 receptor activation, although CB_2 receptor expression is higher in many tumor tissues including prostate tumor compared to corresponding non-malignant tissues. Further, CB_1 receptors are highly expressed in neuronal cells and brain tissues. Therefore, unlike activation of CB_2 receptors, CB_1 receptors activation produces neurobehavioral and psychotropic side effects. Thus, CB_2 receptor-mediated therapeutic intervention of prostate cancer is clinical more relevant. The results from the present study clearly suggest that CB_2 receptor has the potential to be used as a drug target for therapeutic intervention of prostate cancer growth and metastasis. (This study was supported by AHA 0060377Z and BRG grant from North Carolina Biotechnology Center to SM, NIDA U24 DA 12385).

AUTOPHAGY MEDIATES CANNABINOID ANTI-TUMORAL ACTION

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Macro-autophagy, hereafter named autophagy, is a highly-conserved cellular process in which cytoplasmic materials are sequestered into double-membrane vesicles called autophagosomes and delivered to lysosomes for degradation or recycling. Triggering of autophagy relies on the activation of several autophagy proteins (Atg) involved in the initial phase of membrane isolation, nucleation and elongation of autophagosomes.
The participation of autophagy in the promotion or inhibition of tumor cell survival is a widely debated issue. Moreover, despite the potential implication of autophagy manipulation in cancer therapy, the molecular mechanisms by which the antitumoral agents regulate this process remain obscure.

Here we demonstrate that Δ^9-tetrahydrocannabinol (THC), the main active component of marijuana, induces cancer cell death through stimulation of endoplasmic reticulum (ER) stress and autophagy.

Electron microscopy, immunofluorescence and Western blot experiments show that cannabinoid treatment induces an early endoplasmic reticulum dilation and eIF2α phosphorylation that precedes the formation of autophagosomes in tumor cells. Pharmacological inhibition of autophagy as well as knock-down and knock-out of essential autophagy genes demonstrate that autophagy is involved in cannabinoid-induced tumor cell death. Analysis of tumor xenografts generated in mice with the human glioma cell line U87MG as well as of tumor samples obtained from two patients with recurrent gliomas proves that THC administration is associated with activation of the autophagic cell death pathway in vivo. Finally, analysis of tumor xenografts derived from ras^{V12}/Tlarge-$Atg5^{+/+}$ and ras^{V12}/Tlarge-$Atg5^{-/-}$ MEFs shows that autophagy is essential for cannabinoid antitumoral action, since THC administration reduced the growth of tumors derived from wild-type but not autophagy-deficient cells.

These findings define a new route for promoting the autophagic death of tumor cells and support that its activation constitutes a potential therapeutic strategy for inhibiting tumor growth.

INHIBITION OF HUMAN GLIOMA CELL MIGRATION AND INVASIVENESS INDUCED BY CANNABIDIOL, A NON-PSYCHOACTIVE CANNABINOID

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Malignant glioma is the most common primary brain tumor, and its high ability to invade the surrounding brain parenchyma is a leading cause of tumor recurrence and treatment failure. We recently demonstrated that the non-psychoactive cannabinoid compound cannabidiol (CBD) can be effective, both *in vitro* and *in vivo*, in limiting tumor cell growth and triggering apoptosis in human glioma cells (1) through an oxidative stress-based mechanism and modulation of LOX pathway and endocannabinoid system (2,3). We were also able to provide the first demonstration of CBD-induced inhibition of cell migration in Boyden chamber assay (4). Since tumor cell motility represents a fundamental aspect in tumor invasion, in the present work we were interested in analyzing further the ability of CBD in inhibiting glioma cell migration and invasiveness. Among the various factors involved in the acquisition of increasing levels of malignancy, matrix metalloproteinases (MMPs) are a group of enzymes that play a pivotal role in promoting tissue breakdown and remodelling during angiogenesis and invasiveness through degradation of extracellular matrix components.

Therefore, since MMP-2 is one of the most important MMPs in the spreading of glioma, we investigated the influence of CBD on MMP-2 production and activity. We found that U87 glioma cells exposed *in vitro* for 24 h to different concentrations of CBD showed a significant inhibition of MMP-2 release in the supernatants of cell cultures, as evaluated by ELISA assay. CBD was also able to alter the MMP-2 gelatinolytic activity, as detected by gelatine zymography analysis. Moreover, using a scratch wound healing assay, we found that the *in vitro* exposure to CBD for 16 and 24 h, induced a significant inhibition in the rate of glioma cells invasion into the artificial wounded areas.

In conclusion, the present investigation adds further insights into the antitumoral action of the non psychoactive CBD, showing multiple mechanisms through which the cannabinoid inhibits glioma cells growth/invasiveness. Considering that CBD is a natural compound without psychotropic and side effects, these data lead us to consider CBD to have high potential as a new anticancer drug alone or in combinatory therapy.

1)Massi et al., JPET, (2004), 308, 838-845
2)Massi et al., Cell.Mol. Life Sci. (2006), 63, 2057-2066
3)Massi et al., JNC (2008), 104, 1091-1100
4)Vaccani et al., BJP (2005), 144, 1032-1036

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CANNABIDIOL (CBD) AMELIORATES COGNITIVE IMPAIRMENTS ASSOCIATED WITH A MODEL OF CHRONIC LIVER DISEASE IN MICE

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Background: Hepatic encephalopathy (HE) is a major neuropsychiatric complication of both acute and chronic liver failure, but its pathogenesis is still unknown. It has been suggested that the cognitive deficits characterizing this state result, at least in part, from an inflammatory response in the brain. Cannabidiol (CBD) is a non-psychoactive ingredient of the plant *Cannabis sativa* known for its anti-inflammatory properties. Also, its structure resembles that of resveratrol, which is found in red wine and has anti-inflammatory activity. Resveratrol has also been shown to decrease liver oxidative stress in a model of chronic liver disease induced by ligation of the bile duct in rats (BDL). On the basis of these findings, we hypothesized that CBD may have therapeutic potential in chronic liver disease through anti-inflammatory actions.

Methods: Female Sabra mice were subjected to ligation of the bile duct (BDL). Sham operated animals were used as controls. Three weeks post-surgery, animals receiving either vehicle or 5mg/kg CBD daily were evaluated for cognitive and motor function using the eight arm maze and the open field tests, respectively. The animals were sacrificed and their hippocampi were analyzed for mRNA levels of brain derived neurotrophic factor (BDNF) by RT-PCR analysis, while their striata were analyzed for mRNA levels of the dopamine D_2 receptor.

Results: BDNF expression in the hippocampus and D_2 expression in the striatum decreased two-fold and 1.5-fold, respectively, in BDL mice after 3 weeks, and were fully normalized by CBD. Cognitive function was significantly impaired and activity in the open field decreased two-fold in BDL mice and both were partially restored by CBD.

Conclusion: These results indicate that CBD improves cognitive function by elevating the level of BDNF, which is a neurotrophic factor involved in synaptic plasticity and contributes to normal learning. The decrease in BDNF may result from neuroinflammation. In the striatum, the normalization of D_2 receptor expression may explain the improvement in motor function since this receptor is involved in the control of movement. Further studies are required in order to elucidate the action of CBD by using antagonists of potential pathways of its activity, such as the serotonergic and the adenosine systems.

CANNABINOID CB1 RECEPTOR MANIPULATION AT BIRTH AFFECTS ADULT FUNCTIONS: IMPLICATIONS FOR 2-ARACHIDONYL GLYCEROL AS A FOOD SUPPLEMENT FOR INFANTS WITH 'FAILURE-TO-THRIVE'

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Approximately 4% of infants suffer from 'non-organic failure-to-thrive' (NOFTT, low weight and short height for age, due to an inability to ingest sufficient amounts of food). Thus far, no biological mechanism has been found to explain this phenomenon.
We have demonstrated previously that cannabinoid CB1 receptor blockade in neonatal mice, mimics the NOFTT syndrome in humans. While the endocannabinoid 2-arachidonyl glycerol (2-AG) is abundant in neonatal rodent brain and in maternal milk, this first animal model for NOFTT highlights the importance of the endocannabinoid system in feeding and growth during early life.
METHODS: we have now investigated:
1. the implications of CB1 receptor blockade at birth (SR141716; 5,10 or 20 mg/kg), for adult physiology and behavioral parameters (motor activity, performance in the 'forced swim test' (FST) for anti-depressant-like behavior, anxiety-like behavior in the 'elevated plus maze' and 'prepulse inhibition of the startle response' for adaptability (PPI)).
2. Effects of 2-AG administration in neonatal mice (0.5, 1 or 5 mg/kg on postnatal days 1 through 5) on early development and adult performance (motor activity, FST, plus maze, PPI).
3 The presence of 2-AG in various types of milk and infant formulae was assessed by fractional extraction of various milks using gas chromatography mass spectrometry.
RESULTS: 1. Similarly to children with NOFTT, 'NOFTT mice' exhibit behavioral abnormalities (hyperactivity and impaired PPI) at later stages of development, suggesting that the consequences of CB1 receptor blockade at birth persist into adulthood. 2. Administration of 2-AG during the neonatal period results in greater weight gain and adult weight in undernourished mice, but not in mice which were adequately fed as pups. Further, neonatal 2-AG has positive effects on mood regulation (in the plus maze and FST) and improved behavioral adaptability (PPI). These effects were mostly present in males. 3. 2-AG seems to be absent, or present at low concentrations, in various types of infant formulae (dairy and vegetarian), as opposed to considerably higher levels in maternal milk.
SUMMARY and CONCLUSION: We are presenting a first animal model for infant 'failure-to-thrive', based on a dysfunctional endocannabinoid system and suggesting to alleviate this condition by endocannabinoid (2-AG) or endocannabinoid-enhancing supplementation.

MODULATION OF NEUROPATHIC PAIN BY ENDOCANNABINOIDS

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Introduction: Neuropathic pain develops as a consequence of peripheral nerve injury or degeneration. Because it is difficult to treat even with potent analgesic drugs there is a great medical need for novel pharmacotherapies. Recent studies suggest that CB2 selective ligands may fill that need, as they seem to be effective in animal models of neuropathic pain. In this study, we have investigated CB2 mediated mechanisms in the modulation of neuropathic pain.

Methods: We used genetically modified mice lacking or overexpressing cannabinoid CB2 receptors to evaluate the contribution of these receptors in the development of neuropathic pain. We performed partial sciatic nerve ligation followed by nociceptive, histological and expression profiling studies. INF-γ and CB2 receptor double knockout mice, as well as irradiated wild type mice receiving bone marrow transplantation from CB2 knockout animals were used to address the role of inflammatory responses.

Results: CB2 knockout animals subjected to a partial nerve injury developed mechanical allodynia and thermal hyperalgesia to a similar extend as wild type control animals on the ipsilateral side of the nerve injury. However, unlike wild type mice, they also displayed increased pain sensitivity on the contralateral side. In contrast, transgenic mice overexpressing CB2 receptors had attenuated neuropathic pain responses. Expression profiling studies revealed an enhanced INF-γ response in the absence of CB2 receptors. Analysis of double knockout animals confirmed that the enhanced INF-γ response caused the expansion of the hyperalgesic area in the absence of CB2 receptors.

Discussion: These results suggest that CB2 mediated mechanisms contribute to the local containment of the hyperalgesic response after peripheral nerve injury. CB2 receptors thus represent an interesting therapeutic target for the treatment of neuropathic pain.

FUNCTIONAL SUPRA-SPINAL CB_2 RECEPTORS IN NEUROPATHIC MODEL OF PAIN

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There is growing evidence for the presence of cannabinoid CB_2 receptors in the CNS in addition to the periphery (Van Sickle *et al.,* 2005; Gong *et al.,* 2006). In neuropathic states, CB_2 receptors are up-regulated in the spinal cord and their activation attenuates evoked responses of spinal neurones (Sagar *et al.,* 2005). Similarly, activation of CB_2 receptors in the ventral posterolateral thalamus (VPL) of neuropathic rats also attenuates evoked responses of VPL neurones (Jhaveri *et al.,* 2008). The aim of this study was to investigate whether activation of supraspinal CB_2 receptors modulates neuropathic pain behaviour.

Spinal nerves L5 and L6 of male Sprague-Dawley rats (100-125 g) were tightly ligated to induce peripheral mononeuropathy. An intracerebral guide cannula was stereotaxically implanted for injection into the VPL nucleus of the thalamus or the, basolateral amygdala (BLA) and lateral ventricle, 7-8 days following spinal nerve ligation (SNL). Fourteen to eighteen days post-SNL surgery, effects of intracerebral or intracerebroventricular (icv) injection of the opioid morphine, the cannabinoid agonist CP55940 and the selective CB_2 receptor agonist JWH133 on mechanical paw withdrawal threshold, a measure of mechanical allodynia, were studied. Data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test.

In SNL rats, mechanical paw withdrawal threshold was significantly ($P < 0.001$, $n = 26$) reduced in the nerve-injured hindpaw (2.6 ± 0.2 g), compared to the contralateral paw (14.5 ± 0.2 g). Intra-VPL injection of morphine (20 µg in 500 nl) increased ($P < 0.001$, $n = 7$) paw withdrawal thresholds for 15-90 min (9.1 ± 1.6 g *vs* 2.3 ± 0.3 g) post-injection compared to vehicle. Similarly, intra-VPL injection of CP55940 (15 µg in 500 nl) also significantly ($P < 0.001$, $n = 7$) increased paw withdrawal threshold for 15-120 min (7.9 ± 1.5 *vs* 2.3 ± 0.3 g) post-injection, compared to vehicle. Intra-VPL injection of JWH133 (46 ng in 500 nl) did not alter paw withdrawal threshold ($P > 0.05$, $n = 5\text{-}8$), compared to vehicle controls. By contrast, intra-BLA injection of JWH133 (46 ng in 500 nl) increased ($P < 0.05$, $n = 6$) ipsilateral paw withdrawal threshold at 15 min post-injection (9.2 ± 1.4 g *vs* 3.3 ± 0.4 g), compared to pre-injection baseline values. ICV injection of CP55940 (40 µg in 1 µl), but not JWH133 (40 µg in 1 µl), significantly ($P < 0.05$, $n = 7$) attenuated changes in paw withdrawal threshold at 15 min post-injection (7.1 ± 2.2 *vs* 1.5 ± 0.3 g) compared to pre-injection values.

These data indicate that activation of cannabinoid CB_2 receptors in the basolateral amygdala, but not the VPL thalamus or periventricular brain regions, may be able to modulate nociceptive processes in neuropathic pain. Our data suggest that further studies are required to elucidate fully the potential central sites of action of cannabinoid ligands acting at CB_2 receptors.

Gong *et al.,* 2006, Brain Res 1, 10-23
Jhaveri *et al.*, 2008, Eur J Neurosci, in press
Sagar *et al.,* 2005, Eur J Neurosci 22, 371-379
Van Sickle *et al.,* 2005, Science 310, 329-332

IDENTIFICATION OF THE THIAZOLYLIDENE AMIDE A-836339 AS A POTENT AND SELECTIVE CB_2 RECEPTOR AGONIST FOR PAIN MANAGEMENT

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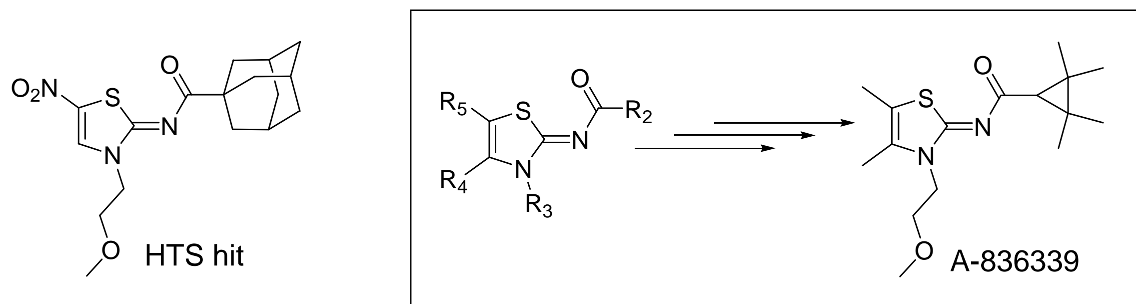
Research directed toward the design of highly selective CB_2 receptor ligands for the treatment of pain continues to gain increased attention. A variety of structurally distinct CB_2-selective agonists has demonstrated efficacy in preclinical models of inflammatory and neuropathic pain. Specifically targeting CB_2 receptors is anticipated to minimize CNS-mediated events, such as sedation, euphoria, and appetite stimulation, which are elicited by activation of the CB_1 receptor and have plagued the development of non-selective cannabinoid agonists. A 5-nitro thiazolylidene derivative was identified as a hit from a high throughput screen with good CB_2 receptor affinity and binding selectivity versus the CB_1 receptor. Thiazolylidene benzamides possessing potent CB_2 receptor activity have been independently disclosed by Taisho, although no *in vivo* efficacy has been reported for this compound class. We explored structural modifications of the thiazole imine acyl moiety, pendant N(3)-sidechain, and C(4)- and C(5)-substituents. Potency and selectivity were assessed by radioligand binding assays performed in cell lines that express recombinant human CB_2 or CB_1 receptors. Functional efficacy at the CB_2 receptor was measured in a calcium flux (FLIPR) assay. Numerous full agonists were found to possess the combination of potent CB_2 receptor activity and high selectivity versus the CB_1 receptor binding site. These SAR investigations led to the identification of A-836339, which exhibits robust efficacy in a rat model of chronic inflammatory thermal hyperalgesia induced by complete Freund's adjuvant (CFA) injection into the hind paw. Efficacy was completely blocked by pretreatment with a selective CB_2 antagonist, and not by CB_1 or μ-opioid receptor antagonists, suggesting that the observed *in vivo* activity is mediated through activation of CB_2 receptors. Among several analogs in this thiazolylidene series, a strong correlation exists between *in vitro* potency in CB_2 receptor functional assays and *in vivo* activity in the CFA model.



MOLECULAR ARCHITECTURE OF ENDOCANNABINOID SIGNALING AT NOCICEPTIVE SYNAPSES

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The striking antinociceptive effect of cannabinoid receptor agonists indicates that endocannabinoid signaling may have a pivotal role in the regulation of nociception. Endocannabinoids are well known retrograde synaptic signaling molecules in the brain, but the underlying molecular basis of endocannabinoid signaling involved in pain modulation is largely unknown at the level of the spinal cord. Since 2-arachidonoylglycerol (2-AG) is emerging as the predominant candidate molecule for synaptic endocannabinoid signaling, we studied the detailed anatomical distribution of diacylglycerol lipase-alpha (DGL-α), a biosynthetic enzyme of 2-AG and its receptor, the CB_1 cannabinoid receptor, in the dorsal horn of spinal cord. Dorsal horn neurons expressed high levels of DGL-α mRNA demonstrated by non-radioactive *in situ* hybridization, while riboprobes directed against CB_1 mRNA visualized only a small population of local neurons. Peroxidase-based immunocytochemistry revealed dense distribution of CB_1 and DGL-α, especially in the superficial dorsal horn, the first site of modulation of the ascending pain pathway. Finally, presynaptic localization of CB_1 and postsynaptic positioning of DGL-α on the two sides of nociceptive excitatory synapses were demonstrated by high-resolution immuno-electronmicroscopy. These data provide a neuroanatomical basis for 2-AG-mediated suppression of nociceptive transmission at the spinal level and contributes to the understanding of spinal mechanisms of pain control. In addition, the striking postsynaptic position of DGL-α at excitatory synapses formed by incoming nociceptive fibers suggests that pharmaceutical agents regulating intrinsic 2-AG levels may have important therapeutic potential in the treatment of pain.

ACTIONS OF THE MAGL INHIBITOR URB602 IN CHRONIC PAIN MODELS

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Despite multiple pharmacological agents have been employed, the clinical need for pharmacotherapies against pain is still a matter of debate. Nowadays, the therapeutic potential of cannabinoids as anti-inflammatory and analgesic agents was largely described. However, the search of cannabic drugs that are effective and devoid of unwanted side effects remains predominant. The enhancement of the endocannabinoid tone, for example through the inhibition of the enzyme MAGL responsible for 2-AG hydrolysis, could be an alternative strategy to avoid undesirable CNS effects and treat pain. We recently demonstrated that the inhibition of MAGL by URB602 showed an anti-inflammatory and anti-nociceptive effect in the carrageenan model of acute inflammation (Comelli et al., 2007, *Br. J. Pharmacol.* 152, 787). Based on this, the present study wanted to extend the knowledge of URB602 analgesic actions investigating its anti-nociceptive properties in different murine models of chronic pain. The dose employed (10 mg/kg i.p.) was the maximal one devoid of cannabimimetic activity, as we previously demonstrated (Comelli et al., 2007, *Br. J. Pharmacol.* 152, 787). URB602 was primarily tested in the mouse formalin test. A single preventive administration of URB602 abolished both the early and the late phase of formalin-evoked nociceptive behaviours. Subsequently, URB602 was tested in the neuropathic pain model of chronic constriction injury of the sciatic nerve (CCI). Only the daily treatment with URB602 for 7 days abolished thermal hyperalgesia and mechanical allodynia, assessed by Plantar test and Dynamic Plantar Aesthesiometer, respectively. The relieve of neuropathic pain behaviour was in fact absent when URB602 was given acutely. Collectively, these results showed for the first time that URB602 systemic administration was able to counteract not only the symptoms associated with an acute inflammation but also those associated with a well-established pain behaviour. Antagonism studies, performed in order to investigate URB602 mechanism of action, suggested that both CB1 and CB2 receptors contributed to the anti-nociceptive effects evoked by URB602 in both animal models. Thus, 2-AG, whose central and peripheral levels increased in presence of URB602, activated on one hand CB1 receptors determining an inhibition of pain transmission, and on the other hand, it activated CB2 receptors on immune cells inhibiting proinflammatory and proalgogen mediator release, modulating negatively pain transmission. It is not excluded the involvement of AEA in the effect elicited by URB602, whose presence probably determined a shift of 2-AG metabolism towards FAAH, which consequently decreased AEA hydrolysis increasing in this way its levels.

FAAH MODULATION OF NEUROPATHIC PAIN: A DISSOCIATION BETWEEN PHARMACOLOGICAL AND KNOCKOUT APPROACHES

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Cannabinoids have been used for thousands of years as therapeutic analgesics. Recent work has focused on the endogenous cannabinoid system in modulating pain, stress, and inflammation. URB597, an irreversible inhibitor of fatty acid amide hydrolase (FAAH), increases concentrations of endocannabinoids by reducing their rapid degradation by FAAH. In the present study, chronic constrictive injury of the sciatic nerve (CCI), a common model of nerve injury, was used to test the hypothesis that neuronal pain is modulated by endogenous cannabinoids. Male C57BL/6 mice were subjected to CCI and tested for pain sensitivity behaviors. The Hargreaves plantar stimulator test was used to assess hyperalgesia to noxious heat stimuli. Mechanical and cold allodynia were measured using the Von Frey and acetone-induced cold allodynia tests, respectively. The nerve injured paw displayed thermal hyperalgesia as well as mechanical and cold allodynia, with no effect on the contralateral paw. URB597 (10 mg/kg i.p.) significantly attenuated both mechanical (mean threshold VEH: 0.58 g; URB: 1.26g) and cold allodynia (mean paw withdrawal VEH: 11.58s; URB: 4.43s), but had no effect on CCI-induced hyperalgesia. These anti-allodynic effects were entirely reversed by pretreatment with the CB_1 antagonist SR141716 (rimonabant), indicating that the observed analgesic effects of URB597 are mediated by a CB_1 receptor mechanism. Surprisingly, FAAH (-/-) mice that had undergone the CCI procedure displayed a similar magnitude of increased nociception as wild type mice. This lack of a phenotypic decrease in hyperalgesia or allodynia in the knockout mice may result from accommodation due to chronically elevated endogenous cannabinoids or a decrease in receptor expression and/or sensitivity. The mechanisms of the disparity between pharmacological treatment and the knockout model are targets of current investigation and may help elucidate the role of endocannabinoid regulation of pain.

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EFFECTS OF THE FAAH INHIBITOR URB597 ON FEAR-CONDITIONED ANALGESIA AND ASSOCIATED ALTERATIONS IN SIGNAL TRANSDUCTION PROTEINS, LEVELS OF 2-AG AND FATTY ACID AMIDES

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The endocannabinoid system regulates nociception and aversion and mediates fear-conditioned analgesia (FCA). We investigated the effects of the fatty acid amide hydrolase (FAAH) inhibitor, URB597, on expression of FCA, and fear- and pain-related behaviour *per se*. We also examined associated alterations in signal transduction proteins Akt and ERK 1/2 in the periaqueductal gray (PAG) and levels of 2-arachidonylglycerol (2-AG), oleoylethanolamide (OEA), and palmitoylethanolamide (PEA) in the basolateral amygdala.

Rats received an intra-peritoneal injection of URB597 (0.3 mg/kg) or vehicle 30 minutes prior to intra-plantar injection of formalin or saline. Pain- and fear-related behaviour were assessed 30 min post-formalin for a 15 min period in a context paired 24 hr earlier with mild footshock (0.4 mA x 1s x 10) as described previously (Finn *et al*. 2004, *Eur J Neurosci* **20**, 848-852). Following the 15 minute behavioural testing, brains were removed and frozen. Tissue from the PAG and basolateral amygdala was obtained using Pavlovits punch; relative levels of phosphorylation of ERK1/2 and Akt in the PAG were determined using a PhastSystem Western blotting protocol. 2-AG, OEA and PEA were quantified by LC-MS. Statistical analysis employed ANOVA followed by Fisher's LSD.

URB597 enhanced FCA and attenuated a formalin-evoked reduction in fear-related behaviours. Fear-conditioning increased Akt phosphorylation in the PAG and URB597 reduced PAG Akt and ERK1/2 phosphorylation in fear-conditioned rats. Formalin injection reduced the fear-induced increase in Akt phosphorylation. Formalin-evoked nociceptive behaviour was associated with reduced levels of 2-AG and PEA in the basolateral amygdala of non-fear-conditioned rats but not fear-conditioned rats. Fear-conditioning was associated with reduced levels of 2-AG and an increase in OEA in the basolateral amygdala. Effects of fear-conditioning on PEA were dependent on the presence or absence of nociceptive tone.

These data provide evidence for enhancement of FCA by the FAAH inhibitor URB597 and differential modulation of fear-related behaviour, kinase phosphorylation in the PAG, and levels of 2-AG, OEA and PEA in the basolateral amygdala, in the presence or absence of formalin-evoked nociceptive tone.

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A RANDOMIZED DOUBLE-BLINDED CROSSOVER STUDY ASSESSING THE EFFECT OF CANNABINOIDS ON SPASTICITY IN SPINAL CORD INJURED PERSONS: A PILOT STUDY

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Objectives: To determine whether nabilone (CESAMET®), a synthetic oral cannabinoid, can alleviate spasticity in people with spinal cord injury.

Methods: 12 subjects were enrolled in this randomized double-blind, placebo-controlled crossover parallel group study. They were randomized to receive either nabilone 0.5 mg OD or placebo during the first two-week period of the study. Subjects were then given the option to increase the dosage of their treatment for the next two weeks to 0.5 mg PO BID, or to maintain at 0.5 mg OD. The subject could drop back to 0.5 mg OD at any time. At the 4th week of treatment, outcome measures and tolerance were assessed. After a two-week washout period, subjects were crossed-over to the opposite arm for 4 weeks, then again were assessed for tolerance and outcome measures.

The primary outcome measure was the Ashworth scale for spasticity assessment in the most involved muscle group of the body, as chosen by the subject and clinician. The secondary outcome measures include Spasm Frequency Scale, Visual Analog Scale, the Wartenberg Pendulum test, sum of the Ashworth Scale in eight muscle groups of each side of the body, including upper and lower limbs, and the Subject's and Clinician's Global Impression of Change.

Results: One subject dropped out of the study, after taking 4 doses of the placebo, secondary to be diagnosed with urinary tract stricture. Eleven subjects completed the study. For the primary outcome measure, tone measurement via Ashworth in most spastic muscle group, the subjects had a significant decrease while on active treatment, with a mean difference of 0.909, (SD=0.85, p=0.0039). Additionally, the active treatment arm had significant decrease in the total Ashworth score (p=0.0010). Visual analog scale only trended towards significant difference after active treatment (p=0.0762). There was no statistically significant difference in Spasm frequency scale, the Clinician and Subject Global Impression Scores, or the Pendulum test. Side effects were generally mild and tolerable.

Conclusion: Nabilone may be beneficial to improve spasticity in people with spinal cord injury, with tolerable side effects, although larger studies are needed.

DETERMINATION OF RIMONABANT THIOPHENE ANALOGUES IN RAT PLASMA AND BRAIN BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

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Introduction: Rimonabant and other pyrazole derivatives have been widely studied for their CB1 antagonism in different in vitro and in vivo models. The lead compound has been successfully employed in human as anti-obesity drug. In order to evaluate structure-activity relationship due to the modification of the different substituents at the various position of the pyrazole core, a new series of cannabinoid pyrazole derivatives have been recently obtained by PharmaNess. Among these new class of compounds, we have synthesized thiophene substituted analogues of Rimonabant (i.e. NESS006A, and NESS014A).
To evaluate pharmacokinetic profile and brain absorption of these compounds, we have developed in this study new HPLC-MS/MS based methods.

Methods: A HPLC system (Waters separation module Alliance 2695) was used to inject 5µl aliquots of processed samples. The chromatographic was performed using Xbridge C18 2.1*100, 3.5um, at 35°C. HPLC mobile phase flow rate was set at 0.2 ml/min, with gradient elution starting at 10% acetonitrile and 90% water (0.1% formic acid), followed by a linear increase of acetonitrile composition to 100%. Quantification was achieved by multiple reaction monitoring (MRM) detection in the positive ion electrospray, using a Quattro Micro triple quadrupole mass spectrometer (Micromass, Waters). The source condition were as follows: Capillary voltage 1kV, Source temperature 120°C, Desolvation temperature 350°C, Desolvation gas flow 800L/min, Cone gas flow 50L/h, Collision cell pressure 3.2 e^{-3} mbar, Dwell time 0.1 sec, Inter-Scan delay 0.1 sec, Inter-Channel delay 0.02 sec. The precursor –product ions pairs monitored were: 463.3> 362.9, 469.1> 368.8 and 469.1> 382.9 respectively for SR141716A, NES006A, and NESS014A. Standard stock solutions (1mg/ml) of SR141716A, NESS006, and NESS014 were prepared in methanol. Analytical standard samples were prepared by spiking known quantity of standard solutions into blank rat plasma and brain. The plasma and homogenate brain samples were purified using protein precipitation technique and solid-phase extraction.

Results: LC-MS/MS identified methods demonstrate no interfering peak from endogenous components in studied complex biological samples. For all the assayed derivatives the method is sensitive and specific starting from both rat plasma and brain.

Conclusion: HPLC-MS/MS based procedures have been optimized to determine cannabinoid thiophene-pyrazole derivative levels in both rat plasma and brain.

DEVELOPMENT OF CONFORMATIONAL CONSTRAINED ANALOGS OF SR141716: SYNTHESIS, COMPUTATIONAL ANALYSIS AND BIOLOGICAL EVALUATIONS

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Numerous derivatives of SR141716, the first drug that selectively blocks both the *in vitro* and *in vivo* effects of cannabinoids mediated by the CB1 receptor, have been developed and their structure-activity relationships have been widely studied. However, the conformational properties of SR141716 and its analogs have only recently received attention. In efforts to determine the ligand and receptor conformations for optimal receptor recognition and inverse agonist activity, we have designed and synthesized a number of derivatives of SR141716 that have ortho substituents on the aryl rings at the 1 and 5 positions, including a four carbon-bridged molecule, to further constrain the conformational mobility of the diaryl ring systems. 2D and variable temperature NMR studies were carried out to study their conformational properties. The results on the four carbon-bridged compound suggested the existence of atropisomers, which would allow for the study of the orientation of aryl rings when interacting with the CB1 receptor. Computational analysis on the rotational energy barriers performed in Sybyl and Spartan suggested a ~20 kcal/mol energy barrier for the rotation of the two aryl rings in the four carbon-bridged compound. The affinity of the compounds was determined by competitive displacement assays with [^{3}H]CP55940 and [^{3}H]SR141716 as ligands. Most compounds showed nM affinity for the CB1 receptor, but all lower than SR141716. The decreased affinity of these conformationally-constrained compounds as compared to SR141716, indicates that our approaches either constrained the ring systems in an orientation less optimal for interaction than that of SR141716; or introduced steric bulk that leads to disfavored steric interactions with the receptor.

QUINOLYL, ISOQUINOLYL, AND QUINOXALINYL PHENYL AMINES AS CB2 RECEPTOR AGONISTS

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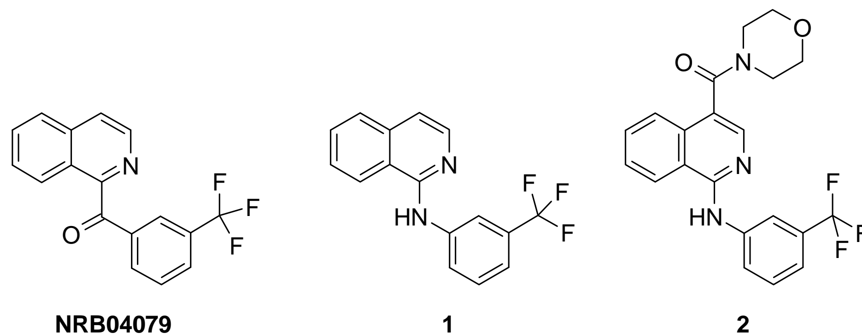
Recent research evidence has demonstrated the therapeutic potential for the selective CB2 receptor ligands. CB2 selective cannabinoids are expected to devoid of undesired CB1-mediated psychotropic side effects and would have therapeutic value in the pain relief, inflammation, osteoporosis, and in treating cancers.

Various CB2 selective compounds have been developed recently [1]. Here, we present series of quinolinyl, isoquinolinyl, and quinoxalinyl phenyl amines, which were synthesized and their CB2 receptor-dependent G-protein activities were determined using the [35S]GTPγS binding assay. Our previously reported CB2 agonist **NRB04079** (isoquinolin-1-yl-[3-(trifluoromethyl)phenyl]methanone) served as a lead structure in our search for more potent CB2 agonists [2]. **NRB04079** was found in a molecular database search study using a CB2 receptor model and it act as a partial agonist at the human CB2 receptor (-logEC50 = 5.3 ± 0.2; *E*max = 53 ± 4 %).

Most of the prepared quinoline, isoquinoline, and quinoxalinyl phenyl amines showed low-potency partial CB2 receptor agonists activity. The isoquinolin-1-yl(3-trifluoromethylphenyl)amine **1** and 4-morpholinylmethanone derivative **2** appear to be the most promising ligands for further development. The amine **1** showed to be a high efficacy CB2 agonist (-logEC50 = 5.8 ± 0.1; *E*max = 128 ± 4 %) and the derivative **2** show considerable potency at CB2 (-logEC50 = 7.4 ± 0.2; *E*max = 75 ± 2 %). All the synthesized compounds were also screened at 10 μM concentration for their CB1 agonist activity and for their ability to antagonize agonist (10 nM HU-210) response in rat cerebellar membranes as previously described [3]. No significant CB1 receptor activation or inactivation was shown in these studies. These ligands serve as novel templates for the development of selective CB2 receptor agonist.



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SYNTHESIS, SAR EVALUATION AND MOLECULAR MODELING OF MODIFIED PHENANTHRIDINES: NOVEL AND SELECTIVE CB2 AGONISTS

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Non-selective cannabinoid (CB) agonists are known to exhibit potent analgesic and anti-inflammatory effects. However, these therapeutic benefits are often accompanied by undesirable side effects which restrict their clinical use. Since the unwanted side effects of CB agonists are mostly CNS-dependent and attributed to activation of CB1 receptors, our aim was to identify compounds selective for CB2 receptors. High throughput screening of our in-house library of compounds resulted in the identification of a number scaffolds that showed potent CB2 agonist activity in the nanomolar range. A modified phenanthridine scaffold was chosen for further exploration, due to ease of synthesis and promising CB2 selectivity in addition to potency. A short SAR program yielded compounds with cAMP $EC_{50} < 10$ nM for hCB2 and $EC_{50} > 10$ μM for hCB1. We will describe the synthesis and SAR of this series and provide a rationale for the activity and selectivity, based on molecular modeling comparison studies with known CB2 selective agonists. These compounds may be further optimized for drug development, and they may provide useful tools for the investigation of CB2-specific pharmacology.

IDENTIFICATION OF THE HEMOPRESSIN BINDING SITE AT CB1

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Introduction: Hemopressin (**HP**) and a truncated version of HP have been reported to function as cannabinoid CB1 inverse agonists (Heimann et al. PNAS 104, 20588 (2007)). **HP** has been shown previously to have a hypotensive effect *in vivo* (Blais et al. Peptides 26, 1317 (2005)), in addition to its antinociceptive activity against inflammatory pain (Dale et al. Peptides 26, 431 (2005)). The **HP** peptide sequence, Pro-Val-Asn-Phe-Lys-Phe-Leu-Ser-His, contains charged N- and C-termini in addition to a positively charged lysine at position five. Most cannabinoid ligands are very lipophilic, yet **HP** is readily dissolvable in saline. In a companion abstract (J.Norris et al.), we report that both HP and the truncated version of **HP** (Pro-Val-Asn-Phe-Lys-Phe) *self-neutralize* two of their three charges in a non-polar environment leaving only a charged N-terminus.

Methods: Output from a Monte Carlo/simulated annealing technique, Conformational Memories (**CM**) study of Pro-Val-Asn-Phe-Lys-Phe (see companion abstract, J. Norris et al.) was used as input for receptor docking studies. The peptide was docked in our CB1 receptor model (including extracellular and intracellular loops, N- and C-termini) that had been pre-equilibrated in 50 ns NAMD molecular dynamics simulations in a POPC bilayer. The peptide in each of its two highly populated conformations (extended vs. bent) was docked in the CB1 model and the complex was energy minimized using the CHARMM force field.

Results: The following interactions were identified for the HP peptide: Pro 1 hydrogen bonds with D2.63 and D6.58. Asn 3 hydrogen bonds with K3.28 and D6.58 and the backbone carbonyl of Val2. Phe 4 has an aromatic stacking interaction with W6.48 and its backbone N hydrogen bonds with M6.55. Lys 5 hydrogen bonds to the peptide C terminus and also to the backbone carbonyl of Y5.39. Phe 6 has an aromatic stacking interaction with F3.25. In this docked position, Phe 4 blocks W6.48 from undergoing the $\chi 1$ g+→trans transition associated with receptor activation.

Conclusions: The ability of the HP peptide to block the W6.48 $\chi 1$ g+ →trans transition is likely key to its inverse agonism at CB1. [Support: NIDA DA03934 and DA021358]

COMPARATIVE MODELING OF THE TRANSMEMBRANE HELICAL BUNDLE OF THE CB_1 RECEPTOR

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Sequence alignment of 19 subgroups of rhodopsin-like GPCRs suggested that most helices of the CB_1 receptor would be aligned well by the highly conserved residues within the TM helices. However, TM helix 5 was an exception partly due to the absence of the highly conserved Pro residue in the middle of the helix. In the present study, two different sequence alignment rules, the one without a gap within TM helix 5 and the other with a gap within TM helix 5, were applied for the construction of four separate TM helical bundle models of the CB_1 receptor: two (**Models 1** & **2**) using the x-ray structure of rhodopsin (Okada et al., *J. Mol. Biol.* **2004**, *342*, 571) as template; and two (**Models 3** & **4**) using the x-ray structure of the human β2-adrenergic receptor (β2-AR) (Cherezov et al., *Science* **2007**, *318*, 1258) as template. The loops connecting the neighboring helices were generated by using the comparative loop building program *Modeller* (Fiser et al., *Prot. Sci.* **2000**, *9*, 1753). The entire system composed of each CB_1 receptor model embedded in a POPC lipid bi-layer was simulated by the *NAMD* simulation package (Phillips et al., *J. Comput. Chem.* **2005**, *26*, 1781) for 30 ns in the NPAT ensemble at 1 atm and 310 K, using the *CHARMM* force field (Feller and MacKerell, *J. Phys. Chem. B.* **2000**, *104*, 7510).

It was revealed that these receptor TM helical bundles within the membrane bi-layer became structurally stabilized in approximately 20 ns of simulations, as indicated by the convergence with small rmsd (< 3 Å) compared with the initial structures. Comparison of the receptor TM helical bundles of the CB_1 receptor showed that they shared a similar overall topology but with some distinct local structural features. The most plausible TM helical bundle model of the receptor was determined by evaluating the degree of the inter-helical interaction, such as the helical extent, the number of the salt bridge and the number of the hydrogen bond. It was **Model 3** that formed more salt bridges than any other TM helical bundle models. Identified TMH bundle model, providing the definitive helical boundary of each helix, would be valuable in determining the loop conformation for the construction of a reliable homology model of the CB_1 receptor.

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MODELING STUDIES OF THE CB1/DOPAMINE D2 $_{short}$ HETERODIMER USING CORRELATED MUTATION ANALYSIS

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Kearn and Glass first reported evidence that CB1/Dopamine D2 receptor complexes exist, are dynamic, and are agonist regulated with highest complex levels detected when both receptors are stimulated with sub-saturating concentrations of agonist. [1] Recently Marcellino and co-workers reported evidence for antagonistic CB1/D2 receptor-receptor interactions within CB1/D2 heteromers.[2] Only the $D2_{short}$ receptor (like CB1) is expressed pre-synaptically. Therefore we have constructed a model of the $D2_{short}$ receptor, including extracellular and intracellular loops, N and C-termini. This model is being used together with our current CB1 model to identify the most likely sites for formation of CB1/$D2_{short}$ heterodimers. To this end, we are employing a BioInformatics approach that uses an algorithm to calculate correlated mutations (Correlated Mutation Analysis, CMA).[3] Multiple sequence alignments performed with the CLUSTALW program, [4] used the human sequence for each receptor as reference. Each of these multiple sequence alignments was then used as input to the CMA computational procedure that automatically calculates correlated mutations on the lipid-exposed faces of the transmembrane (TM) helices.[5] Solvent accessibility values were calculated from the atomic coordinates of our current 3D models of the CB1 receptor and the $D2_{short}$ receptor, both refined from an initial rhodopsin template.[6] Possible heterodimer interfaces are currently being identified by considering only residues that form "interaction neighborhoods". These are regions of the receptor sequence where at least three residues identified by CMA appear close to each other, i.e., within i+7 on the same alpha helix.

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CANNABINOID RECEPTOR INTERACTING PROTEIN ($CRIP_{1a}$) AFFECTS CB_1 SIGNALLING

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A recently discovered protein, Cannabinoid Receptor Interacting Protein ($CRIP_{1a}$) interacts with the C-terminal tail of the CB_1 receptor (aa 418-473). Initial studies found that $CRIP_{1a}$ decreased the effects of the inverse agonist SR141716A (SR1) on Ca^{2+} currents in superior cervical ganglion neuronal expression system, while leaving the effects of the full agonist WIN 55,212-2 unchanged, suggesting a decrease in constitutive activity (Niehaus et. al. Molecular Pharmacology (2007) 72:1557-1566). To examine $CRIP_{1a}$, previous studies in this laboratory have characterized HEK cells transfected with CB_1, with and without $CRIP_{1a}$ co-transfection. Stable co-transfection of $CRIP_{1a}$ had no effect on CB_1 expression levels in HEK cells stably expressing CB_1 receptor (hCB_1-HEK) or CB_1 receptor and $CRIP_{1a}$ (hCB_1-HEK $CRIP_{1a}$) (B_{MAX} (pmol/mg); hCB_1-HEK: 1.87 ± 0.26, hCB_1-HEK $CRIP_{1a}$: 2.01 ± 0.29). $CRIP_{1a}$ protein expression was verified using immunoblot analysis. The effect of $CRIP_{1a}$ on the efficacy of various full, partial and inverse agonists were examined using [^{35}S]GTPγS binding assays. Significantly lower E_{max} values were found with the full agonists WIN 55,212-2 (79 ± 2.4 with $CRIP_{1a}$, 111 ± 6.6 without) and CP 55940 (75 ± 7.6 with $CRIP_{1a}$, 100 ± 9.3 without) in hCB_1 HEK cells co-expressing $CRIP_{1a}$ compared to hCB_1-HEK cells. Importantly, the inverse antagonism by SR1 was greatly reduced in the presence of $CRIP_{1a}$ (-7.3 ± 1.2 with $CRIP_{1a}$ versus -13.3 ± 1.6 without), in agreement with observances by Neihaus et. al. $CRIP_{1a}$ did not affect receptor stimulation by methanandamide, Δ^9-tetrahydrocannabinol, or levonantradol. The effect of $CRIP_{1a}$ on downstream signaling was examined. hCB_1 HEK cells (±$CRIP_{1a}$) were treated in culture and lysates were analyzed for cAMP levels via [^{3}H] cAMP kit or phosphorylation of MAPK via immunoblot analysis. $CRIP_{1a}$ did not affect inhibition of cAMP generation or MAPK phosphorylation for full or partial cannabinoid agonists. However, there was a definite trend toward a decrease in the ability of SR1 to act as an inverse agonist, suggesting a decrease of constitutive activity in the CB_1 receptor. Studies are currently underway to measure cAMP generation and MAPK phosphorylation in more sensitive testing protocols, including competitive immunoassay for quantitative determination of protein levels. Studies are also underway to examine the affect of $CRIP_{1a}$ on agonist affinity states by varying Na^+ concentrations in [^{35}S]GTPγS binding studies.

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SYNERGISTIC EFFECTS OF CB_1, MUSCARINIC AND δ-OPIOID RECEPTOR AGONISTS ON INTRACELLULAR Ca^{2+} MOBILIZATION IN SH-SY5Y CELLS

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CB_1 receptors are coupled to variety of intracellular signalling pathways including activation of inwardly rectifying potassium channel and inhibition of both voltage-operated Ca^{2+} channels and adenylyl cyclase activity. However, like with other $G_{i/o}$-coupled receptors, CB_1 receptor activation can also significantly elevate intracellular free Ca^{2+} ($[Ca^{2+}]_i$) although the mechanisms underlying this phenomenon are still not well understood. In human neuroblastoma SH-SY5y cells, $G_{i/o}$-coupled receptors like δ- and μ-opioid receptors are known to exert this effect in a significantly more efficacious way after stimulation of $G_{q/11}$-coupled M_3 muscarinic receptors with carbachol (CCh) (Connor & Henderson, *Br. J. Pharmacol*., 1996; 117, 333-40). We report here that also CB_1 activation weakly elevates $[Ca^{2+}]_i$ in SH-SY5y cells, and that co-stimulation with carbachol causes a dramatic enhancement of this effect.

In SH-SY5y cells, activation of CB_1 receptors with ACEA slightly increased the intracellular free Ca^{2+} mobilization (maximal effect=20.9 % of the maximal effect observed with 10^{-3}M CCh, EC_{50}: $2.04\pm0.77\times10^{-7}$M), measured in the absence of extracellular Ca^{2+} and using Fura-2AM as intracellular Ca^{2+} fluorescent probe. However, when an almost inactive concentration of ACEA (10^{-9} M) was applied 5 min after CCh (10^{-6}M), it produced a dramatically stronger (~6.7-fold) elevation of $[Ca^{2+}]_i$ than without CCh. This effect was attenuated by antagonists of both CB_1 (AM251) and muscarinic (atropine) receptors. The elevation of ACEA effect by CCh was inversely correlated with ACEA concentration (87.3%, 108.8, 153.8 and 201.5% of the maximal effect of ACEA 10^{-6} M *per se*, with ACEA 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M, respectively, and 10^{-6}M CCh), and directly correlated with CCh concentration (EC_{50} $1.6\pm1.2\times10^{-8}$M with 10^{-9} M ACEA). CCh enhanced in a similar way the effect on $[Ca^{2+}]_i$ of the selective agonist of the δ-opioid receptor, DPDPE. We also investigated the effect on Ca^{2+} mobilization by DPDPE after stimulation of CB_1. DPDPE elevated intracellular Ca^{2+} with an EC_{50} of $1.12\pm0.57\times10^{-7}$M. When DPDPE was applied 5 min after ACEA (10^{-6}M) its EC_{50} increased to $2.26\pm1.04\times10^{-8}$M, and its maximal effect on $[Ca^{2+}]_i$ by ~3-fold. Thus, unlike the stimulatory actions of CCh, the effect of ACEA on DPDPE was directly correlated to the concentration of both ACEA and DPDPE, and represents the first example of the enhancement of $[Ca^{2+}]_i$ in the presence of concomitant stimulation of two $G_{i/o}$-coupled receptors (δ-opioid and CB_1). All the stimulatory effects described above were inhibited by selective antagonists of the corresponding receptors involved, as well as, to a lower extent, by the phospholipase C (PLC) β inhibitor, U73122, and by pertussis, but not cholera, toxin. Therefore, the convergent activation by CB_1 and M_3 muscarinic receptors, and by CB_1 and δ-opioid recptors, of intracellular Ca^{2+} mobilization in SH-SY5y cells might involve G protein βγ subunits released upon $G_{i/o}$-coupled receptor activation. The partial inhibitory effect of U73122 suggests that further sites of interaction might occur also down-stream of PLC activation, or involve non-U73122-sensitive PLC isoforms.

The present data might be relevant to the previously observed pharmacological synergic interactions between CB_1 receptor and muscarinic or δ-opioid agonists in the control of synaptic plasticity or nociception, reward and anxiety, respectively.

DIFFERENTIAL COUPLING OF G PROTEINS TO CB1 RECEPTORS IN HIPPOCAMPAL GLUTAMATERGIC AND GABAERGIC NEURONS

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The discovery that CB1-expressing cells can be divided into distinct neuronal subpopulations raised questions about their involvement in different physiological and pathological processes as well as about differences in the pharmacological actions of cannabinoids. In order to precisely distinguish between the functions of GABAergic and glutamatergic neurons expressing CB1, two conditional mutant mouse lines have been generated in our lab: $CB1^{f/f;dlx5/6-Cre}$ and $CB1^{f/f;NEX-Cre}$ mice. In $CB1^{f/f;dlx5/6-Cre}$ mice, CB1 receptor is specifically deleted in all forebrain GABAergic neurons, while $CB1^{f/f;NEX-Cre}$ mice lack CB1 expression on cortical glutamatergic neurons. CB1 expression levels in both mouse lines were determined by *in situ* hybridization and immunostaining. The aim of this study was to quantitatively determine the expression and function of the CB1 receptor in these conditional mutant mice. In order to analyze the expression pattern of the CB1 receptor in the hippocampal formation of $CB1^{f/f;NEX-Cre}$ mice, double *in situ* hybridization studies were performed using a radioactive riboprobe for CB1 and a non-radioactive riboprobe for GAD65 (65kD isoform of glutamic acid decarboxylase, a marker for GABAergic neurons) to detect CB1 and GAD65 mRNA expressing cells, respectively. CB1 expressing GABAergic interneurons and total number of GABAergic cells were counted in brain sections of 3 mutant and 3 wild-type mice. 27% of the GAD65-positive cells expressed CB1 in both $CB1^{f/f;NEX-Cre}$ and in wild-type littermates. For the functional analysis of the CB1 receptor in conditional mutant mice, [^{35}S]GTPγS binding assays were performed in hippocampal and cerebellar tissue homogenates of $CB1^{f/f;dlx5/6-Cre}$ and $CB1^{f/f;NEX-Cre}$ mice and their wild-type littermates. We found no significant differences in the cerebellar preparations, however, in both mutants half of the CB1 coupled G protein signalling activity was lost in the hippocampus. Measuring the amount of G proteins coupled to CB1 receptors by [^{35}S]GTPγS saturation showed that in the $CB1^{f/f;NEX-Cre}$ mutants a greater proportion of CB1 coupled G protein signalling is lost than in $CB1^{f/f;dlx5/6-Cre}$ mutants, each compared to their wild-type littermates. In summary, we conclude that deleting CB1 from forebrain GABAergic neurons leads to a major loss of CB1 expression at both mRNA and protein levels. $CB1^{f/f;NEX-Cre}$ mice showed no Cre-mediated CB1 recombination in the GABAergic neurons. While only a small amount of CB1 mRNA and protein is missing in the hippocampus of $CB1^{f/f;NEX-Cre}$ mice, a large fraction of the CB1-coupled G protein signalling is lost. Therefore, our results indicate a differential coupling of G proteins to CB1 receptors in hippocampal glutamatergic and GABAergic neurons.

ALLOSTERIC MODULATION OF THE CANNABINOID CB_1 RECEPTOR

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In 2005, the first evidence was obtained indicating that the cannabinoid CB_1 receptor contains an allosteric binding site (Price et al, 2005). The modulators (Org27569, Org27759 and Org29647) display a markedly divergent effect on orthosteric ligand affinity versus efficacy; they are allosteric *enhancers* of agonist binding *affinity* and allosteric *inhibitors* of agonist signalling *efficacy*.

Because Org27569 is highly aromatic, we sought an interaction site that could provide aromatic stacking interactions with the allosteric ligand and found that Org27569 could find more aromatic stacking interactions in its extended conformation. We identified a binding site for Org27569 between TMH5 and TMH6 on the lipid face of CB_1 in its R* state. At this site, Org27569 has aromatic stacking interactions with F6.60 and W6.48. The interaction with W6.48 stabilizes this complex in the activated (R*) state because W6.48, together with F3.36, is one of the toggle switch residues that controls the R to R* transition in CB_1 (McAllister et al, 2004). This binding site is consistent with experimental findings (Price et al, 2005) that CP55940 affinity increases in the presence of Org27569, whilst the affinity of SR141716A is reduced. Org27569 is stabilizing the state for which the agonist, CP55940 would have higher affinity, but the state for which the inverse agonist SR141716A would have lower affinity, the R* state. The binding of Org27569 at this TMH5/6 binding site would inhibit movement of TMH6 and thereby produce interference with CP55940 signalling.

Here we investigate the effects of the Org compounds on HEK cells expressing various mutations in the amino acid residues of the cannabinoid CB_1 receptor using a [^{35}S]GTPγS binding assay in CB1-HEK293 cell membranes. We find that in hCB1-HEK293 (wild type) and the W6.48A and F3.36A mutants the Org27569 behaves as an inverse agonist, inhibiting basal [^{35}S]GTPγS signalling. In line with the molecular modelling hypotheses, the Org2769 is significantly less potent as an inverse agonist in these mutants as compared to the wild type CB_1-HEK293 (see Table 1).

Table 1: pEC_{50} and E_{max} values for inverse efficacy of Org27569 in a [^{35}S]GTPγS binding assay.

Membranes	E_{max} (%) (95% confidence limits)	pEC_{50} (95% confidence limits)
CB_1 wild type	26 (20 – 33)	8.88 (8.08 – 9.68)
W6.48A	32 (21 – 44)	6.54 (5.76 – 7.31)
F3.36A	40 (27 – 54)	6.86 (6.13 – 7.60)

These findings raise the possibility that these allosteric modulators may trap the CB_1 receptor in a high affinity coupled non-signalling state (RG_{GDP}).

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INVESTIGATION OF THE EFFECTS OF CANNABINOID LIGANDS IN BRAIN MEMBRANES DERIVED FROM $CB_1^{-/-}$ AND $CB_2^{-/-}$ MICE

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The cannabinoid CB_2 receptor exists mainly peripherally in immune cells and the cannabinoid CB_1 receptor mainly in the CNS. However, there is evidence to suggest that CB_2 receptors are present in the brain (Onaivi *et al*, 2006, Van Sickle *et al*, 2005). This raises the possibility that the CB_2 receptor may have a role in mediating the effects of cannabinoids in addition to the CB_1 receptor. Furthermore, evidence is emerging for the existence of novel, non-CB_1, non-CB_2 cannabinoid receptors in the mammalian brain, an example being the orphan G-protein coupled receptor GPR55 (Ryberg *et al*, 2007). Despite there being evidence for the existence of CB_2 and non-CB_1, non-CB_2 cannabinoid receptors in the brain, their functional significance and pharmacological profile is still not clear.

We investigated the ability of several cannabinoid ligands to increase $[^{35}S]GTP\gamma S$ binding in brainstem, cerebellum and forebrain membranes derived from wild type C57bl/6, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice.

In the brainstem, cerebellum and forebrain membranes prepared from the wild type C57bl/6 mice, neither potency nor E_{max} values differed significantly across the different brain regions for CP55940, anandamide or Δ^9-THC. CP55940 produced E_{max} values in the range of 77-80%. Anandamide had a lower efficacy, with E_{max} values in the range 22-33%, and Δ^9-THC produced E_{max} values of 7-24%.

In all brain regions, the stimulation of $[^{35}S]GTP\gamma S$ binding by CP55940, anandamide and Δ^9-THC were either significantly attenuated or abolished when membranes were obtained from $CB_1^{-/-}$ mice, suggesting that the CB_1 receptor is solely or predominantly involved in mediating this effect of these ligands. However, in membranes derived from $CB_2^{-/-}$ mice, the efficacy of CP55940 was also significantly attenuated in both the cerebellum (E_{max} 56 %, 95% confidence limits 46-66) and forebrain (E_{max} 36%, 95% confidence limits 26-45%) in comparison to that seen in the same brain regions of the membranes derived from wild type mice (80%, (95% confidence limits 67-93%) and 77% (95% confidence limits 60-93%) respectively). However, in the brainstem of the $CB_2^{-/-}$ mice, CP55940 E_{max} was not significantly altered (90%, (95% confidence limits 81-99%)) in comparison to that seen in the wild type mice (71%, (95% confidence limits 58-83%)). Furthermore, the efficacies of anandamide and Δ^9-THC were not significantly altered in membranes prepared from any of the three brain regions of the $CB_2^{-/-}$ mice in comparison to those from wild type mice.

The results indicate that either the CB_2 receptor selectively contributes in some way to the effects of CP55940 in certain brain regions, or that CB_1 receptor pharmacology is altered by this particular genetic deletion of the CB_2 receptor from mouse brain.

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STUDIES IN TISSUES FROM $CB_1^{-/-}$ AND $CB_2^{-/-}$ MICE REVEAL JWH-015 IS AN AGONIST AT A NOVEL CANNABINOID RECEPTOR

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The cellular actions of cannabinoids are thought to be primarily mediated by the CB_1 and CB_2 subtypes of cannabinoid receptor. In recent years there has been an increasing amount of evidence for the existence of novel cannabinoid receptors; several cannabinoid compounds have been identified which bind to and activate GPR55 using a number of different approaches. In this investigation we have shown that the CB_2-selective agonist JWH-015 displays agonist properties at a site distinct from both CB_1 and CB_2 in native tissues. In this study we employed the $[^{35}S]GTP\gamma S$ binding assay using brain membranes and the electrically-stimulated isolated vas deferens in tissues taken from wildtype C57Bl/6, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. In brain membranes from wildtype mice, JWH-015 stimulated $[^{35}S]GTP\gamma S$ binding with an EC_{50} of 253 nM and an E_{max} of 31%. In brain membranes from $CB_1^{-/-}$ mice, JWH-015 stimulated $[^{35}S]GTP\gamma S$ binding with an EC_{50} of 308 nM and an E_{max} of 29.7%. Similarly, in $CB_2^{-/-}$ membranes, JWH-015 stimulated $[^{35}S]GTP\gamma S$ binding with an EC_{50} of 138 nM and an E_{max} of 29.5%.

We also investigated the ability of JWH-015 to inhibit electrically-evoked contractions of the isolated vas deferens, comparing tissue taken from wildtype, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. We found that JWH-015 retained the ability to inhibit electrically-evoked contractions in vas deferens taken from both $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. In wildtype C57Bl/6 tissue, JWH-015 inhibited electrically-evoked contractions with an EC_{50} of 8 nM and an E_{max} of 81%. In $CB_1^{-/-}$ tissue, the EC_{50} was 36 nM and the E_{max} was 35.8%, demonstrating partial inhibition of electrically evoked contractions. In the $CB_2^{-/-}$ vas deferens, JWH-015 inhibited electrically-evoked contractions with an EC_{50} of 10 nM and an E_{max} of 93%.

Taken together, these data suggest that neither the CB_1 nor CB_2 receptor alone is solely responsible for the actions of JWH-015 in these native tissue assays.

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THE GPR55 LIGAND, L-α-LYSOPHOSPHATIDYLINOSITOL, PROMOTES RHO-DEPENDENT CA^{2+} SIGNALING

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Recently it has been suggested that the orphan G protein-coupled receptor, GPR55, is a novel endocannabinoid receptor that may also be activated by the lyso-phospholipid, L-α-lysophosphatidylinositol (LPI). GPR55 mRNA is expressed widely throughout the body with high levels found in the adrenal glands, spleen, CNS and the gut, suggesting that, like the CB_1 receptor, it may play a role in regulating a wide range of physiological processes. In addition, endogenous lipid amides and phospholipids are key signaling intermediaries, controlling many aspects of cellular function. Thus, the proposed identification of GPR55 as a novel molecular target for these ligands suggests an important role for this receptor in lipid signaling.

So far a number of inconsistencies have been documented with respect to GPR55 pharmacology and its downstream signalling pathways, despite the fact that all the studies to date have been performed in the same cellular background (HEK293 cells). The sensitivity of GPR55 to cannabinoid ligands has been contested and there is also no consensus on GPR55 coupling to downstream signaling pathways. For instance, LPI mediated GPR55 signaling is associated with the activation of ERK Map-kinases and a modest increase in cytosolic Ca^{2+}, whereas cannabinoid ligands induce $G\alpha_{13}$ and RhoA activation via this receptor.

Thus, we set out to investigate LPI-mediated signaling in a HEK293 cell line stably over-expressing the recombinant human GPR55. In these cells we find that LPI results in a dramatic change in the cellular localization of GPR55 and stimulates a novel Rho dependent, Ca^{2+} signaling pathway. We confirm that LPI is a potent and efficacious ligand at GPR55, which is likely to influence cell function via activation of Rho-family GTPases.

THE NOVEL CANNABINOID RECEPTOR GPR55 IS FUNCTIONALLY EXPRESSED IN BRAIN

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Cannabinoid ligands have been understood to function through two well-characterized receptors termed CB1 and CB2. CB1 receptors have been found to be abundantly expressed in brain tissues and are believed to mediate endocannabinoid ligand signaling and pharmacology. While abundant CB1 receptors are likely to represent the principal brain sites for the action of endogenous and exogenous cannabinoids, a number of lines of evidence support possible brain roles for other as yet unidentified cannabinoid receptors. The studies that provide support for the existence of non-CB1/CB2 receptors have been performed with CB1 -/- and/or CB2 -/- mice. Recent work has described GPR55 as a novel cannabinoid receptor but has not demonstrated evidence for functional GPR55 receptors in tissues. We demonstrate using radioligand binding that specific binding of [^{3}H]-CP55940 is found not only in wild type mouse brain but also in both CB1-/- and GPR55-/- mouse brain when HU210 is the competitor ligand used to determine specificity. In contrast WIN55,212-2 only revealed specific binding in wild type and GPR55-/- animals whilst O-1602 revealed specific binding in wild type and CB1-/- animals only. Furthermore, when using GTPγS, HU210 stimulated binding in WT, CB1-/- and GPR55-/- mouse brain, whilst WIN55,212-2 only stimulated GTPγS binding in WT and GPR55-/- mouse brain and O-1602 and virodhamine only in WT and CB1-/- mouse brain. These data demonstrate that GPR55 is functionally expressed in the mouse brain and that GPR55 selective ligands can functionally distinguish these receptors. It is therefore proposed that both these receptors contribute to cannabinoid activity in the CNS.

PROSTANOID FP RECEPTOR VARIANT/WILD TYPE COMPLEXES EXHIBIT PRONOUNCED FUNCTIONAL RESPONSES TO THE PROSTAMIDE F2A ANALOG BIMATOPROST

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Anandamide is oxygenated by COX-2 to form prostamides (PG-ethanolamides) , which appear to be pharmacologically unique. The recent discovery of compounds that selectively and potently block prostamide effects suggests the existence of a receptor that preferentially recognizes prostamides. Nevertheless, to date, prostamide activity has been observed only in PGF_{α}-sensitive cells and tissues. It, therefore , seemed reasonable to adopt a prostamide receptor cloning strategy based on the premise that prostamide and prostanoid FP receptors are similar and encoded by the same gene.

We identified six novel alternative splicing variants of FP receptor mRNA (altFPs) in human and monkey ocular tissues . Compared to wt FP receptors, alternatively spliced FP receptors lack a 7TM domain and the carboxyl-terminus is extracellular. We investigated the physical interaction of FP and altFP receptors co-expressed in HEK293/EBNA cells.

A cells co-expressing FP and altFP receptors using immunoprecipitation and western blot analyses. The FP receptor dimerized or oligomerized with altFP receptors forming FP complexes. Ca^{2+} signaling studies (FLIPR) showed that both $PGF_{2\alpha}$ and bimatoprost (10^{-7}M) activated the FP/altFP receptor dimer . For FP/altFP , the fluorescence counts for bimatoprost treatment were very close to those of $PGF_{2\alpha}$, whereas bimatoprost only slightly activated wt FP receptors expressed as a single entity . The kinetic profile for Ca^{2+} mobilization produced by bimatoprost was different from that for $PGF_{2\alpha}$ in cells co-expressing FP and altFP receptors. $PGF_{2\alpha}$ elicited a robust and rapid increase in $[Ca^{2+}]_i$ followed by a steady state phase. In contrast, bimatoprost elicited an immediate and robust increase in $[Ca^{2+}]_i$, followed by a secondary phase Ca^{2+} wave. A prostamide antagonist, AGN 211335, selectively and dose-dependently inhibited the bimatoprost-induced secondary Ca^{2+} phase in cells co-expressing FP and altFP receptors but did not block the steady state phase produced by $PGF_{2\alpha}$. Moreover, AGN 211335 blocked bimatoprost-induced MLC phosphorylation and Cyr61 mRNA upregulation in cells co-expressing FP and altFP receptors but did not block the MLC phosphorylation and Cyr61 mRNA upregulation induced by $PGF_{2\alpha}$. It appears that bimatoprost may interact with FP receptor complexes, such as wtFP receptor dimerizing or oligomerizing with FP spliced variants, which may result in new ligand recognition sites and altered Ca^{2+} signaling.

CB_1 RECEPTOR AND NON-CB_1/CB_2 ANANDAMIDE RECEPTOR-MEDIATED DIFFERENTIAL S-NITROSYLATION OF MMP: A NOVEL ANGIOGENIC SWITCH FOR REGULATION OF ANGIOGENESIS

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Previously we showed that in HUVEC cells endogenous cannabinoid analog methanandamide produced angiogenesis and an increase in MMP (matrix metalloprotease; MMP2 and MMP9) activity either in the presence of CB_1 receptor antagonist rimonabant or following the knockdown of CB_1 receptor. In the present study we have found that in EAhy926 endothelial cells (that does not express CB_1 receptor) methanandamide produces pro-angiogenic responses in *in vitro* assay which could not be mimicked by the CB_1 cannabinoid receptor agonists WIN55212-2 or CP55940. CB_1 receptor antagonist rimonabant or CB_2 receptor antagonist SR144528 failed to block methanandamide-mediated angiogenesis suggesting the involvement of a non-CB_1/CB_2 anandamide receptor in this response. Further, we have found that in transiently CB_1 receptor expressing EAhy926 cells, CB_1 receptor agonist CP55940 inhibits sphingosine-1-phosphate (S1P)-induced angiogenesis. Taken together these results suggest that in endothelial cells CB_1 receptor and non-CB_1/CB_2 anandamide receptor activation produces anti-angiogenic and pro-angiogenic responses respectively.

In further investigation to determine the molecular mechanisms of these angiogenic responses, we have found that activation of CB_1 receptors and non-CB_1/CB_2 anandamide receptors produces differential phosphorylation of eNOS protein. Activation of CB_1 receptors produces higher degree of S-nitrosylation of MMP-9 in endothelial cells compared to that produced by the activation of non-CB_1/CB_2 anandamide receptors. We have also found that activation of CB_1 receptors inhibits VEGF (vascular endothelial growth factor) signaling.

Thus, results from this study suggest that endogenous cannabinoid anandamide receptors (CB_1 and non-CB_1/CB_2 anandamide receptor)-mediated differential S-nitrosylation of MMP9 regulates MMP9 activity and thereby regulate angiogenesis. These results also indicate that S-nitrosylation can play an important modulatory role in the regulation of angiogenic switch in endothelial cells. (This study was supported by AHA 0060377Z and BRG grant from North Carolina Biotechnology Center to SM, NIDA U24 DA 12385)

LIGAND AND REGION SPECIFIC ACTIVATION OF G-PROTEINS BY CB1 RECEPTORS AND NON-CB1 SITES IN THE 3D RECONSTRUCTED MOUSE BRAIN

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Receptor-mediated G-protein activity has previously been examined in specific brain regions thought to mediate the effects of cannabinoids (CBs). However, the relationship between ligand type and localization of receptor-mediated G-protein activity has not been examined using a novel whole-brain unbiased image analysis approach.

The ability of different CB agonists, 25 μM methanandamide (m-AEA), 3 μM CP-55,940 (CP) and 10 μM WIN55,212-2 (WIN), to activate G-proteins was first assessed in wild-type and CB1 knockout mice. Because WIN appeared to activate G-proteins via non-CB1 sites, naive mouse brains were then processed using 10 μM WIN in the presence and absence of the CB1 selective antagonist SR141716A (0.5 μM SR1). Both studies used agonist-stimulated [^{35}S]GTPγS autoradiography to localize G-protein activity in 3D reconstructed brain images, derived from coronal sections that were collected throughout the neuroaxis with an interslice distance of 200 μm. Each autoradiographic section was digitized, realigned to its neighbor using an intensity-based registration algorithm, and aligned sections were stacked into a volumetric image array and quantitated. Reconstructed brain volumes were spatially normalized into a common coordinate space as defined by a linear combination of brain templates derived from each condition (no agonist (basal), m-AEA, CP, or WIN). We used a voxel-based whole brain analysis strategy, Statistical Parametric Mapping (SPM), to localize differences in magnitude of [^{35}S]GTPγS binding in the reconstructed mouse brain.

All three agonists stimulated [^{35}S]GTPγS binding in brains from wild-type mice. No significant stimulation was found using CP in any region of brains from CB1 knockout mice, and stimulation by m-AEA was significant only in hypothalamus and dorsal tegmental nuclei in CB1 knockout mice. In contrast, WIN significantly stimulated G-protein activity in CB1 knockout mice mouse brains in several regions, including cortex, hippocampus, hypothalamus, amygdala, and dorsal tegmental nuclei. No significant WIN stimulation was found in basal ganglia and cerebellum. Further, WIN stimulation in wildtype brains in the presence of SR1 showed a similar regional profile of G-protein activation as in CB1 knockout mice. SPM analysis revealed a unique profile of CB1 receptor-mediated G-protein activity, varying both in magnitude and neuroanatomical localization, when using a maximally effective concentration of different CB agonists. These results also revealed WIN-stimulated activity mediated by non-CB1 sites that could be spatially localized and mapped throughout the reconstructed mouse brain.

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PROTEOME-WIDE CHARACTERIZATION OF MOUSE BRAIN 2-AG HYDROLASES

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Endocannabinoid signaling is terminated by enzymatic hydrolysis *in vivo*. Inactivation of anandamide by fatty acid amide hydrolase (FAAH) has been well characterized using both selective FAAH inhibitors and FAAH-deficient mice. Previous studies have demonstrated that monoacylglycerol lipase (MAGL) likely plays a significant role in 2-arachidonoyl glycerol (2-AG) degradation *in vivo*, but multiple enzymes can hydrolyze 2-AG *in vitro*, and the mechanism of 2-AG inactivation in the nervous system has yet to be fully elucidated. To address this issue we employed functional proteomics method to characterize all of the 2-AG hydrolases present in the mouse brain [Blankman et. al. 2007, Chem Biol 14:1347-1356] and we are working to relate these results to endocannabinoid signaling.

In order to identify 2-AG hydrolases expressed in the brain we utilized the activity-based probe fluorosphosphonate-biotin (FP-biotin), which inhibits >99% of mouse brain 2-AG hydrolase activity. We assembled a list of 32 candidate enzymes using FP-biotin enrichment of mouse brain homogenates and the advanced liquid chromatography-mass spectrometry (LC-MS) method ABPP-MudPIT (activity-based protein profiling-multidimensional protein identification technology). Each of these enzymes was recombinantly expressed and assayed for 2-AG hydrolase activity *in vitro*, resulting in the identification of several proteins that could hydrolyze 2-AG. Correcting the *in vitro* data for relative brain expression using a spectral counting method suggested that >98% of mouse brain 2-AG hydrolysis is catalyzed by three enzymes: MAGL (85%) and two uncharacterized alpha/beta hydrolases ABHD12 (~9%) and ABHD6 (~4%). These relative activities were confirmed in mouse brain homogenates using a panel of pharmacological inhibitors targeting the relevant enzymes.

Our results confirm that MAGL is the major 2-AG hydrolase in the brain and identify two novel 2-AG hydrolases, ABHD12 and ABHD6, which are also highly express in the CNS. Interestingly, initial characterization of MAGL, ABHD12, and ABHD6 suggests that these enzymes occupy distinct subcellular localizations, which may allow them to regulate distinct subcellular pools of 2-AG. Currently we are working to clarify the role that each protein plays in endocannabinoid mediated signaling by developing pharmacological and genetic tools to selectively disrupt their function. These studies should help to determine if MAGL, ABHD12 or ABHD6 are appropriate targets for pharmaceuticals that that may provide some of the therapeutic benefits of THC without the psychotropic side effects.

ROLE OF FAAH-2 IN ENDOCANNABINOID METABOLISM

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Fatty acid amide hydrolase (FAAH) is the principle enzyme in mice that degrades the endocannabinoid anandamide (AEA) and the anti-inflammatory lipid palmitoylethanolamide (PEA), among other fatty acid amides (Deutsch and Chin, Biochem Pharmacol (1993) 46; 791-6; Cravatt et al., Proc Natl Acad Sci U S A (2004) 101; 10821-6). Recently, a novel FAAH enzyme was identified (termed FAAH-2) that is expressed in higher mammals but not in mice and rats (Wei et al., J Biol Chem (2006) 281; 36569-78). To facilitate its characterization, Wei et al. incorporated a FLAG epitope tag at its N-terminus since the C-terminally tagged variant did not yield an active enzyme. Using the tagged FAAH-2, the authors demonstrated that FAAH-2 is a luminal membrane protein whose expression is six fold lower compared to FAAH. FAAH-2 catalyzed the hydrolysis of several N-acylethanolamines including AEA and PEA *in vitro*, albeit with 30 and 10-fold lower specific activity compared to FAAH, respectively.

Given the involvement of FAAH-2 in endocannabinoid and fatty acid amide metabolism, we decided to further characterize FAAH-2 and examine its capability to hydrolyze AEA and PEA in intact cells. To monitor the expression of FAAH-2, we independently tagged the enzyme with several C-terminal epitope tags and surprisingly found that these constructs yielded active enzymes whose activities were slightly lower compared to untagged FAAH-2. In agreement with the observations of Wei et al., we found that FAAH-2 was expressed at a significantly lower level compared to FAAH. In transfected cell homogenates, PEA served as a better substrate for FAAH-2 than AEA (V_{max} = 1.21 vs. 0.65 nmol/mg/min; Km = 4.3 vs. 7.9 μM, respectively), while the specific activity for FAAH was significantly higher for both substrates.

The contribution of FAAH-2 towards AEA and PEA metabolism was further examined by following the uptake and hydrolysis of these substrates in intact COS-7 and HeLa cells expressing either FAAH or FAAH-2. In contrast to the *in vitro* assays, FAAH-2 efficiently hydrolyzed AEA and PEA in intact cells. Following a one minute incubation, FAAH-2 metabolized approximately 30% of the AEA and PEA taken up by cells, a level approximately one-half that of FAAH. Preliminary immunofluorescence studies indicate that FAAH-2 localizes to perinuclear areas in COS-7 cells, suggesting that FAAH and FAAH-2 may both reside on the endoplasmic reticulum, while attaining different membrane orientations. More comprehensive immunolocalization studies using subcellular markers will be presented. Collectively, these data suggest that despite its lower expression level and enzymatic activity compared to FAAH *in vitro*, the ability of FAAH-2 to efficiently hydrolyze AEA and PEA in cells and its wide distribution in human tissues suggest that it may be an important regulator of endocannabinoid and fatty acid amide signaling.

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CALCIUM-INDEPENDENT FORMATION OF ENDOCANNABINOIDS IN RAT BRAIN SLICES

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Endocannabinoids (ECs) are believed to be synthesized in cell membranes, on demand, via calcium–sensitive phospholipases, although the objective evidence for this is limited. The aim of the present study was to investigate whether EC synthesis and release in rat cerebral cortical slices is driven by increased intracellular Ca^{2+} concentration. We have, therefore, examined the effects of different excitatory stimuli KCl, Ca^{2+} ionophore (ionomycin), Ca^{2+}-mobilising agents (glutamate and the cholinergic agonist carbachol), the Ca^{2+}-channel blocker (verapamil), non-selective calcium channel blockers Lanthanum hydrochloride, manganese sulphate ($MnSO_4$) and removal of Ca^{2+} from the medium on EC levels in rat cortical slices.
Brain slices from male Lister hooded rats (>250 g) were prepared as previously described (Sarmad *et al*., 2007) and EC levels measured by LC/tandem mass spectrometry. (Richardson *et al*., 2007). Statistical analysis (one way ANOVA, Kruskal-Wallis or Dunnett's multiple comparison test, compared basal levels with those following drug exposure.
Depolarising levels of KCl (50 mM) stimulated anandamide (AEA) synthesis significantly by 2.3±0.26 fold (mean±s.e.m) (n=6, P<0.05) and oleoylethanolamine (OEA) by 1.75±0.21fold (n=6, P<0.05) but had no effect on *N*-palmitoylethanolamine (PEA) and 2-arachidonoylglycerol (2-AG). Stimulation of excitatory amino acid receptors by glutamate (10 mM) and ionomycin slightly increased AEA levels (non significantly) but no changes were observed for OEA, PEA and 2-AG. The cholinergic receptor agonist carbachol (1mM) had no effect on ECs. Removal of $Ca^{2+}$from the medium (±EGTA, 300μM) did not altered either EC basal levels or URB597 stimulated levels suggesting that absence of calcium did not affect the ECs turnover and existence of other calcium-independent metabolic routes for EC formation. Lanthanum and $MnSO_4$ had no effect on EC levels while, Verapamil (100 μM) significantly increased 2-AG levels in cortical slices (P<0.05, n=12) by 2.5±0.33 fold compared to basal but had no effect on other ECs. Verapamil decreased elevations of AEA, OEA and PEA due to the FAAH inhibitor URB597 (1μM) but significantly (P<0.001, n=12) enhanced URB597-elevated 2-AG by 3.69±0.74 fold.
In conclusion, we observed little evidence for Ca^{2+} driving EC formation in rat brain slices. Also, verapamil may be a useful lead compound in the search for novel agents interfering with EC turnover.

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REGULATION OF *N*-ACYLETHANOLAMINE-HYDROLYZING ACID AMIDASE (NAAA) BY PROTEOLYSIS AND GLYCOSYLATION

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N-Acylethanolamine-hydrolyzing acid amidase (NAAA) is a lysosomal enzyme catalyzing the hydrolysis of various *N*-acylethanolamines including anandamide. Our previous studies revealed that the N-terminus of NAAA purified from rat lung is Cys-131, suggesting the proteolytic maturation of NAAA. In addition, we found that NAAA is modified with glycans. However, physiological roles of these posttranslational modifications of NAAA remained unclear. Thus, we examined the mechanism and importance of proteolytic cleavage and the location of glycosylation sites with recombinant human NAAA expressed in HEK293 cells. Western blot analysis using anti-NAAA antibody revealed that the major population of NAAA in the cell homogenates is the cleaved 30-kDa form. We also detected NAAA in the culture media, which was mostly the uncleaved 48-kDa form. When incubated at pH 4.5, the 48-kDa form was converted to the 30-kDa form in a time-dependent manner, and this conversion was well correlated with increase in its *N*-palmitoylethanolamine-hydrolyzing activity. Interestingly, this cleavage hardly occurred at pH 7.4 or in the presence of an SH-blocker, PCMB. When Cys-126, corresponding to Cys-131 of rat NAAA, was substituted with serine, the mutant existed exclusively as a catalytically inactive 48-kDa form. These results suggested that the proteolytic cleavage of NAAA at acidic pH is a critical step for its maturation. We also examined glycosylation sites of human NAAA by site-directed mutagenesis on six potential *N*-glycosylation sites. The results showed that Asn-37, Asn-107, Asn-309 and Asn-333 are actual glycosylation sites. The modification with glycans appeared to be required for the stabilization of NAAA.

THE TRANSCRIPTIONAL REGULATION OF DAGLα EXPRESSION

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Gene expression can be controlled at many levels in the pathway leading from DNA to protein and one principle mechanism is that of transcriptional regulation. The requirement of transcription factors for eukaryotic gene expression provides a huge level of complexity for cell type specific spatial and temporal control. Promoter analysis is a useful tool in understanding the molecular mechanisms of transcription regulation and has been used in this study as a novel approach to understanding and characterising the expression pattern of DAGLα to investigate the role of this enzyme in adult neurogenesis.

The sn1-diacylglycerol lipases, DAGLα and DAGLβ catalyse the hydrolysis of diacylglycerol (DAG) to the endocannabinoid 2-arachidonoylglycerol (2-AG). The pattern of expression of the DAGLs in the brain correlates with the proposed function of 2-AG, being spatially restricted to axonal tracts during development to synthesize 2-AG for axonal growth and guidance, and to dendrites in the adult to provide retrograde 2-AG signalling for regulating neurotransmitter release. Evidence of a dynamic expression pattern in the subventricular zone (SVZ) of the adult brain and the loss of proliferating cells following the addition of a DAGL inhibitor to the SVZ, suggests that the DAGLs may also play a role in the maintenance of a self-renewing neural stem cell population in adult neurogenesis.

The aim of this study is to characterise the promoter region of DAGLα to address the hypothesis that the dynamic expression pattern of DAGLα is due to transcriptional control. A promoter was predicted by bioinformatic analysis and cloned, such that a luciferase assay could be optimised to study promoter activity. The predicted DAGLα promoter was found to be active in a range of cell lines, both non-neural cell types, 3T3, Cos and HEK cells, and also in the neural stem cell Cor-1, NS5 and CGR8 lines. Deletion analysis of the full length promoter has identified a core promoter element required for maximal activity and a core region for suppression of activity. Conservation between species and transcription factor binding site predictions has enabled the identification of putative regulatory elements within these regions. Site-directed mutagenesis of a predicted Sp1 binding site from the core active region has revealed a role for Sp1 in a neural stem cell-specific regulation of DAGLα expression. Gel-shift analysis has confirmed that this predicted Sp1 site is a true Sp1 binding site.

This approach in the characterisation of DAGLα will enable the elucidation of putative regulatory pathways functioning upstream of DAGLα expression that can be further investigated to better understand the function of the endocannabinoid system in neurogenesis.

PROTEOMIC CHARACTERIZATION OF HUMAN MONOACYLGLYCEROL LIPASE OVEREXPRESSED IN *E.COLI*

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The serine hydrolase monoacylglycerol lipase (MGL) is primarily responsible for the efficient deactivation of 2-arachidonoylglycerol (2-AG), an endocannabinoid with full agonist activity at both cannabinoid receptors. Although MGL is recognized as a potential therapeutic target, the paucity of structural information on this enzyme has hindered the development of MGL-selective inhibitors. To provide a sufficient quantity of this enzyme for biochemical assays and for structural studies the recombinant hexa-histidine-tagged human MGL (hMGL) was overexpressed in *E.coli* and purified in a single step by Immobilized metal affinity chromatography (IMAC). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the hMGL identified: 1) an absence of intramolecular disulfide bridges in the functional, recombinant enzyme and 2) the post-translational removal of the protein's N-terminal methionine. The hMGL and the homologous native enzyme prepared from rat brain were shown to be similar in biochemical assays using both natural 2-AG and the novel fluorogenic arachidonoyl, 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) substrates. Two chemically distinct inhibitors: 5-((biphenyl-4-yl)methyl)-*N,N*-dimethyl-2*H*-tetrazole-2-carboxamide (AM6701) and *N*-arachidonylmaleimide (NAM) were used to characterize proteomically the hMGL active site. Suitable conditions were established for hMGL inhibition by AM6701, and the inhibitor-treated enzyme was subjected to trypsin digestion. MALDI-TOF and tandem MS analysis of the tryptic digest of AM6701-inhibited hMGL showed that the serine residue in a GXSXG motif of the putative MGL catalytic triad was carbamylated. These results provide the first direct confirmation of the essential role of this serine residue for catalysis and establish the mechanism underlying the potency and high selectivity of AM6701 as a covalent hMGL inhibitor. When applied this ligand-assisted proteomic approach to NAM-treated hMGL, we revealed a partial alkylation of cysteine residues 215 and/or 249 that was sufficient to achieve ~ 80% hMGL inhibition. Further alkylation at cysteine 39 did not increase the extent of enzyme inhibition. These data conclusively demonstrate a sulfhydryl-based mechanism underlying MGL inhibition by this fatty alkyl-maleimide substrate analog and allow us to propose a mechanism for MGL inhibition by maleimides. In addition to defining the molecular mechanisms of MGL inhibition by AM6701 and NAM, proteomic identification of hMGL amino acids critical to catalysis provides information useful in the design of selective MGL inhibitors as potential drugs.

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THE PHYSIOLOGICAL ACTIVITY OF 2-ARACHIDONOYLGLYCEROL IN THE STRIATUM IS REGULATED BY LIPID RAFTS

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Several G protein-associated receptors and synaptic proteins function within lipid rafts, which are subdomains of the plasma membranes that contain high concentrations of cholesterol. In the present study we addressed the possible role of lipid rafts in the control of endocannabinoid system (ECS) in the striatum.

Disruption of lipid rafts following cholesterol depletion with methyl-β-cyclodextrin (MCD) failed to affect synthesis and degradation of anandamide, while it caused a marked increase in the synthesis and levels of 2-arachidonoyl-glycerol (2-AG), as well as in the expression and function of cannabinoid CB1 receptors in the striatum. These effects, however, did not result in potentiated endocannabinoid-mediated control of synaptic transmission, since neither basal nor 3,5-DHPG-stimulated 2-AG produced measurable neurophysiological effects on striatal GABAergic mIPSCs. Along with the observations that elevation of 2-AG concentration with 3,5-DHPG caused a significant CB1 receptor-mediated inhibition of GABA transmission in control slices, and that synaptic responses to the direct CB1 receptor agonist HU210 were intact in MCD-treated slices, these data indicate that lipid rafts are essential for the maintenance of the correct localization and orientation of cannabinoid CB1 receptors with respect to the site of production of 2-AG.

Understanding the regulatory mechanisms of endocannabinoid metabolism and physiological activity is essential for the development of pharmacological interventions aimed at treating neurodegenerative disorders associated with abnormal activity of the ECS.

CIRCADIAN CHANGES IN REGIONAL FATTY ACID AMIDE HYDROLASE ACTIVITY IN THE MOUSE BRAIN

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Fatty acid amide hydrolase (FAAH) is a widely expressed serine hydrolase that metabolizes several fatty acid amides including anandamide and oleamide. It is the primary metabolizing enzyme of anandamide, since transgenic mice lacking FAAH have elevated endogenous anandamide levels, and do not efficiently metabolize exogenously administered anandamide (Cravatt *et al.* 2001 PNAS 98: 9371-9376). FAAH activity plays a role in regulating endocannabinoid tone, since the administration of FAAH inhibitors such as URB597 (Kathuria *et al.* 2003 *Nat. Med.* 9:76-81) and OL-135 (Lichtman *et al.* 2004 *JPET* 311:441-448) reduces nociception in mice.

Despite the wide distribution of this protein throughout the CNS and the central role it plays in modulating fatty acid amide tone, there has been only one study examining circadian changes in FAAH activity (Cell. Mol. Life Sci. 61 (2004) 945–950). Valenti *et al.* reported an increase in endogenous anandamide levels in the nucleus accumbens, prefrontal cortex, striatum, and hippocampus of the rat brain at night. This increase in endogenous anandamide tone complemented a decline in FAAH activity in the striatum and hippocampus at night.

Given the large physiological contributions of FAAH in the CNS, we studied circadian changes in FAAH activity and expression in the mouse brain. Wild-type C57Bl/6 mice were kept in LD12:12 and tested at the midpoint (sixth hour) of the light and dark cycles. At each time point, regional tissues, including the hippocampus, cortex, striatum, thalamus, and cerebellum, were collected and processed for quantitative real-time PCR, *in vitro* FAAH activity assays, and western blot analysis. In addition, other mice were intravenously administered [3H]-anandamide and their brains processed for *ex vivo* imaging (according to the protocol first described in Glaser *et al.* (2006) *JPET* 316:1088-1097).

Similar to the rat, FAAH activity declines in several regions of the mouse brain at 'midnight'. Western blots and real-time PCR confirm the reduction in protein levels and expression in several regions. *Ex vivo* imaging studies provide further evidence of regional circadian changes in FAAH activity. These data indicate FAAH's contribution in mediating endocannabinoid tone in the mouse brain varies both temporally and regionally.

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OMEGA, AND (OMEGA -1) - HYDROXYLATED METABOLITES OF *N*-ARACHIDONOYL DOPAMINE, ACTIVATE RECOMBINANT HUMAN $TRPV_1$ RECEPTORS

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Introduction: *N*-arachidonoyl dopamine (NADA) is an endogenous lipid that modulates signal transduction in nociceptive pathways. NADA activates the non-selective cation channel, transient receptor potential vanilloid type 1 ($TRPV_1$) and binds cannabinoid receptor 1 (CB_1). To investigate the metabolism of NADA through the cytochrome P450 (CYP450) metabolic pathway, we studied the *in vitro* rat liver microsomal production of hydroxylated metabolites and their activity at recombinant human $TRPV_1$ receptors.

Method: Rat liver microsomal fractions were isolated using differential centrifugation. The microsomal metabolites of NADA were partially purified from methanolic extracts on solid phase C-18 cartridges. Metabolites were identified by HPLC/quadrupole time-of-flight mass spectrometry and compared to synthesized standards. The most abundant hydroxylated metabolites were quantified by tandem mass spectrometry and tested for calcium influx on human recombinant $TRPV_1$ receptors over-expressed in human embryonic kidney cells (HEK293).

Results: Following microsomal activation in the presence of NADA, omega and (omega-1) hydroxylated metabolites were formed *(*19-HETE dopamine, 20-HETE dopamine). These hydroxylated NADA metabolites were active at recombinant human TRPV1 receptors, inducing a dose-dependent calcium influx (20-HETE dopamine, EC50= 1.5 μM; 19-HETE dopamine, EC50= 1.6 μM). Both metabolites exhibited lower potencies compared to NADA (EC50 = 650 nM).

Conclusions: CYP450 enzymes are capable of metabolizing several fatty acids (e.g. arachidonic acid) and fatty acid-containing lipids (e.g. *N*-arachidonoyl dopamine) forming a larger family of neuromodulators with potential activity at various lipid receptors. The current study shows that NADA metabolism occurs through microsomal enzymes, and that these metabolites are active at recombinant $TRPV_1$ receptors.

A NOVEL TYPE OF PROSTAMIDE/PROSTAGLANDIN F SYNTHASE, BELONGING TO THE THIOREDOXIN-LIKE SUPERFAMILY

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The resultant products of arachidonyl 1-ethanolamide (anandamide) are prostaglandin (PG) ethanolamides (prostamides), which are a pharmacologically novel class of substances. Prostamide F_{2a} potently stimulated cat iris contraction with a potency closely approaching that of the corresponding PGs, however, prostamide F_{2a} exhibited no meaningful interaction with the cat recombinant FP receptor. Although the physiological and pharmacological roles of prostamide $F_{2\alpha}$ appear to be distinct from those of $PGF_{2\alpha}$, the nature of the enzyme responsible for the synthesis of prostamide F_{2a} from prostamide H_2 has not yet been clarified. Prostamide F synthase, which catalyzed the reduction of prostamide H_2 to prostamide $F_{2\alpha}$, was found in mouse and swine brain. The enzyme was purified from swine brain, and its amino acid sequence was defined. The mouse enzyme consisted of a 201-amino acid polypeptide with a molecular weight of 21,669. The amino acid sequence placed the enzyme in the thioredoxin-like superfamily. The enzyme expressed in *E. coli* as well as the native enzyme catalyzed not only the reduction of prostamide H_2 to prostamide $F_{2\alpha}$ but also that of PGH_2 to $PGF_{2\alpha}$. The Vmax and Km values for prostamide H_2 were about 0.25 μmol/min.mg of protein and 7.6 μM, respectively; and those for PGH_2, about 0.69 μmol/min.mg of protein and 6.9 μM, respectively. Neither PGE_2 nor PGD_2 served as a substrate for this synthase. Based on these data, we named the enzyme prostamide/PG F synthase. Thioredoxin preferentially served as a reducing equivalent donor for this enzyme. Moreover, Northern and Western blot analyses in addition to the prostamide F synthase activity showed that the enzyme was mainly distributed in the brain and spinal cord, suggesting that prostamide/PG F synthase may play an important functional role in the central nervous system.

DEVELOPMENT OF A NOVEL MONOGLYCERIDE LIPASE ASSAY

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Monoglyceride lipase is a major player in the control of the endocannabinoid 2-arachidonoylglycerol levels. Inhibition of this presynaptic enzyme results in increased 2-arachidonoylglycerol levels and in enhanced retrograde signaling in the hippocampus. However, through study of both the enzyme and its substrate (patho)physiological roles are hampered by the paucity of inhibitors available. This prompted us to develop a new enzymatic assay, in a 96-well format, allowing for the high-throughput detection and characterization of putative monoglyceride lipase inhibitors.

We therefore looked for an ester-based substrate, that upon hydrolysis by monoglyceride lipase would release a chromophore group allowing for the quantification of the reaction. Two additional criteria were the λ_{max} of the product which needed to be different from the substrate one, and the solubility of both the substrate and its product. Meeting these criteria, a series of 4-nitrophenol esters were selected as putative substrates. As expected the 4-nitrophenol esters are hydrolyzed by our highly purified monoglyceride lipase demonstrating that they are indeed substrate of the enzyme. Because of the limited solubility of some substrates, we selected 4-acetoxynitrobenzene for further investigations. When adapted to a 96-well format this continuous assay allows for the easy determination of the kinetic parameters of the enzymatic reaction. 4-Acetoxynitrobenzene is hydrolyzed in a time and protein dependent manner along a michaelian kinetic.

To confirm the relevance of the inhibition data obtained, several known monoglyceride lipase inhibitors were tested in parallel using the well known radiolabeled assay and this novel spectrophotometric assay. For instance, the maleimide derivative *N*-ethylmaleimide, as well as the recently reported disulfiram showed IC_{50} values of the same magnitude in both assays.

Thus, the novel monoglyceride lipase assay reported here represents a useful alternative to the classic and more expensive radiolabeled assay, allowing for both the easy determination of the kinetic parameters of the enzyme and the screening for novel inhibitors using a 96-well format.

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THE HORMONE-SENSITIVE LIPASE INHIBITOR CAY10499 INHIBITS MONOGLYCERIDE LIPASE

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Hormone-sensitive lipase (HSL) is a vital enzyme involved in lipid metabolism. HSL is a serine hydrolase responsible for the hydrolysis of stored triglycerides into monoglycerides and non-esterified fatty acids, thus playing an essential role in providing fatty acids as an energy source for most tissues in mammals.[1,2] It has been reported to hydrolyze also various of di- and monoglycerides, as well as cholesteryl esters.[2] HSL is primarily expressed in adipose tissue.[2]

CAY10499 (Figure 1) has been reported to be a potent inhibitor of human HSL exhibiting an IC_{50} of 90 nM for the recombinant enzyme.[3,4] The present study shows that CAY10499 can also inhibit human recombinant monoglyceride lipase (MGL) and rat brain fatty acid amide hydrolase (FAAH) with IC_{50} values of 58 and 86 nM, respectively. These results indicate that CAY10499 may be utilized as a useful scaffold for further design of more potent inhibitors of both MGL and FAAH.

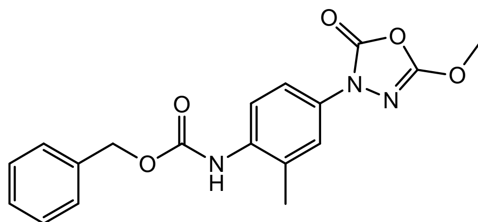

Figure 1. Chemical structure of CAY10499

[1] Raclot, T. *Prog. Lipid Res.* **2003**, *42*, 257-288
[2] Kraemer, F. B.; Shen, W. *J. Lipid Res.* **2002**, *43*, 1585-1591
[3] Ben Ali, Y.; Müller, G.; Petry, S., Verger, R.; Carrière, F.; Abousalham, A. *Abstracts / Chem. Phys. Lipids* **2005**, *136*, 85-166
[4] www.caymanchem.com

DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF NEW MONOGLYCERIDE LIPASE INHIBITORS

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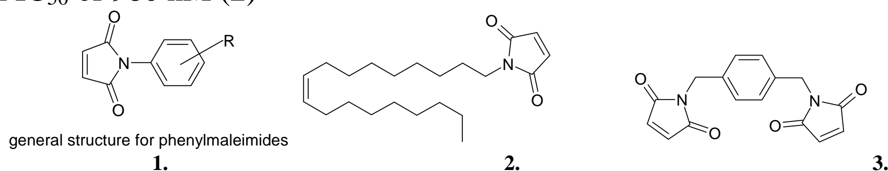
2-arachidonoylglycerol (2-AG), the most abundant endocannabinoid in the brain, is a lipid transmitter which activates the cannabinoid receptors and is involved in neurotransmitter release control. The useful pharmacological properties of 2-AG in neuroprotection, appetite regulation, or cell proliferation are limited by a rapid metabolisation by specific degradation enzymes. Among the enzymes known to hydrolyse 2-AG, monoglyceride lipase (MGL) is thought to be of prime importance in the regulation of 2-AG signalling.

Recently, a few maleimides were shown to inhibit MGL, but demonstrated only moderate inhibition potency. Thus, three series of maleimides were designed, synthesized and tested on a recombinant human MGL. Their activity towards human Fatty Acid Amide Hydrolase (FAAH) was also monitored.

Two series of phenylmaleimides and bismaleimides were synthesized from maleic anhydride and the appropriate amines. A third series was synthesized following the Mitsunobu procedure, using maleimide and the appropriate alcohol.
Among the phenylmaleimide derivatives synthesized (**1**), some compounds presented moderated inhibition of MGL with IC_{50} values in the low micromolar range, but interesting selectivity for MGL vs FAAH. Among the third series, the *N*-oleylmaleimide was the best inhibitor with an IC_{50} of 980 nM (**2**)



The 1,4-bismaleimidoxylene (**3**) constitutes, so far, the best inhibitor of our series, with an IC_{50} value of 138 nM on h-MGL.
As no selective and potent MGL inhibitor has been reported so far, these series of compounds constitute promising new inhibitors that may provide key information on MGL activity and therapeutic potential.

CHARACTERIZATION OF MAGL ACTIVITY AND ITS INHIBITION BY *N*-ARACHIDONYLMALEIMIDE (NAM) USING A COLORIMETRIC ASSAY

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The principle enzyme responsible for 2-arachidonylglycerol (2-AG) hydrolysis is monoacylglycerol lipase (MAGL). MAGL is a serine hydrolase belonging to a family of proteins that contain a catalytic triad with serine, aspartate, and histidine residues. A colorimetric assay was employed as an alternative to costly radio-labeled 2-AG or time-consuming mass spectrophotometric assays for measuring MAGL hydrolytic activity to measure the effects of mutations and an inhibitor upon activity.

MAGL obtained from transiently transfected COS7 cells was incubated with arachidonoyl-1-thio-glycerol, a thioester-containing analog of 2-AG, for 3-5 minutes at 37ºC. The thioglycerol that is reacted with 5,5'-dithiobis(2-dinitrobenzoic acid) (DTNB, Ellman's Reagent) results in the release of a thiolate ion (TNB) that emits a yellow color and has measurable absorbance at 412 nm that is used to calculate the rate of substrate hydrolysis by MAGL.

Incubation of cell lysate protein (5µg) with varying concentrations of arachidonoyl-1-thio-glycerol yielded a K_m= 67.96 ± 20.67 µM and V_{max}= 681.1 ± 66.91 nmol/min/mg (mean± SEM; n=3). This K_m is similar to the K_m of 67.8 ± 4.0 µM for hydrolysis of 2-AG by MAGL in rat cerebellar membranes (Saario et al., Biochem. Pharmacol. (2004) 67:1381-7). Membrane and cytosolic fractions contributed about equally to hydrolysis with 49.2±1% and 50.8±1% activity respectively (n=3). The rate of MAGL hydrolysis increased linearly with up to 11 µg COS7 cell lysate protein. These data suggest that this assay is feasible as a means to measure MAGL hydrolytic activity.

A competitive inhibition assay revealed that arachidonoyl-1-thio-glycerol is a slightly better substrate for MAGL than 2-AG. Four hundred micromolar 2-AG inhibited arachidonoyl-1-thio-glycerol (70 µM) hydrolysis by 63.7±10% (n=3). Mutation of the catalytic serine (Ser122) to an alanine residue reduced MAGL activity by 95.5±2% (n=3; P<0.0001). This loss of enzyme activity was seen in membrane and cytosolic fractions. Saario et al. (Chem. Biol. (2005) 12:649-656) tested the potency of several *N*-ethylmaleimide analogs and discovered that *N*-arachidonylmaleimide (NAM) was the most efficient inhibitor. Cys208 and Cys242 were proposed as potential residues near the catalytic site that react with NAM. A Cys208Ala mutant was indistinguishable from the wild-type. It was fully active and inhibited by NAM (10 µM) indicating that this cysteine is not targeted by NAM (n=3).

FAAH INHIBITION BY CNS-ACTIVE NATURAL PRODUCTS

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Fatty acid amide hydrolase (FAAH) recently emerged as an exciting new target for anxiety, depression and potentially other psychiatric disorders. As numerous plants have been used by natural medicine practitioners to treat patients suffering from anxiety and depression, we postulated that some of these natural products might act through the inhibition of FAAH.

Using the scientific literature, we selected medicinal natural products having either clinical validation data or significant pre-clinical validation data in animal models to obtain the following, non-exhaustive, list: 1) Grape seed (Vitis vinifera), 2) St John's Wort (Hypericum perforatum), 3) Wood leaf (Isatis indigotica), 4) Ginseng panax root (Panax ginseng), 5) Valerian root (Valeriana officinalis), 6) Lemon balm (Melissa officinalis), 7) Jujube fruit (Ziziphus jujube), 8) Passionflower (Passiflora incarnate), 9) Gotu kola leaf (Centella asiatica), 10) Gingko leaf (Gingko biloba), 11) Chinese Skullcap (Scutellaria baicalensis), 12) Ashwagondha root (Withania somnifera) and 13) Kava root (Kava kava).

To adequately extract compounds possessing a variety of properties we used Snyder's solvent classification model to select five solvents with different characteristics: water, ethanol, acetonitrile, ethylene chloride and methyl t-butyl ether. Powdered natural product material (0.9g) was extracted using 2.7mL of each solvent by sonicating the sealed mixture for 2h. The supernatant was then filtered when necessary and transferred to a glass vial for storage. Activity of the extracts was measured in parallel using two distinct assays. These were a biochemical assay using recombinant hFAAH-containing cell membranes hydrolyzing ^{3}H-AEA and a cell-based assay measuring endogenous hFAAH activity in the T84 cell line using AA-AMC as the substrate. Promising activity (>80% inhibition) was observed with 1.5% extract for the following conditions: extracts #1, 3, 10, 12 and 13 (ethanol) and extracts #2, 3, 9, 10 and 11 (acetonitrile). The potency of these extracts was further documented with concentration-response curves. Additional mechanism of action studies are also shedding light on whether these results might be physiologically relevant. In summary, extracts of medicinal natural products were demonstrated to inhibit FAAH activity in biochemical and cell-based assays.

SALVINORIN A INTERACTION WITH THE CANNABINOID SYSTEM

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The body of knowledge of *Salvia Divinorum* and its main active ingredient, Salvinorin A, is still at an early stage. Although very recent papers suggest that Salvinorin A could act as a k-opioid receptor selective agonist, some discrepancies between in vitro and in vivo results raise the possibility that other pharmacological targets, in addition to the k-opioid receptor, could exist. At this regard, some observations suggest a possible interaction of salvinorin A with the cannabinoid system. For example *Salvia divinorum* has been used as a marijuana substitute by Mexican youths; the structure of salvinorin A is lipid-like, and endocannabinoids are lipids; finally, recent papers support the involvement of the cannabinoid system in some behavioural effects of salvinorin A (Braida et al. Psychopharmacology 190:441, 2007, Braida et al. Biol Psychiatry. 63(3):286, 2008).

On these basis, in the present work we tested the hypothesis of possible interaction of salvinorin A with the cannabinoid system. To this aim we studied the ability of salvinorin A to bind cannabinoid receptor through competition binding experiments with [^{3}H]CP55,940 in striatal membranes. In parallel, spiradoline, a k-opioid agonist and CP-55,940 were also tested in the same binding conditions. As expected, unlabeled CP-55,940 was able to compete with the labelled compound for CB1 receptors. In contrast spiradoline did not alter [^{3}H]CP55,940 binding even when added at high molar excess. Salvinorin A did not alter the labelled compound binding within the range 3 pM-10μM, however at higher concentrations (30μM-1mM) it bound CB1 receptor with a curve that fit to one- site binding model. Since both CB1 and k-opioid receptors are coupled to G proteins we studied the stimulation of [^{35}S]GTPγS binding induced by Salvinorin A in comparison with the one induced by CP-55,940 and spiradoline. In order to discriminate the different involvement of CB1 and k-opioid receptors in Salvinorin induced GTPγS binding co-incubation with SR141716A or nor-BNI (the specific CB1 and k-opioid receptor antagonists respectively) have been used. SR141716A was not able to reverse Salvinorin A induced GTPγS binding whereas nor-BNI antagonized it. These results together suggest that Salvinorin A mainly acts as a k-opioid agonist. Finally, to test whether this compound could indirectly modulate cannabinoid system acting on FAAH enzyme we tested the ability of Salvinorin to alter FAAH activity. Different Salvinorin A concentrations (1nM, 10nM, 30 μM) were able to reduce FAAH activity on striatal membranes although in a dose-independent manner.

These results suggest that beside k-opioid receptor stimulation Salvinorin A could reduce FAAH activity increasing the endocannabinoid tone. This dual behaviour might explain the discrepancy between Salvinorin and the other k-opioid agonists and the ability of SR141617A to antagonize some behavioural effects.

DEVELOPMENT OF NEW TETRAZOLES THAT SELECTIVELY INHIBIT ANANDAMIDE CELLULAR UPTAKE

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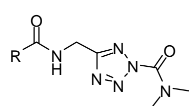
The endocannabinoid signaling system is involved in an increasing number of physiological and pathological conditions. Inhibitors of endocannabinoid degradation might offer a therapeutic approach to a variety of diseases in which elevation of endocannabinoid levels represent an adaptive reaction to restore normal homeostasis when this is pathologically perturbed. The levels of the two most studied endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are regulated in different, and sometimes even opposing ways. AEA is assumed to be transported into the cell by a specific mechanism and then rapidly hydrolyzed by the enzyme fatty acid amide hydrolase (FAAH), whereas a monoacylglycerol lipase (MAGL) is involved in degrading 2-AG. However, the existence of an AEA cellular uptake process partly independent of FAAH activity remains controversial and several hypotheses have been proposed, including passive diffusion, endocytosis, and intracellular sequestration.

We have investigated here the effect of the substitution of the *N,N*-dimethylaminocarbonyl portion of previously reported carbamoyl tetrazolic ureas that were found to inhibit AEA uptake (Moore et al., *P.N.A.S. USA*, **2005**, *102*, 17852), although with little selectivity over FAAH and monoacylglycerol and diacylglycerol lipases (MAGL and DAGL), the metabolic enzymes of 2-AG (Ortar, G. et al *Eur. J. Med. Chem.* **2008**, *43*, 62). From the previous compounds, the 4-biphenyl and biphenyl-4-carboxamido derivatives were selected since they were already lacking inhibitory activity against MAGL and DAGL. Groups entirely devoid of inherent carbamylating activity were inserted instead of the *N,N*-dimethylaminocarbonyl moiety, which is likely responsible for the covalent modification of hydrolases. As expected, this substitution yielded compounds with no significant inhibitory activity on FAAH, MAGL and DAGL at concentrations up to 50 μM. However, five compounds retained the ability to inhibit efficaciously [^{14}C]AEA cellular uptake by RBL-2H3 cells with IC_{50} values in the low micromolar range (2.3 – 5.1 μM), a result which seems to be in agreement with the existence of a FAAH-independent mechanism for AEA uptake. As also observed previously by us, tetrazole 1,5-isomers were generally more potent inhibitors than the corresponding 2,5-isomers. The inhibitory activity seems to depend also on the presence of some functional groups that might reinforce the interaction with the putative specific protein(s) involved in AEA uptake.

In conclusion, we reported the development of non-carbamylating tetrazole AEA uptake inhibitors that might be useful in the elucidation of the question of the existence of an AEA transporter.



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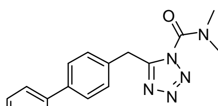
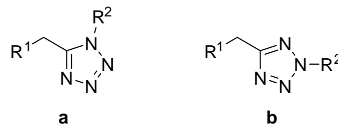


R=biphenyl-4-carboxamido-derivatives

Structures of non-selective carbamoyl tetrazole inhibitors of AEA cellular



R[1] = 4-biphenyl or biphenyl-4-carboxamido
a = 1,5 isomers ; b = 2,5 isomers
R^2 = $CH_2CON(CH_3)_2$, CH_2COCH_3, $CH_2CH(CH_3)_2$, CH_2CN, CH_2CO_2CH3

New tetrazole inhibitors

MODULATION OF THE CELLULAR PROCESSING OF ANANDAMIDE BY NITRIC OXIDE DONORS

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Anandamide (AEA) is metabolised by a process of cellular uptake followed by FAAH-catalyzed hydrolysis. It has been reported that following incubation of RBL2H3 basophilic leukaemia cells with AEA, its downstream hydrolysis products are recovered in lipid rafts (McFarland *et al., J Biol Chem* 279 [2004] 41991-7). It is not known whether this process of recycling of the arachidonate from FAAH to the plasma membrane is constitutive or regulated by signalling molecules. In the present study, the ability of nitric oxide (NO) donors to modulate the FAAH-dependent incorporation of label into cell membranes following incubation of intact cells with AEA has been investigated.

Cells in suspension were incubated with [^{3}H]AEA and then ruptured by rapid filtration through filters with distilled water. The tritium label retained by the filters represents the amount of label in the cell membranes. Two cell-lines where used: RBL2H3 cells, containing a large FAAH component, and 3T3-L1 fibroblasts known to be rich in caveolae but which have a low FAAH activity. Treatment of RBL2H3 cells with URB597 and other established FAAH inhibitors decreased the membrane retention of tritium by about 70%. The membrane labelling of the 3T3-L1 cells was approximately the same as seen for the FAAH-inhibited RBL2H3 cells, and was not affected by URB597. Jurkat cells, which naturally exist in suspension and express low FAAH activity, showed no accumulation of label.

The NO donors, S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) increased in a concentration-dependent manner the amount of tritium retained by the membranes from both cell-lines but the effect was not when intact adherent cells were studied. The FAAH activity was not affected by the NO donors and pretreatment of FAAH inhibitors prior to the NO donors prevented the increase of accumulation, suggesting that the effect produced were downstream of FAAH. Experiments using 3T3-L1 cells indicated that the increased accumulation in response to SNAP and SNP was not affected by the CB receptor inverse agonists AM251 or AM630, but was blocked by the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and the reducing agent dithiothreitol. Treatment of the cells with 8-bromo-cGMP did not mimic the effects of SNAP and SNP.

It is concluded that treatment with the NO donors SNAP and SNP increases the labelling of membranes with the downstream products of AEA after its metabolism by FAAH. This may be the result of actions both involving guanylyl cyclase and nitric oxide radicals.

SKIN PERMEATION OF ANANDAMIDE UPTAKE INHIBITOR N-(4-HYDROXYPHENYL)ARACHIDONYLAMIDE (AM404)

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Drug delivery research involving enhancers of endocannabinoid action, such as fatty-acid amide hydrolase and anandamide (AEA) uptake inhibitors, is necessary in order to optimize the duration of the beneficial properties. AM404 is an inhibitor of reuptake of the endogenous CB1 receptor agonist AEA and has been shown to increase the therapeutic effects of endogenous and administered AEA *in vivo*. AM404 has also been shown to have therapeutic value in animal studies for reducing inflammation and neuropathic pain, and decreasing alcohol self-administration. Administering AM404 by the transdermal route may increase its therapeutic efficacy by potentially decreasing the extent of AM404 metabolism after administration and increasing the circulation time of AM404 in the blood via controlled release. Local topical administration of AM404 directly to the site of action may also be therapeutically beneficial.

In this study, AM404 was investigated for its skin permeation rate in order to evaluate its capability for systemic transdermal and/or local delivery. *In vitro* diffusion studies of AM404 were completed using skin from hairless guinea pig (dermatomed to 0.2µm) in flow-through diffusion cells. The receiver solution consisted of HEPES-buffered Hanks' balanced salt solution with 40%v/v polyethylene glycol 400 (PEG 400) or 4%w/v bovine serum albumin (BSA). AM404 was prepared as a saturated solution, with excess solid, in a 3:1 mixture of propylene glycol and water. At the end of each experiment, drug disposition in the skin was assessed. The amounts of AM404 in donor, receiver, and skin samples were determined using high pressure liquid chromatography with UV detection and a new analytical method.

The *in vitro* fluxes of AM404 for receiver solutions containing 40% PEG and 4% BSA in guinea pig skin were 2.3±0.6 $nmol/cm^2/h$ and 1.7±0.6 $nmol/cm^2/h$, respectively. The mean drug content in the skin for the experiments using 40% PEG and 4% BSA receiver solutions were calculated to be 16.96±2.36 µmol/g of skin over 96 hours and 2.44±0.82 µmol/g of skin over 48 hours, respectively. The results indicated that a significant level of AM404 could be delivered via the transdermal route and that there is a potential for local delivery due to its retention in the skin. We expect to improve the skin penetration of AM404 for systemic delivery through formulation optimization and/or chemical modifications in future studies and also to assess skin penetration of AM404 *in vivo*. This work was supported by the American Cancer Society (RSG-00-027-04-CDD).

OLEAMIDE & PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA: A NOVEL SITE OF ACTION

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Oleamide (ODA) is a fatty acid primary amide first identified as an endogenous lipoamide in the cerebrospinal fluid of sleep-deprived cats. ODA is a sleep-inducing factor when administered *in vivo*, also eliciting hypothermia, analgesia and hypo-locomotion. *In vitro*, ODA has also been reported to induce vasorelaxation in the rat small mesenteric artery. While the biological effects of ODA are well documented, the molecular mechanisms and site of action remain elusive. However, i*n vitro*, ODA can inhibit gap junction formation, modulate GABA and 5-HT receptors and bind to CB_1 cannabinoid receptors. We have investigated whether ODA is able to act through the nuclear receptor subfamily of peroxisome proliferator-activated receptors (PPARs)

We have previously observed that the FAAH inhibitor URB597 was able to activate PPARγ in HeLa human cervical carcinoma cells, albeit at relatively high concentrations and without significant occupancy of PPARγ ligand binding site. A potential route for indirect enhancement of PPARγ activity would be through elevation of calcium ion concentrations, $[Ca^{2+}]_i$. Given that URB597 is reported to activate TRPA1 at these concentrations, we investigated the potential for URB597-evoked $[Ca^{2+}]_i$ elevations in these cells. Although carbachol and ATP evoked significant elevations in $[Ca^{2+}]_i$ in HeLa cells, URB597 was without effect. A further possibility for indirect effects of URB597 would be the elevation of endocannabinoid levels through inhibition of FAAH activity. We quantified mRNA levels for FAAH-1 in these cells and observed a much lower expression compared to SH-SY5Y human neuroblastoma cells. However, RT-PCR analysis revealed the expression of mRNA encoding FAAH-2 in HeLa cells. Enzymatic hydrolysis of anandamide in particulate preparations from HeLa cells was not different from background and was unchanged in the presence of URB597. FAAH-2 is reported to have a slight preference for ODA as a substrate compared to FAAH-1. Investigating enzymatic hydrolysis of ODA in these cells revealed a much lower level of activity compared to rat liver, but which was however inhibited by URB597. We investigated whether ODA was able to bind and activate PPARγ *in vitro*. Indeed, ODA was able to displace a fluorescent competitor ligand from the PPARγ binding domain in a concentration-dependent fashion. Moreover, ODA evoked a low potency concentration-dependent fold activation of PPARγ in a reporter gene assay.

In summary, we have identified a novel site of action of ODA, through PPARγ. We are currently engaged in quantifying levels of ODA in biological samples to establish whether URB597 is able to enhance levels of ODA in intact cell preparations.

HEMOPRESSIN: THE PARADOXICAL LIGAND

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Introduction: Recently, an exciting study characterized hemopressin (**HP**) as a new inverse agonist to the CB1 receptor. Earlier work illustrated the hypotensive effect of HP *in vivo,* in addition to antihyperalgesic activity in a model of experimental pain. The peptide sequence, Pro-Val-Asn-Phe-Lys-Phe-Leu-Ser-His, contains charged N- and C-termini in addition to a positively charged lysine at position five. Most cannabinoid ligands are very lipophilic, yet hemopressin is readily dissolvable in saline. We wanted to use the technique of Conformational Memories (**CM**) to investigate the structural properties of hemopressin that allow it to be both water soluble and yet bind to a G protein-coupled receptor that prefers very hydrophobic ligands.

Methods: The **CM** technique was applied to four separate systems, consisting of the full peptide and a truncated version of **HP** (Pro-Val-Asn-Phe-Lys-Phe) in two different implicit solvents representing water and non-polar environments. Briefly, **CM** consists of two phases, pre-biased and biased. Each complete **CM** run resulted in an output of 96 structures which were further analyzed using tools in the Maestro Suite. The output structures were clustered into conformationally similar families. Each family group was then further characterized by measuring centroid-centroid distances between the two phenylalanine residues and the distance between the lysine residue and the charged C-terminus.

Results: The results are summarized in Table 1. In all four systems, the same two major families (1(extended) and 2(bent)) were always present. A third family was present only in the truncated –non-polar system.

TABLE 1	**WATER**						**NON-POLAR**					
FAMILIES	**1**		**2**		**3**		**1**		**2**		**3**	
***L**ong/**S**hort*	L	S	L	S	L	S	L	S	L	S	L	S
% Members	17	16	45	60	0	0	27	41	43	39	0	20
Centroid Distance Å	10.00	10.39	8.04	7.25	-	-	9.97	10.33	7.23	6.82	-	10.30
Lysine-Cter Distance Å	15.00	8.36	15.20	7.93	-	-	3.31	3.37	3.29	3.33	-	3.37

Conclusions: The dual personality of hemopressin is evident in the above results.
When placed in a water environment, the peptide forms two major families and the lysine is readily solvated by water. Placing this same peptide in a non-polar environment results in different proportions of the same families. Most interestingly, the peptide *self-neutralizes* two of its three charges in a non-polar environment leaving only a charged N-terminus. [Support: NIDA DA03934 and DA021358]

ON THE "TOXIFICATION" OF ANANDAMIDE BY PATHOGENIC FUNGI: FORMATION OF 3-HYDROXY-ANANDAMIDE AND ITS ACTIVITY ON CANNABINOID AND TRPV1 RECEPTORS

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Pathogenic fungi like *Candida albicans* use their fatty acid β-oxidation pathway to transform arachidonic acid into 3-hydroxy-arachidonic acid (3-HETE), a metabolite with several biological actions (Ciccoli, et al. *Biochem J*. 2005, 390, 737-747.). We investigated whether *Dipodascopsis uninucleata*, a non-pathogenic but still 3-HETE-overproducing fungal species belonging to the same family of *C. albicans*, can transform anandamide into 3-hydroxy-anandamide (3-HAEA), and if such putative metabolite is still capable, like anandamide, to activate cannabinoid and vanilloid TRPV1 receptors.

Arachidonic acid or anandamide were incubated with *D. uninucleata* under conditions leading to 3-HETE formation, using 2 x 50 ml of culture and 100 microM of each compound. Incubation was carried out for 6 and 12 hr at 37°C, after which the reaction was stopped by extraction of cells plus media with methanol chloroform. Lipid purification was carried out by reverse phase HPLC. The major HPLC peaks were then submitted to liquid chromatography coupled to an ion trap-time of flight mass (IT-TOF) spectrometric analyser for LC-MS-MS analysis. Both enantiomers of 3-HEAE were also synthesised starting from 3(*R*)- and 3(*S*)-HETE and ethanolamine. Their activity at human recombinant CB_1 and CB_2 receptors was assessed by binding assays, and their capability to activate the human recombinant TRPV1 receptor was investigated by means of intracellular Ca^{2+} assays, as previously described (De Petrocellis, et al. *J Biol Chem*. 2001, 276, 12856-12863).

D. uninucleata was shown to convert anandamide into a compound that was identified by LC-MS-MS as 3-HEAE. The conversion proceeded less efficaciously than with arachidonic acid (~4% conversion *vs.* ~10% with arachidonic acid after 12 hr incubation). Both 3(*R*)-and 3(*S*)-HAEA were as potent as anandamide at activating TRPV1 receptors (EC_{50} = 0.44, 0.40 and 0.28 microM, respectively), but exhibited significantly lower affinity at both CB_1 (K_i = 1.85, 1.46 and 0.02 microM, respectively) and CB_2 (K_i = 6.4, 4.9 and 0.11 microM, respectively) receptors. Conversely, 3(*R*)-and 3(*S*)-HETE and arachidonic acid were inactive at cannabinoid receptors (K_i > 10 microM) and weakly active or inactive at TRPV1 receptors (EC_{50} = 13.40, 7.03 and >20 microM, respectively).

Based on these results, we hypothesize that pathogenic fungi like *C. albicans* and other 3-HETE producing species use anandamide produced by host cells at the site of infection and convert it into 3-HAEA, a compound significantly less active at cannabinoid receptors and yet still active at TRPV1 receptors. In view of the anti- and pro-inflammatory effects, and analgesic and hyperalgesic actions, of cannabinoid and TRPV1 receptors, respectively, this transformation might be responsible for part of the inflammatory and pro-nociceptive response caused by infections with pathogenic fungi.

[†]This work is dedicated to the beloved memory of Prof. Santosh Nigam, who passed away in October 2007.

IDENTIFICATION OF TWENTY-THREE MORE FATTY ACID AMIDES USING LC/MS/MS

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A variety of novel acyl amino acids related to anandamide have been discovered and found to activate GPCRs (e.g. NAGly, N-arachidonoyl serine) and TRP channels (e.g. *N*-arachidonoyl dopamine, *N*-acyl taurines), which suggested that many more novel *N*-acyl amino acids could serve as signaling molecules. Thirty novel acyl amino acids were identified two years ago. Here we used a different purification and two mass spectrometers and identified twenty-three more acyl amino acids in mammalian tissue.

We homogenized, extracted rat brains in methanol, and partially purified the supernatant using solid phase extraction column and semi-preparative HPLC, which separates the methanolic brain extract into more than 10 fractions. We analyzed fractions using nano HPLC-ESI-triple quadrupole mass spectrometer (Qstar) and LTQ-FT, which are two of the best MS instruments for identification of novel compounds and whose advantages are complementary to each other. LTQ-FT provides mass error of less than 1 ppm and Qstar generates a fragmentation pattern including the low mass ions.

Using semi-preparative HPLC, Qstar Pulsar, and LTQ-FT enabled us to identify twenty-three acyl amino acids, besides thirty-three acyl amino acids identified without semi-preparative HPLC. It is possible that these novel endogenous compounds have functions similar to those of endocannabinoids. The structures identified in these experiments, too many to list in this abstract, will be provided.

Acknowledgements: The authors are grateful for the support of the National Institute on Drug Abuse (DA-018224, DA-020402), the Gill Center for Biomolecular Science, Indiana University, Bloomington, and the Lilly Foundation Inc, Indianapolis, IN.

PLASMA ENDOCANNABINOID LEVELS INCREASE IN MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS which is characterized by autoimmune responses against myelin proteins eventually leading to impairment of neurological function. The different forms of MS include relapsing-remitting (RRMS), secondary-progressive (SPMS), and primary-progressive (PPMS) which all vary in rate of disease progression over time. Sensory and motor symptoms related to these MS stages can be improved through therapies modulating the activation of the endocannabinoid system [1]. The aim of this study was to determine how levels of endocannabinoids anandamide (AEA), 2-arachidonoyl glycerol (2-AG), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are altered in MS patients. Blood plasma samples were collected and processed from 24 MS (10 RRMS; 8 SPMS; 6 PPMS; 19 females; 25-66 years) and 17 control subjects (10 females; 22-62 years) and were gender- and age-matched. Endocannabinoids were quantified by using a liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) based on a previous method [2]. Our results show MS is associated with significant changes in levels of the endocannabinoids. In RRMS, AEA levels were found to be increased 2-fold ($p=0.001$) and 0.2-fold ($p=0.027$) for PEA, while no significant changes were observed for OEA and 2-AG. SPMS showed elevated concentrations of AEA (2-fold, $p=0.001$), PEA (0.25-fold, $p=0.004$), and OEA (0.25-fold, $p=0.005$), but not 2-AG, while only AEA concentrations were significantly increased in PPMS (0.6-fold, $p=0.009$). The highest concentrations of endocannabinoid were measured in the SPMS group (AEA=1.65 ± 0.70 nmol/L; PEA=12.30 ± 3.65 nmol/L; OEA= 15.76 ± 3.96 nmol/L). Our study demonstrates that levels of endocannabinoids are elevated in MS, which may impact upon symptoms and be neuroprotective.

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2. Richardson D., Ortori C. A., Chapman V., Kendall D.A., Barrett D.A. (2007). "Quantitative profiling of endocannabinoids and related compounds in rat brain using chromatography-tandem electrospray ionization mass spectrometry." Anal. Biochem. **360**: 216-226.

MECHANISM OF THE STIMULATION OF ENDOCANNABINOID PRODUCTION BY ATP IN CEREBELLAR PURKINJE CELLS

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The CB_1 cannabinoid receptor is typically localized on axon terminals and its activation leads to presynaptic inhibition of neurotransmission (Szabo & Schlicker, Handb Exp Pharmacol 168: 318–56, 2005). During the process of retrograde signaling, the presynaptic CB_1 receptor is activated by endogenous cannabinoids (endocannabinoids) synthesized by postsynaptic neurons (Chevaleyre et al., Ann Rev Neurosci 29:37-75, 2006). The production of endocannabinoids in postsynaptic neurons is usually triggered by an increase in calcium concentration and by activation of $G\alpha_{q/11}$ protein-coupled receptors. The hypothesis of the present work was that activation of calcium-permeable ligand-gated ion channels can also lead to endocannabinoid production and retrograde signaling. For testing the hypothesis, we studied whether activation of P2X purinoceptors leads to endocannabinoid production.

Cerebellar slices were prepared from mouse brain and Purkinje cells were patch-clamped. Glutamatergic excitatory postsynaptic currents (EPSCs) were elicited by stimulation of parallel fibers. P2X receptors on Purkinje cells were activated by ejections of ATP from a pipette. ATP ejection elicited inward currents in Purkinje cells, which were frequently accompanied by calcium spikes. Fluorometric calcium imaging showed strong increases in intracellular calcium concentration when calcium spikes were occurring; without spikes, the calcium increases were negligible. In experiments in which ATP led to calcium spikes, the subsequent EPSCs were inhibited by 92 ± 3 %; in the presence of the CB_1 antagonist rimonabant (10^{-6} M), EPSCs were inhibited only by 42 ± 15 %. In those experiments in which ATP did not lead to calcium spikes, ATP inhibited EPSCs only by 56 ± 10 % and this inhibition was not sensitive to rimonabant. PPADS (10^{-4} and 10^{-3} M), an antagonist of some P_{2X} receptors, did not change the ATP-evoked currents in Purkinje cells. The suppression of EPSCs by ATP was also not affected by PPADS.

The primary effect of ATP on Purkinje cells was probably mediated by $P2X_4$ purinoceptors, because these receptors are known to be present in Purkinje cells (Rubio and Soto, J Neurosci 21:641-653, 2001), and are insensitive to PPADS (Gever et al., Eur J Physiol 452: 513-537, 2006). Our results show that ATP can elicit endocannabinoid-mediated retrograde signaling between Purkinje cells and parallel fibers. Calcium entering the Purkinje cells via P2X receptor channels is probably not sufficient for triggering endocannabinoid production. Very likely, P2X receptors lead to depolarization, and calcium entering the neurons via voltage-gated calcium channels is the trigger for endocannabinoid production. One component of the ATP-evoked suppression of EPSCs was not mediated by endocannabinoids and CB_1 receptors; this suppression may have been mediated by P2Y receptors localized on axon terminals of parallel fibers.

INVESTIGATING THE ROLE OF THE ENDOCANNABINOID SYSTEM IN THE REGULATION OF ADULT NEUROGENESIS

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Adult neurogenesis, the formation of new neurons from resident stem or progenitor cells, is now widely accepted to occur in two neurogenic regions of the adult human brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. Neurogenesis is a tightly regulated process and evidence suggests it is involved in hippocampal-dependant memory, as well as certain disease states. The endocannabinoid system, which is known to play a role in neuroprotection, has recently been shown to be involved in neurogenesis. CB1 knockout mice showed a decrease in neurogenesis while cultures treated with HU-210, a CB1/CB2 cannabinoid agonist showed an increase in BrdU incorporation by 25%, which was blocked by cannabinoid receptor antagonists, confirming increased proliferation (Jiang *et al.*, J Clin Invest. 115 (2005) 3104-3116; Molina-Holgado *et al*., Eur J Neurosci. 25 (2007) 629-634). These results suggest that cannabinoid receptor activation may promote hippocampal neurogenesis.

Using three different neural stem cell lines, Cor-1, NS-5 and CGR-8, derived from mouse embryonic stem cells and foetal forebrain (Conti *et al*., PloS Biology. 3 (2005) 1594-1606), we were able to investigate the role of the endocannabinoid system in neurogenesis. Using the CellTiter 96® AQ_{ueous} Cell Non-Radioactive Cell Proliferation Assay (Promega) to measure cell number, the neural stem cells were treated with various pharmacological drugs to both stimulate and inhibit the endocannabinoid system. A general serine lipase inhibitor, THL, as well as a more specific DAGLα inhibitor, RHC80267, significantly decreased cell number in a dose-dependant manner in all cell lines. In addition, CB1 and CB2 receptor antagonists also decreased cell number in a dose-dependant manner, although inhibition of the CB2 receptor led to a greater decrease than inhibition of the CB1 receptor. Finally, various receptor agonists, for CB1 and CB2 receptors, separately as well as together, had no effect on cell number.

These results suggest that inhibition of the endocannabinoid system, either at the level of one of its main synthesizing enzymes, DAGLα, or at the level of its receptors, CB1 and CB2, decreases cell proliferation in a dose-dependant manner. However, the endocannabinoid system could not be further stimulated induce an increase in cell proliferation. Future work will involve using RNA interference as an additional tool to knock down DAGLα in order to further study the role of the endocannabinoid system in neural stem cell proliferation.

THE CB_1 RECEPTOR ANTAGONIST AM251 ACTS VIA A CALCIUM-DEPENDENT PATHWAY TO INCREASE INHIBITORY NEUROTRANSMISSION AT MOUSE CEREBELLAR PURKINJE CELLS

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G-protein coupled cannabinoid CB_1 receptors play an important role in the modulation of inhibitory transmission at interneurone-Purkinje (IN-PC) synapses in the mammalian cerebellum. We have recently reported that the CB receptor agonist WIN55 decreases miniature inhibitory postsynaptic current (mIPSC) frequency at IN-PC synapses, and that the effect of WIN55 was reversed by the CB_1 receptor antagonist AM251, which overall caused an increase in mIPSC frequency beyond control levels (Ma *et al*., Br. J. Pharmacol; in press). In order to investigate mechanisms underlining the additional action of AM251, we investigated the potential role of endocannabinoid release in acute cerebellar brain slices

Cerebellum brain slices were prepared from 3- to 5-week-old male TO mice. Slices were continuously perfused with artificial cerebrospinal fluid aerated with 95% O_2/5% CO_2. Whole-cell patch clamp recordings were performed with 3-7 MΩ resistance electrodes filled with a CsCl-based intracellular solution. Inward, bicuculline-sensitive mIPSCs were isolated in the presence of TTX, NBQX and CGP 55845. Changes in mIPSC frequency induced by drugs were normalised against control, and the data are presented as mean ± S.E.M.

We first examined the effects of AM404, a blocker of endocannabinoid uptake. AM404 (1-10 μM) had no effect on mean mIPSC frequency (1.04 ± 0.03; n = 6; $P<0.05$) or amplitude (1.04 ± 0.08; n = 6; $P<0.05$). In a subset of cells, subsequent application of WIN55 was still able to reduce mIPSC frequency. These data suggest that blockade of cannabinoid uptake is either unable to increase local endocannabinoid levels to a sufficient extent to activate CB_1 receptors or that such uptake systems are present only at low density/absent at IN-PC synapses. We next examined the effects of the Ca^{2+} chelator BAPTA-AM on AM251 action. BAPTA-AM (200 μM) alone had no overall effect on mIPSC frequency (0.92 ± 0.03; n = 5; $P < 0.05$); in the presence of BAPTA-AM, AM251 (2 μM) now failed to increase the mIPSC frequency beyond control level (0.95 ± 0.08; n = 5; $P < 0.05$).

These findings are consistent with AM251 acting via a Ca^{2+}-dependent pathway to increase presynaptic GABA release. These results contrast with our previous findings that BAPTA-AM had no effect on the $GABA_B$ receptor-mediated inhibition of GABA release at IN-PC synapses (Stephens & Harvey, 2004 *Eur J Neurosci* 20: 684-690) and suggest differential modes of action of presynaptic G protein-coupled receptors at IN-PC synapses.

SYNAPTIC LOCALIZATION OF THE ENZYMATIC MACHINERY RESPONSIBLE FOR 2-ARACHIDONOYLGLYCEROL SYNTHESIS AND DEGRADATION IN THE PRIMATE HIPPOCAMPUS

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Endocannabinoid signaling in the hippocampus is known to be involved in synaptic plasticity and memory. Although the specialized molecular architecture of the endocannabinoid system modulating synaptic neurotransmission has already been revealed in rodents, whether it is an evolutionarily conserved feature of hippocampal synapses has remained elusive. To address this issue, we studied the subcellular localization of enzymes synthesizing and degrading endocannabinoids in the monkey and human hippocampus. Immunostaining for diacylglycerol lipase-α (DGL-α), the main biosynthetic enzyme of the endocannabinoid, 2-arachidonoyl-glycerol (2-AG), visualized a similar staining pattern in post mortem human and perfused rhesus monkey (Macaca mulatta) as found earlier in the rodent hippocampus. At higher magnification, the dense granular staining often outlined immunonegative main apical dendrites of pyramidal cells suggesting that DGL-α is present in dendritic spines in primates. Remarkably, at the light microscopic level, the immunostaining for 2-AG's predominant degrading enzyme, monoacylglycerol lipase (MGL) resulted in very similar overall staining pattern, namely an intense punctuated staining was present throughout the neuropil. However, further electron microscopic analysis revealed that MGL was accumulated in axon terminals suggesting that elimination of 2-AG occurs presynaptically.

These findings show that the localization of metabolic enzymes involved in synaptic 2-AG signaling is similar in rodents and in primates. Thus, the molecular architecture of synaptic endocannabinoid signaling, which seems to be precisely designed to subserve retrograde regulation of synaptic neurotransmisson is an evolutionarily conserved feature of synapses and may have a fundamental physiological role as a negative feed-back signal protecting synapses from excess presynaptic activity.

NEUROPROTECTIVE EFFECT OF CANNABIDIOL AFTER OXYGEN AND GLUCOSE DEPRIVATION OF NEWBORN MICE FOREBRAIN SLICES.

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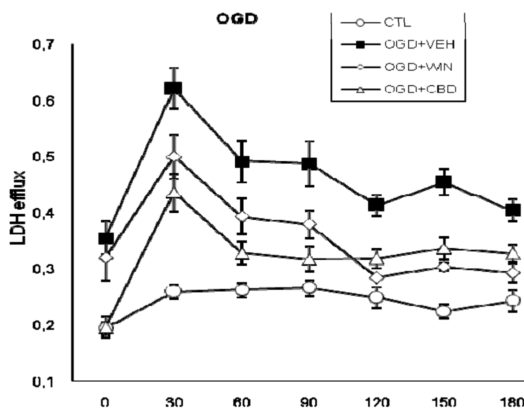
Introduction: cannabidiol (CBD) affords neuroprotection in adult animal models of ischemic brain damage. We aimed to test CBD neuroprotection in a novel in vitro model of newborn hypoxic-ischemic brain damage (NHIE) –oxygen and glucose deprivation (OGD) of mice forebrain slices-. Effects of CBD were compared with those of the CB1/CB2 agonist WIN55212, for which we demonstrated a neuroprotective effect in a similar model in newborn rats (Fernández-López D et al, Pediatr Res 2006).

Methods: 500 µm brain slices obtained from 7-day-old C57BL6 mice were exposed to OGD for 30 min. The effect of the incubation of OGD slices with CBD 100 µM or WIN 50 µM was studied using the quantification of LDH efflux by spectrophotometry as indicator of hypoxic-ischemic damage in the brain. Levels of Nitric oxide (NO), nitro-Tyrosine (N-Tyr) or Superoxide Dismutase (SOD) were measured in slices supernatant by ELISA.

Results: OGD (■) led to severe tissue damage, as reflected by the increase in LDH efflux (Figure). Incubation of slices with CBD (Δ) reduced that increase in LDH efflux; this effect was better than WIN (◊) over the first 90 min post-insult. OGD led to an increase of NO production, which was blunted by CBD. OGD led to a decrease of SOD levels, likely due to SOD consumption because of oxidative stress; CBD reduced that effect. Finally, OGD led to an increase of tyrosine nitration, an effect prevented by CBD. Incubation with WIN led to similar results to CBD.



Conclusions: 1) OGD of mice forebrain slices is a reliable in vitro model of NHIE; 2) in this model CBD affords neuroprotection; 3) this effect is likely related to toxic NO production and oxidative stress modulation.

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IN VIVO PET BRAIN IMAGING OF THE TYPE 1 CANNABINOID RECEPTOR IN HUNTINGTON'S DISEASE

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Objective: The endocannabinoid system has been proposed to be involved in the pathogenesis of HD. Post-mortem studies have reported a profound loss of CB1 in HD brains. Studies in transgenic mouse models of HD have shown that mutant huntingtin suppresses *CB1* transcription. The aim of this study was to characterize the expression of the cannabinoid-type 1 receptor (CB1) in vivo in early and advanced symptomatic Huntington's disease (HD) patients with PET imaging using the novel high-affinity, high-selectivity CB1 radioligand ^{18}F-MK9470.

Methods: Twenty HD patients (mean age 53.3 yrs, range 32-83 yrs; 8 M/12 F) in different stages of disease severity (median Total Functional Capacity [TFC] score 6.5, range 0-13) and 14 healthy age- and gender-matched controls (mean age 54.2 yrs, range 30-70 yrs; 6M/8F) were included. All subjects underwent PET scanning with 302±30 MBq ^{18}F-MK9470. Parametric standardized uptake value images reflecting receptor availability were constructed and corrected for partial volume effects. Statistical parametric mapping and subcortical volume-of-interest statistical analysis were performed. Striatal volumes were determined on T1 MPRAGE MRI.

Results: HD patients showed a profound reduction of cerebral CB1 binding compared to controls (-24 ± 9% , $p<0.00001$). The reduction of CB1 binding in HD patients was remarkably uniform across gray matter regions. Regional CB1 binding did not correlate with disease severity parameters (Unified Huntington Disease Rating Scale [UHDRS] motor and TFC scores), age, disease duration, CAG repeat length or CAG x age. UHDRS motor and TFC scores were significantly correlated with caudate and putamen volume loss (all $|r|>0.74$, $p<0.001$).

Conclusion: CB1 availability is strongly and uniformly decreased across gray matter regions of HD brains in vivo. The findings are consistent with a model where mutant huntingtin represses CB1 transcription from very early disease stages onwards. This study provides the first in vivo evidence for disturbance of the endocannabinoid system in a human neurological disease, and reveals that the anatomic distribution of the disease process in HD is much more widespread than previously suspected.

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THE CANNABINOID AGONIST JWH-015 STIMULATES BETA AMYLOID REMOVAL *IN SITU* AND *IN VITRO*

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A possible role for the endocannabinoid system (ECS) in the treatment of Alzheimer's disease has been recently proposed. Studies carried out in animal models of this disease as well as with human samples have provided the rationale for this hypothesis. Among the different elements of the ECS that are under study, cannabinoid CB_2 receptors present in glial cells may be of remarkable importance, as their activation would be devoid of psychotropic effects. We have recently shown that a specific CB_2 agonist (JWH-015) is capable of inducing beta-amyloid removal from human frozen tissue sections by a human macrophage cell line (THP-1). Remarkably, this effect was achieved at low doses (max. effect at 5nM) and was specific for this type of cells, as U373MG astrocytoma cells did not respond to the treatment. The effect was CB_2-mediated, as the selective CB_2 antagonist SR144528 prevented the JWH-015-induced plaque removal.

In order to expand these observations, we have developed an *in vitro* model to precisely quantify the amount of beta amyloid removed by phagocytic cells. Thus, 200ng of Cy3-labelled fibrillar beta amyloid (1-42) were allowed to dry at the bottom of 96-well cell plates. Afterwards, THP-1 macrophages were seeded and allowed to adhere. Beta amyloid removal in the absence or presence of several concentrations of the CB_2 cannabinoid agonist JWH-015 was quantified by fluorimetric detection of remaining Cy3-beta amyloid in the wells as well as in the cellular fraction. Our results show that JWH-015 is able of inducing a remarkable enhancement of phagocytic properties of THP-1 macrophages. Thus, the treatment with 100nM JWH-015 induced the removal of 40% of synthetic fibrillar beta amyloid, as measured by remaining fluorescence per well. Furthermore, the amount of labeled beta amyloid incorporated into the macrophages increased as a consequence of the treatment with the cannabinoid agonist. Finally, morphological analysis by confocal microscopy revealed that beta amyloid was not only bound but indeed phagocyted by human macrophages. These data suggest that CB_2 cannabinoid specific chemicals may enhance the removal of beta amyloid and thus be of interest for the development of novel therapeutic approaches in the treatment of Alzheimer's disease.

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β-AMYLOID PROTEIN EVOKES LYSOSOMAL DESTABILISATION VIA P53 AND BAX ACTIVATION: THIS IS PREVENTED BY TREATMENT WITH THE ENDOCANNABINOID, 2-ARACHIDONOYLGLYCEROL

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Alzheimer's disease (AD) is a neurodegenerative disease associated with cognitive decline and neuronal loss due to inappropriate apoptosis. The presence of senile plaques composed of β-amyloid (Aβ), a 1-42 amino acid peptide, which induces neuronal apoptosis, is a consistent feature of the AD brain. Whilst the precise intracellular mechanisms responsible for apoptosis are not fully understood, it is thought increased lysosomal permeability contributes to the apoptotic pathway by releasing lysosomal enzymes. Within the brain, the endocannabinoid system regulates a range of neuronal processes, including neuronal survival. There is mounting evidence linking the endocannabinoid system to the pathology and cognitive decline associated with AD. Thus, the aim of this study is to investigate i) the effect of Aβ on lysosomal permeability ii) the ability of 2-AG to afford protection against Aβ-induced toxicity.

Cultured cortical neurones were prepared from Wistar rats and maintained in neurobasal medium. Neurones were treated with $A\beta_{1-40}$ (10μM) for 48 hr ± 2-AG (0.01μM). The TUNEL (Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling) method was used to quantify apoptotic cells. Neurones were then incubated with acridine orange (AO) for 10 minutes followed by treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ (2μM) ± a bax inhibitor, V5 (50μM) for 6 hours. Neurones were also pre-treated with p53 siRNA for 48 hours followed by AO incubation for 10 minutes and treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ for 6 hours. Lastly, neurones were treated with $A\beta_{1-40}$ ± 2-AG (0.01μM) subsequent to incubation with AO. Lysosomal stability was observed by fluorescent confocal microscopy under 63X magnification, and AO was excited at 488nm.

When cells were exposed to $A\beta_{1-40}$ (10μM) for 48 hr, the % of apoptotic cells was significantly increased from 7.80 ± 0.75% to 24.54 ± 0.96% ($p<0.001$, ANOVA, n=6), and this was reduced to 8.1 ± 1.07% by 2-AG (0.01μM). Lysosomal permeability was measured by loss in fluorescent intensity. This was reduced from 325.3 ± 8.752 in control cells to 289.1 ± 5.86 and 275.2 ± 7.28 in cells treated with $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively. Co-treatment with the bax inhibitor prevented the Aβ-induced increase in lysosomal permeability. siRNA-mediated knockdown of p53 increased the fluorescent intensity from 261.3 ± 4.019 to 299.3 ± 8.323 and 248.1 ± 11.06 in cells treated with $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively. Co-treatment of $A\beta_{1-40}$ and 2-AG prevented the Aβ-induced increase in lysosomal permeability.

We conclude that Aβ induces apoptosis, and impacts on the lysosomal system via p53 and bax. We also report that the endocannabinoid, 2-AG, prevents the Aβ induced increase in DNA fragmentation and also the earlier occurrence of lysosomal rupture. This suggests that Aβ may induce apoptosis through a lysosomal-mitochondrial pathway that is initiated by lysosomal destabilisation. It also highlights the neuroprotective role of 2-AG in alleviating Aβ-induced cell death, and suggests a cannabinoid-based therapy may have potential value in the treatment of Alzheimer's disease.

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$CB_2^{-/-}$ MICE ARE HIGHLY SENSITIVE TO MALONATE TOXICITY WHICH EMPHASIZES THE IMPORTANCE OF THIS RECEPTOR IN STRIATAL PATHOLOGY: RELEVANCE FOR HUNTINGTON'S DISEASE

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We have recently obtained pharmacological and histological evidence supporting that CB_2 receptor agonists may be efficacious in delaying/arresting neurodegeneration in a rat model of Huntington's disease generated by intrastriatal application of the mitochondrial toxin malonate (Fernández-Ruiz et al., Trends Pharmacol. Sci. 28, 39-45, 2007). Here, we have further explored the issue by evaluating the toxicity of malonate when administered to $CB_2^{-/-}$ mice. Compared to wild-type animals, $CB_2^{-/-}$ mice presented a higher number of degenerating cells, labelled with fluoroJade-B, in the striatal parenchyma after the malonate application, thus confirming the neuroprotective effect exerted by this receptor type. This greater sensitivity of $CB_2^{-/-}$ mice to malonate seems to be caused by a greater recruitment of reactive microglia at the lesioned sites and a possible higher generation of proinflammatory cytokines, confirming the data obtained in rats lesioned with malonate that proved that CB_2 receptors are markedly up-regulated in reactive microglial cells, and also in astrocytes, in the striatum after the lesion with malonate (Sagredo et al., Mol. Neurobiol. 36, 82-91, 2007). In summary, our results provide support to the notion that CB_2 receptors could be a therapeutic target to slowdown neurodegeneration in HD (and/or other neurodegenerative diseases) and that this neuroprotective effect would be exerted through a mechanism involving glial cells, in particular reactive microglial cells.

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PRE-INCUBATION WITH Δ^9-TETRAHYDROCANNABIVARIN INHIBITS SPONTANEOUS EPILEPTIFORM ACTIVITY IN PIRIFORM CORTICAL BRAIN SLICES

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Introduction

Cannabis has been historically used as an anticonvulsant although more recent research has highlighted both pro- and anti-convulsant effects. We have previously shown that Δ^9-tetrahydrocannabivarin (Δ^9-THCV) inhibits Mg^{2+}-free media-induced spontaneous epileptiform activity in acute rat piriform cortical (PC) brain slices (Weston, 2006) at relatively high doses (Brown, 2004). In the present work, we have investigated the effects of low concentration Δ^9-THCV pre-incubation upon the same epileptiform activity.

Methods

Acute transverse slices of adult rat PC were pre-incubated with 10 µM Δ^9-THCV (minimum 30 minutes) before transfer to a recording chamber superfused with Mg^{2+}-free artificial cerebrospinal fluid (aCSF; to induce epileptiform activity) plus 10 µM Δ^9-THCV. As a comparator, non-pre-incubated slices were acutely treated with 10 µM Δ^9-THCV after epileptiform activity had been induced. Activity was electrophysiologically recorded using a multi-electrode array. In n = 39 slices, perfusion with Mg^{2+}-free aCSF alone induced sustained epileptiform activity in 95% of slices.

Results

Measure		**Pre-incubated**	**Acutely treated**
	Mg^{2+}- free aCSF	Mg^{2+}- free aCSF plus 10 µM Δ^9-THCV	
Burst complex incidence (min^{-1})	1.76 ± 0.17	0.61 ± 0.14**	2.2 ± 1.7*
Peak PDS amplitude (µV)	130 ± 11	80 ± 4**	110 ± 17*
Decay constant for best fit to interictal activity ISI plots (sec^{-1})	0.034 ± 0.001	0.054 ± 0.001**	0.038 ± 0.001

Mean±SEM; *P<0.05; **P<0.01 Student's t-test; PDS=paroxysmal depolarising shift; Comparisons made vs Mg^{2+}-free aCSF; n=5 for all data.

Conclusions

These data demonstrate that Δ^9-THCV pre-incubation significantly reduced epileptiform activity in this brain slice model of epilepsy.

Brown SP, Safo PK, Regehr WG (2004). *J Neurosci* 24: 5623-5631.

Weston SE, Constanti A, Stephens G & Whalley BJ (2006). *British Pharmacological Society*; Oxford, UK. 2006.

CANNABINOIDS ALTER AVERAGE FIRING RATE, BURSTING AND CELL SYNCHRONY OF HIPPOCAMPAL PRINCIPAL CELLS.

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Cannabinoid agonists Δ^{9}-THC; WIN 55,212-2 (WIN-2) and HU-210 have been shown to alter hippocampal neuronal activity and disrupt spatial learning and memory tasks (Hampson & Deadwyler, J. Neurosci, 2003; Robinson *et al*. Bri. J. Pharm, 2007).Moreover, *in vivo* recordings of local field potentials and single neurones have demonstrated that Δ^{9}-THC and CP 55,940 disrupt the synchrony of action potentials between hippocampal cells without altering average firing rates. Here we assess how numerous cannabinoid agonists (THC; WIN-2 and HU-210) affect: 1) hippocampal principal cell 'firing' and 'bursting' characteristics and 2) synchronous firing between cell pairs located within and between CA3 and CA1 hippocampal sub-fields. .

Adult, male, Long Evans rats were implanted with multi-electrode arrays to CA3/CA1 regions of hippocampus. On recovery from surgery subjects were anesthetised and principal cells (firing rate-0.25-6Hz) were isolated and 'tracked' following Tween-80 (vehicle); THC (1 & 3 mg/kg); WIN-2 (1 & 3 mg/kg) and HU-210 (50 & 100 mcg/kg) treatments respectively.

Results show that all doses of WIN-2, HU-210 and higher doses of THC produce significant changes in the average firing rate, 'burst' characteristics and de-sychronisations between pairs of CA3 and CA1 hippocampal neurones. In contrast, THC at the low dose failed to show changes in average firing rate and bursting. Moreover, disruptions in synchronous firing between cells in CA3-CA3 and CA1-CA1 but not those between CA3-CA1 sub-fields were seen at this particular dose of THC suggesting that de-synchronisations were localised to given hippocampal sub-fields.

These cannabinoid induced changes in hippocampal function (i.e. changes in firing rate, bursting and disruptions in spike timing) may well contribute towards memory deficits reported in numerous spatial learning and memory tasks in rats.

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CHRONIC CANNABINOID AGONIST AND ANTAGONIST TREATMENT ALTERS THE HIERARCHICAL ENCODING OF TASK-RELATED EVENTS BY HIPPOCAMPAL NEURONS DURING ACQUISITION OF A DELAYED-NONMATCH-TO-SAMPLE TASK

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Cannabinoid receptor (CB1) agonists Δ^9-tetrahydrocannabinol (THC) and WIN 55,212-2 (WIN) have been shown to inhibit hippocampal neural activity and impair behavioral performance in a spatial delayed-nonmatch-to-sample (DNMS) task in rats (Hampson & Deadwyler, J. Neurosci, 2003; Hampson *et al*. Hippocampus, 2000). Likewise, under some conditions, the CB1 antagonist/inverse agonist Rimonabant (Rim., also SR141716) enhances neural firing and facilitates DNMS performance. .These memory altering effects of cannabinoids have been demonstrated in animals that were fully trained to accommodate the delay component. To date no studies have looked at how cannabinoid receptor agonists and antagonists shape behavior and hippocampal ensemble activity when subjects are initially exposed to a delay to meet the demands of the task. Here we use this short-term memory task to assess how chronic Rim. And WIN treatment affect: 1) the acquisition of incorporating discrete delay intervals during performance; 2) the hippocampal cell recruitment during this process and 3) hippocampal ensemble activity during task related events.

10 adult, male, Long Evans rats were pre-trained to perform the DNMS task at 0s delay and implanted with microwire arrays targeting the CA3/CA1 cell layers of hippocampus. Following single neuron isolation and identification procedures, animals were treated daily with WIN (0.35mg/kg), Rimonabant (0.5 mg/kg) or vehicle ($n=5$ per group) and subjected to perform the DNMS task on delays of 1-10s that were subsequently raised to 11-20s and 21-30s on achieving criterion (80% correct responding) at each stage. Both initial and newly recruited cells were isolated and ‘tracked’ throughout treatment.

Preliminary results show that modulation of the CB1 receptor altered the number of days it took each animal to achieve criterion performance at each delay increment as well as altering the hippocampal functional cell types (FCTs) thought to be crucial for successful DNMS performance at each delay interval. These cannabinoid induced morphological and functional alterations in hippocampus may explain the role of cannabinoid receptors and endocannabinoids in “shaping” hippocampal response during learning and memory.

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INHIBITION OF FATTY ACID AMIDE HYDROLASE FACILITATES REVERSAL LEARNING IN THE MORRIS WATER MAZE

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Increasing evidence argues that the endocannabinoid system plays a critical role in tasks requiring cognitive flexibility. Accordingly, deficits in endocannabinoid activity are known to impair both extinction and reversal learning, largely through promoting perseveratory responses. A few recent studies have revealed that facilitation of endocannabinoid neurotransmission may enhance performance in tasks of cognitive flexibility; however, this remains to be fully examined. To this extent, we examine the effects of pharmacological inhibition of FAAH in a standard reversal learning protocol in the Morris water maze. Initially, male rats were trained to find a hidden platform in a fixed location of the pool. Following this acquisition phase, rats were administered the FAAH inhibitor URB-597 (at a dose of either 0.1 mg/kg or 0.3 mg/kg), or vehicle, prior to each reversal learning session for 3 consecutive days. While there was no effect of URB-597 administration on the overall performance in the reversal learning task across all 3 days, rats which had been administered the 0.3 mg/kg of URB-597 were significantly faster at learning the new location of the platform than vehicle-treated animals during the first session of reversal learning. These data indicate that facilitation of anandamide/CB_1 receptor signaling accelerates the acquisition of reversal learning and supports previous studies demonstrating that the endocannabinoid system may promote cognitive flexibility.

SEX-DEPENDENT DEGENERATIVE CHANGES IN THE CEREBELLAR CORTEX OF MATERNALLY DEPRIVED RATS. MODULATORY EFFECTS OF TWO INHIBITORS OF ENDOCANNABINOID INACTIVATION

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Adult animals submitted to a single prolonged episode of maternal deprivation (MD) [24 h, postnatal day 9-10] show behavioural alterations that resemble specific symptoms of schizophrenia. It has been hypothesised that, according to the neurodevelopmental theory, certain functional deficits observed in maternally deprived animals may be related to neurodegenerative processes occurring in early developmental periods (Ellenbroek & Cools 2002, Pharmacol Biochem Behav 73:177-84). We have recently shown that MD-induced increases in plasma corticosterone levels (as measured at PND 13) were accompanied, only in males, by an increase in the number of astrocytes in CA1 and CA3 hippocampal areas. We have also demonstrated that the fatty acid amide hydrolase inhibitor N-arachidonoyl-serotonin (AA-5-HT) and the endocannabinoid reuptake inhibitor OMDM-2 reversed both these endocrine and cellular effects of MD (Llorente et al., 2008 Hippocampus, submitted). Similarly to the hippocampus, the cerebellar vermis has a very high density of glucocorticoid receptors during development (Lawson et al 1992, Neuroendocrinology 55: 695–707) and might, therefore, be especially sensitive to excessive levels of glucocorticoids (McEwen 1999, Ann Rev Neurosci 22: 105–122). Accordingly, we have addressed possible degenerative effects of MD on the cerebellar cortex. To evaluate the presence of degenerated nerve cells we used Fluoro-Jade C (FJ-C) staining (Schmued et al 2005, Brain Res 1035: 24-31) and for the study of astrocytes we employed Glial fibrillary acidic protein (GFAP). Further, we analyzed the modulatory actions of AA-5-HT and OMDM-2. Pharmacological treatment consisted of daily subcutaneous injections during the postnatal period 7-12 and the animals were sacrificed at postnatal day 13. The results indicated that MD induced significant increases in the number of FJ-C positive cells, indicative of degenerating neurons (co-localization studies by confocal microscopy) and astrocytes (more GFAP positive cells) only in males. This gender-dependent differential effect may be attributable to a greater vulnerability of males to maternal deprivation-stress and/or to sex-dependent differences in the onset and/or progression of the effects. In line with our previous findings in the hippocampus (Llorente et al., 2008), the present results show that two drugs that are considered as endocannabinoid system enhancers, i.e. AA-5-HT and OMDM-2, reversed or attenuated the cerebellar neural and astroglial damage induced by MD, thus supporting the view that up-regulation of endocannabinoids might represent an adaptive response aimed at counteracting the consequences of MD-induced stress.

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POTENTIAL SOURCES OF POOR PERFORMANCE OF CHRONIC MARIJUANA USERS ON DECISION MAKING TASKS

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Chronic marijuana users perform poorly on the Iowa Gambling Task which requires choices between small immediate profits/small long-term losses vs. large immediate gains/large future losses (Whitlow et al., 2004). Because this task is complex and involves multiple processes, the poor performance of users could arise from a number of different sources. We have hypothesized that marijuana users persist in selecting disadvantageously because of faulty working memory processes. To test this hypothesis, marijuana users completed a decision making task (Rogers et al., 2003) that minimizes the role of working memory. In this task, participants choose between two gambles on each trial. The gambles differ in the probability of winning and losing and the amounts that can be won on each trial. Each trial is independent and does not require any memory of the outcomes of previous choices. If heavy marijuana users perform poorly on the risky choice task, then working memory deficits cannot account for poor performance on the Iowa Gambling Task by marijuana users.

A total of 15 non-treatment seeking heavy marijuana users and 15 controls served as subjects. All procedures were approved by the Wake Forest University School of Medicine Institutional Review Board and written informed consent was obtained from each participant. The task consisted of 80 trials. On each trial subjects were asked to choose one of two options. One option was the control gamble- equal (50%) chance of winning or losing an equal number of points. The alternative gamble varied in the probability of winning and the possible gains and losses. Thus, to optimize performance subjects were required to consider both the probability of wins and the magnitude of the gains.

Marijuana users and controls did not differ in the total points obtained (502 ± 35 vs. 520 ± 39, respectively). However, the marijuana users were more likely to base their decisions on a single dimension of the stimulus configuration rather than considering both aspects. Whereas controls tended to choose low probability options when the potential gains were high, users tended to base their choices solely on the probabilities rather than the magnitude of gain or loss.
The lack of difference between users and controls on the performance measures of the risky choice decision-making task, a task with no working memory component, suggests that working memory deficits are a contributing factor in the poor performance by users on the IGT. The users' one-dimensional approach to the task suggests that their decisions are limited to the stimuli requiring the least mental calculation. This is consistent with poor performance on other executive function and decision tasks with multiple dimensions. Marijuana users may have limited capacity to consider concurrent plans or processes during complex decision making and reasoning.

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THE EFFECT OF COGNITIVE LOAD ON WORKING MEMORY IN CHRONIC MARIJUANA USERS: A FUNCTIONAL MRI INVESTIGATION

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Marijuana use has extensive effects on executive functioning and information processing in the brain. While memory deficits in marijuana users have been extensively documented, it is unclear whether these impairments are directly associated with working memory load or if they reflect a more global deficit in information processing. The intent of this functional MRI investigation was to determine whether there was a consistent decrease in information processing in marijuana users or whether executive processing deficits were amplified by increasing cognitive load. Functional MRI data was acquired from 19 marijuana users and 20 controls performing a modified version of the N-back working memory task, which has three levels of difficulty (0back, 1back, 2back). For both marijuana users and controls, there was a load-related decrease in performance with more errors associated with higher cognitive loads. Marijuana users performed more poorly than controls at all cognitive loads ($F = 2.19$, $p<0.01$). Load-related decreases in performance declined at the same rate in MJ users and controls. As with the behavioral performance, there were significant changes in neurofunctional activity in both groups as load increased. In contrast to the behavioral data however, the effect of cognitive load on neurofunctional activity differed between the groups. Specifically, during the 2back task marijuana users recruited significantly less dorsolateral and medial prefrontal cortex than controls, and more accessory executive processing areas including the medial temporal cortex and the parietal cortex ($p<0.05$ corrected). The between group differences were less significant in the 1back task, though marijuana users still recruited significantly less of the prefrontal cortex and the hippocampus than controls, areas known to be modulated by working memory load. Taken together, these results demonstrate that while working memory performance degrades at the same rate in marijuana users and controls, the neurofunctional differences between the groups are amplified as the working memory demands increase. This suggests that, in the presence of an overall deficit in information processing, marijuana users recruit more widespread and divergent distribution of brain regions than controls as cognitive load increases.

CHRONIC MARIJUANA USE AND PROSPECTIVE MEMORY TASK PERFORMANCE

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Does chronic marijuana use negatively affect prospective memory? Prospective memory is our ability to make plans, to retain them and to carry them out at the appropriate time or in the appropriate context; it is our ability to remember to perform tasks at a later time. Some everyday examples of prospective memory include remembering to attend an appointment, to pay bills on time and to return a phone call. Only one previous study (Cuttler, McLaughlin & Graf, 2007; ICRS) has examined the relationship between chronic marijuana use and problems with prospective memory. For that study, undergraduate students completed an online survey designed to assess marijuana use and everyday life prospective memory performance. The results revealed very small correlations between the self-reported frequency and quantity of marijuana use and problems with a variety of aspects of prospective memory.

The present study is the first to examine the influence of chronic marijuana use on actual – as opposed to self-rated – prospective memory task performance. We recruited three groups of undergraduate students: individuals who have never used marijuana (nonusers; N=48), individuals who have experimented with marijuana fewer than six times in their lives (experimenters; N=37) and individuals who have used marijuana three or more times a week for at least a year (chronic users; N=25). As part of a larger battery of neuropsychological tests participants were asked to complete three prospective memory tasks. Specifically, they were asked to remind the experimenter to send an email after completing a particular cognitive test, to press the "p" key on the computer keyboard whenever they saw a picture of fruit and to place a phone call to the lab one week after the testing session. Participants also completed a survey designed to assess marijuana use, problems with prospective memory, use of other substances, personality, motivation and mood.

The results show that nonusers, experimenters and chronic users perform similarly on each of the three prospective memory tests. On the questionnaire measures of prospective memory the chronic users reported having more problems than nonusers with episodic and internally cued prospective memory. However, when compared to the experimenters, chronic users only reported experiencing more problems with internally cued prospective memory. The results suggest that chronic marijuana use has few, if any, detrimental effects on prospective memory.

EFFECTS OF CANNABINOID TREATMENT DURING PUBERTY ON SPONTANEOUS ETHANOL CONSUMPTION AND EMOTIONAL BEHAVIOR IN RATS

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The CNS of both humans and rodents shows increased vulnerability for the aversive effects of exogenous cannabinoids during certain phases of development. Furthermore, an involvement of the endogenous cannabinoid system in the regulation of emotional behavior, as well as ethanol consumption and preference has been suggested. In the present study we examined the acute and long-term behavioral effects of the cannabinoid receptor agonist WIN 55,212-2 (WIN) on anxiety-related behavior and voluntary spontaneous ethanol consumption during different postnatal periods in the rat. To identify underlying molecular mechanisms, we addressed WIN-induced differences in the glutamatergic signaling system in a small-scale proteomic approach. Chronic WIN (1.2 mg/kg)/vehicle treatment was extended over 25 days throughout puberty, or for a similar time period in adult rats. Behavioral testing (elevated plus maze, light/dark emergence test, spontaneous ethanol consumption) and brain samples were performed directly after the first injection of WIN (acute effects) and 15 days after cessation of WIN treatment (chronic effects).

Chronic pubertal WIN treatment induced a persistent increase in anxiety-related behaviors and altered ethanol consumption in adult rats, whereas no lasting effects were obtained after chronic treatment in adulthood. Acute WIN administration induced stronger anxiety responses in pubertal than in adult animals, and increased the consumption of higher ethanol solutions specifically in pubertal rats. On the molecular level, these changes were accompanied by the modulation of glutamatergic signaling.

In summary, our data underline the vulnerability of the pubertal period for the aversive effects of cannabinoid agonists. Furthermore, the enhanced intake of higher ethanol concentrations in pubertal cannabinoid treated rats might derive from an increase in anxiety-related behavior.

EFFECTS OF MICROINJECTION OF ENDOCANNABINOIDS INTO THE VENTRAL HIPPOCAMPUS ON PAIN- AND FEAR-RELATED BEHAVIOUR IN RATS

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There is evidence of a role for the ventral hippocampus in both fear conditioning and nociceptive processing. The endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide (AEA) are widely acknowledged to modulate nociception, fear, and fear-conditioned analgesia, however, their role in the ventral hippocampus has yet to be examined. This study investigated the effects of direct administration of 2-AG and AEA into the ventral hippocampus on nociception and on the expression of contextually-induced fear in the presence of nociceptive tone.

Male Lister-Hooded rats (250-330 g, *n*=6-10/group) were bilaterally implanted with stainless steel guide cannulae into the ventral hippocampus (AP -4.6, ML ± 5.0, DV -5.0, Paxinos and Watson, 1997) and allowed to recover for at least 6 days post-surgery. The fear-conditioning paradigm was mild footshock (0.4 mA x 1s x 10) paired with context or no footshock controls. On the day of testing, animals received a bilateral injection of either AEA or 2-AG at two doses (0.5µg/µl and 2µg/µl), or vehicle (DMSO), into the ventral hippocampus 15 minutes prior to re-exposure to the conditioned context. The formalin test was used to evoke nociceptive behaviour (intra-plantar injection of 50µl 2.5% formalin into the right hindpaw) which was scored along with conditioned aversive behaviour (freezing and 22 kHz ultrasonic vocalisation) for a 15 minute period (30-45min post-formalin).

Fear conditioning in formalin-treated rats was associated with increased ultrasonic vocalisations and freezing behaviour, effects that were significantly attenuated by intra-hippocampal injection of high dose AEA (2 µg/µl) and low dose 2-AG (0.2µg/µl) throughout the entire 15 minute trial period. Low dose 2-AG increased formalin-evoked nociceptive behaviour in fear-conditioned, but not non-fear-conditioned, rats. High dose AEA increased nociceptive behaviour in non-fear-conditioned rats but not fear-conditioned rats.

These data suggest that, in the presence of nociceptive tone, 2-AG and AEA may act in the ventral hippocampus to reduce conditioned aversive behaviour. Moreover, the data provide evidence for differential effects of intra-ventral hippocampal administration of endocannabinoids on nociceptive behaviour in the presence or absence of contextually-induced fear.

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DEGRADATION OF ANANDAMIDE IN ADHD AND ITS RELATIONSHIP WITH DOPAMINE SIGNALING IN THE STRIATUM

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Abnormal dopamine (DA) transmission in the striatum has been proposed to play a role in attention-deficit/hyperactivity disorder (ADHD). Since the modulation of the endocannabinoid system is a major effect of DA receptor stimulation, the present study was aimed at investigating the metabolism of the endocannabinoid anandamide (AEA) in ADHD patients. We found a selective reduction of AEA degradation in the peripheral blood of ADHD patients, an alteration that was replicated in the mouse striatum following the stimulation of dopamine D2-class, but not of D1-class receptors. In addition, we provided neurophysiological evidence that the biochemical defect of AEA degradation found in ADHD patients selectively altered glutamate synaptic transmission but not GABA transmission in the striatum, indicating that ADHD symptoms may rely, at least in part, on differential dysregulation of excitatory and inhibitory synaptic transmission in this bran area. On the basis of our results, it can be proposed that pharmacological modulation of the endocannabinoid system might be useful for the treatment of ADHD patients.

MONOAMINERGIC NEUROTRANSMISSION MEDIATES CANNABINOID-INDUCED ACTIVATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

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While a convincing body of research indicates that endogenous cannabinoid signaling inhibits activation of the hypothalamic-pituitary-adrenal (HPA) axis, it has been repeatedly found that administration of high doses of exogenous CB_1 receptor agonists can potently activate the HPA axis. The mechanism by which this occurs is not well characterized but it is likely not due to local CB_1 receptor activation within the hypothalamus as: 1) CB_1 receptors in the paraventricular nucleus of the hypothalamus are found to inhibit glutamatergic excitation of CRH neurosecretory cells; 2) deafferentation of the hypothalamus prevents the ability of exogenous CB_1 receptor agonists to activate the HPA axis and increase glucocorticoid secretion. Both monoaminergic and glutamatergic neurotransmission are known to activate the HPA axis and cannabinoids have been found to modify levels of these neurotransmitters throughout the brain. The aim of the current study was to pharmacologically determine the extent to which specific serotonergic, noradrenergic and glutamatergic receptor subtypes were involved in the ability of exogenous cannabinoids to activate the HPA axis. To this extent, we initially characterized a robust induction of corticosterone secretion following administration of a dose of 100 ug/kg HU-210, a potent CB_1/CB_2 receptor agonist. Pretreatment with antagonists to the serotonergic type 1A (5-HT_{1A}; WAY100635; 0.5 mg/kg) and 5-HT_{2A} (ketanserin; 1 mg/kg) receptors significantly attenuated HU-210 induced corticosterone secretion. Similarly, the increase in corticosterone secretion following HU-210 administration was significantly reduced by pretreatment with antagonists to the alpha1-adrenoreceptor (prazosin; 1 mg/kg) and beta-adrenoreceptor (propanolol; 2.5 mg/kg). Conversely, pretreatment with antagonists to the NMDA (MK-801; 0.1 mg/kg) AMPA/Kainate (DNQX; 10 mg/kg) receptors did not modify activation of adrenocortical secretion evoked by HU-210. These data indicate that acute administration of exogenous cannabinoid ligands likely activates the HPA axis indirectly through an increase in serotonergic and noradrenergic signaling.

FUNCTIONAL CHARACTERIZATION OF A MISSENSE MUTATION IN THE CB1-RECEPTOR GENE INDUCED BY ENU MUTAGENESIS IN RATS

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Treatment with N-ethyl-N-nitrosourea (ENU), a powerful mutagen in rodent spermatogonial stem cells, is a highly efficient tool to generate new mutants in mice and rats. Here, we report the functional characterization of a rat line carrying one ENU-induced missense mutation in the CB1 receptor (Cnr1) gene.

The Cnr1 mutant rat line was generated by target-selected ENU-based mutagenesis, resulting in a point mutation in exon 2 of the Cnr1 gene in a male founder rat (Fischer344 background). In order to ascertain whether this missense mutation affects the functionality of the CB1 receptor, [^{35}S]GTPγS binding studies were performed with different cannabinoid receptor ligands (WIN 55,212-2, HU-210) on dissected and homogenized brain tissue from mutant and wild-type animals. Furthermore, the phenotype of mutant rats and wild-type littermates was assessed in a variety of behavioral paradigms, including locomotor activity, emotional behaviors, reward sensitivity and cognition.

WIN 55,212-2 and HU-210-stimulated [^{35}S]GTPγS binding was found to be increased in Cnr1 mutant rats compared to wild-type controls. In addition, mutant animals showed a highly significant decrease in anxiety-related behavior, enhanced reward sensitivity as well as cognitive and attentional deficits.

In conclusion, the present data indicate that the ENU-induced point mutation in the Cnr1 gene leads to enhanced CB1 receptor signalling. Furthermore, this rat line might serve as a useful animal model for impulsivity and addictive behavior, due to the pronounced phenotype which is mainly characterized by the lack of anxiety-responses and increased sensitivity for rewarding stimuli.

ACUTE EFFECTS OF ORAL Δ^9-TETRAHYDROCANNABINOL AND STANDARDIZED CANNABIS EXTRACT ON THE AUDITORY EVOKED P300 POTENTIAL: INFLUENCE OF GENETIC VARIANTS WITHIN THE CANNABINOID RECEPTOR GENE

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Impaired P300 generation is a robust finding in schizophrenia and substance dependence, as well as in chronic cannabis abuse, indicating deficient attentional resource allocation and active working memory. Moreover, an (AAT)*n* triplet repeat polymorphism within the cannabinoid receptor gene (*CNR1*) has been found to be associated with schizophrenic and addictive disorders. Given the involvement of the endogenous cannabinoid system in the pathogenesis of schizophrenia as well as in mesolimbic reward pathways, we examined the acute effects of oral Δ^9-tetrahydrocannabinol (Δ^9-THC) and standardized cannabis extract, containing Δ^9-THC and cannabidiol (CBD), on P300 generation, and the association between genetic variants within *CNR1* and the cannabinoid-induced alterations of the P300 potential. This study was performed in a prospective, double-blind, placebo-controlled cross-over design. On three consecutive weeks, twenty healthy volunteers were administered either Δ^9-THC (10 mg), cannabis extract (10 mg Δ^9-THC, 5.4 mg CBD), or placebo. P300 waves were recorded during an auditory choice reaction task. Δ^9-THC as well as cannabis extract revealed a significant reduction of P300 amplitude at midline frontal, central, and parietal electrodes. Concerning the AAT >12/>12 genotype, there was a significant decrease of P300 amplitude under the Δ^9-THC condition, and a significant prolongation of P300 latency under the Δ^9-THC and placebo condition. Moreover, the number of AAT repeats was significantly correlated with the P300 amplitude and latency. Our data suggest that Δ^9-THC as well as cannabis extract may lead to acute impairment of attentional functioning and working memory. These cognitive deficits, as assessed by the P300 wave, depend on the number of AAT repeats within the *CNR1* gene. Given the close interaction between the endogenous cannabinoid system and dopamine release and metabolism, that is critically involved in the susceptibility to schizophrenia as well as to substance dependence, it can be speculated whether the above-mentioned effects of Δ^9-THC and *CNR1* may be due to a relevant influence on dopaminergic neurotransmission.

A MOUSE MODEL FOR THE SCHIZOPHRENIA CANDIDATE GENE NEUREGULIN 1 - MECHANISMS BEHIND AN AGE-DEPENDENT AND Δ^9-TETRAHYDROCANNABINOL-SENSITIVE BEHAVIOURAL PHENOTYPE

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Neuregulin 1 (*NRG1*) is one of the most promising susceptibility genes for schizophrenia and is known to influence neurodevelopmental processes in the CNS potentially related to schizophrenia. The neurodevelopmental theory of schizophrenia suggests that interactions between genetic and environmental factors during development are responsible for biochemical alterations leading to schizophrenia.
Earlier, we characterized heterozygous transmembrane domain *Nrg1* mutant (*Nrg1* HET) mice and their wild type-like littermates (WT) at two different ages (3-4 months vs. 4-6 months) and after acute treatment with the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC) using a variety of behavioural paradigms. We discovered an age-dependent hyper-locomotive and anxiolytic-like phenotype for *Nrg1* mutants. These mice were also more sensitive to the locomotor suppressant and anxiogenic effects of acute THC. Using autoradiography, we have now analysed the expression of the cannabinoid receptor 1 (CB1), the dopamine 2 receptor (D2) and the glutamatergic NMDA receptor for *Nrg1* HETs and WT animals in both age groups.
Receptor binding analyses revealed significant genotype and age differences: CB1 expression was significantly increased in the substantia nigra of *Nrg1* HET compared to WT mice. D2 binding was reduced in the caudate putamen. Furthermore, NMDA receptor expression was decreased in the lateral thalamus of mutant mice and the hippocampus exhibited a reduction in NMDA binding in 4-6 months old *Nrg1* HETs compared to younger mutants.
In conclusion, the age-dependent schizophrenia-related behavioural phenotype of heterozygous Nrg1 mutant mice as well as their increased sensitivity to THC seems to be dependent on a disbalance in the cannabinoid as well as the dopaminergic and glutamatergic system.

EFFECTS OF THE CB1 CANNABINOID AGONIST WIN 55,212-2 SELF-ADMINISTRATION ON THE BEHAVIOURAL RESPONSES IN THE PHENCYCLIDINE (PCP) MODEL OF SCHIZOPHRENIA IN RATS

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Recently, on the basis of the still incongruent data on the correlation between high Cannabis consumption in schizophrenics and frequent psychoses episodes in Cannabis users, a cannabinoid hypothesis of schizophrenia has been proposed according to which schizophrenic dysfunctions could be related to dysregulation of the endocannabinoid system. Cannabis abuse is a significant yet complex variable in schizophrenia but whether such comorbidity may be due to a causal relationship is still to be clarified. Some schizophrenics suffer from the deteriorating influence of Cannabis reducing the vulnerability threshold and/or coping resources, in some individuals already vulnerable to schizophrenia Cannabis could act as stress factor triggering psychosis, and others use Cannabis as self-medication against negative and depressive symptoms of schizophrenia.

Objective of the present study was to elucidate the role of the endocannabinoid system in mechanisms underlying psychotic phenomena. To this aim we used the phencyclidine (PCP) model of schizophrenia to investigate the effects of previous history of spontaneous cannabinoid abuse on behaviours symptomatic of schizophrenic traits. To this purpose, rats previously trained to intravenously self-administer WIN 55,212-2 underwent an acute or chronic intermittent PCP treatment (2.5 mg/kg), and their motor responses were compared with those of drug-naïve rats. Moreover, since many schizophrenic are significantly impaired in memory skills and sociability, animals have been also tested for object recognition memory and social interaction. Results obtained 24h after the last injection with PCP showed that: - after chronic intermittent PCP treatment, horizontal, vertical, marginal and central motor activities did not change in drug naïve rats and WIN trained rats. On the contrary, both dug-naive and trained- WIN animals showed higher horizontal motor activity after a challenge of PCP as compared to saline treated animals; - both in the object recognition and social interaction tests, drug-naive rats displayed cognitive and social impairments when acutely or chronically treated with PCP. Conversely, at the end of the self- administration period WIN-trained were not impaired in both memory and sociability, and after chronic intermittent PCP treatment they maintain memory performance, and social contact higher than saline treated rats. These findings seem to suggest that in the PCP experimental model of schizophrenia, a history of spontaneous cannabinoid abuse does not precipitate behavioral traits like resembling human positive symptoms, and concomitantly seems to protect animals from cognitive deficits and social interaction impairments which instead resemble human negative symptoms.

OLFACTORY BULBECTOMY AS A RODENT MODEL OF CANNABINOID DYSFUNCTION

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Introduction: The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) modulate neuronal excitability. Removal of the olfactory bulb in rats results in behavioral and neurobiological symptomatology indicative of dopaminergic dysfunction in the ventral striatum. The present work aims to elucidate the neuromodulatory role of endocannabinoids on two types of behavior known to be influenced by dopaminergic dysfunction in the olfactory bulbectomized (OBX) rat: hyperactive response to novelty and a presensitized motor response to amphetamine.

Methods: Male Sprague-Dawley rats underwent olfactory bulbectomization or sham surgery. Two weeks following surgery, locomotor activity was measured in rats exposed to a novel environment. Liquid chromatography/mass spectrometry (LC/MS) was used to quantify regional changes in endocannabinoid content in OBX and sham rats that underwent exposure to novelty. In a separate experiment, OBX and sham rats received eight daily systemic injections of amphetamine sulfate for the induction of locomotor sensitization. To determine the impact of manipulation of endocannabinoid transmission on sensitization behavior, the FAAH inhibitor URB597, an inhibitor of anandamide hydrolysis, and the CB_1 antagonist rimonabant, were administered prior to amphetamine treatment.

Results: Novelty-induced locomotor activity was greater in OBX rats relative to sham-operated groups. Endocannabinoid content in the ventral striatum was also reduced following olfactory bulbectomy. 2-AG levels were negatively correlated with novelty-induced locomotor activity in sham-operated rats, and positively correlated in OBX rats. OBX rats exhibited increased amphetamine-induced locomotor activity even on the first day of administration. URB597 reduced the development of amphetamine-induced locomotor sensitization in sham but not in OBX rats. The CB_1 antagonist rimonabant blocked the effect of URB597.

Conclusion: Olfactory bulbectomy induces a dysregulation of the cannabinoid signaling in the ventral striatum. The extent of this dysregulation is correlated with activity in response to novelty. The negative correlation between 2-AG accumulation and novelty-induced locomotor activity in sham rats is consistent with the hypothesis that endocannabinoids suppress novelty-induced dopamine release in the ventral striatum in intact animals, and this modulation is dysfunctional following OBX. Amphetamine-induced hyperactivity is also insensitive to inhibition of anandamide deactivation in the OBX rat. Our results suggest that the OBX model may be useful for studying behavioral consequences of endocannabinoid dysregulation.
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NEUTRAL CB1 RECEPTOR ANTAGONISTS DO NOT BLOCK Δ^9-TETRAHYDROCANNABINOL EFFECTS, BUT HAVE ANTIDEPRESSANT POTENTIAL

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SR141716, the first cannabinoid CB1 receptor antagonist, also displays inverse agonist and at high doses, agonist properties. These additional effects of SR141716 make the interpretation of its effects inconclusive. Recently, several neutral CB1 antagonists have been synthesized. In this study we further characterized 5-(4-chlorophenyl)-3-[(E)-2-cyclohexylethenyl]-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole (VCHSR) and (5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-[(E)-piperidinoiminomethyl]-1H-pyrazole) PIMSR. METHODS: In order to investigate the antagonistic effects of VCHSR and PIMSR, 1. they were compared to SR141716 for inhibition of ^{35}GTPγS binding in membranes expressing hCB1 receptors 2. they were injected *i.p.* to female mice, 30 min before Δ^9-tetrahydrocannabinol (THC). After 60 min, mice were tested in the 'tetrad', a series of *in vivo* assays used to characterize cannabinoids (ambulation and rearing in an open field, catalepsy, hypothermia and nociception), and in addition, for THC-induced inhibition of intestinal motility. 3. Therapeutic potential of VCHSR and PIMSR was monitored in the forced swim test (FST) for 'antidepressant-like' effects in and the 'plus maze' for 'anxiolytic' effects. 4. Putative adverse effects were assessed (hyperthermia, acoustic startle response, behavioral adaptability (PPI, 'pre-pulse inhibition of the startle reflex') and hyperactivity). RESULTS: 1. SR141716 reduced GTPγS binding, but VCHSR and PIMSR were inactive, confirming that these compounds are neutral antagonists. 2. SR141716 (5 mg/kg) antagonized THC-induced effects in the tetrad and on intestinal motility. In contrast, except for rearing behavior, none of the THC-induced effects were antagonized by VCHSR (5-10-12.5-25 mg/kg) or PIMSR (11 mg/kg). 3. No 'anxiolytic' potential was recorded for either VCHSR (12.5-25 mg/kg) or PIMSR (5.5-11 mg/kg), but each antagonist displayed significant 'antidepressant' effects at each dose. 4. No adverse effects such as hyperthermia, hyperactivity or 'anxiety' were observed for either VCHSR or PIMSR when given alone. However, the acoustic startle reflex was enhanced by SR141716 and reduced by PIMSR, while PPI was impaired by PIMSR only. CONCLUSIONS: The observation that these neutral CB1 receptor antagonists did not inhibit THC-induced effects in the mouse 'tetrad', suggests that 'silent' blockade of the CB1 receptor is not enough to antagonize the mouse tetrad assays, but rather, inverse agonism is required. On the other hand, endocannabinoid tone may be responsible for depressive-like behavior, since its interruption had antidepressant activity. Finally, the absence of VCHSR-induced adverse effects, suggests that this compound may be developed as a selective antidepressant, with a reduced side effect profile.

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ROLE FOR SEROTONIN ON THE ANTIDEPRESSANT-LIKE EFFECT INDUCED BY Δ^9-TETRAHYDROCANNABINOL, BUT NOT BY RIMONABANT

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Considering the evidences that the endocannabinoid system may modulate serotoninergic transmission, the present work was designed to test the hypothesis that this neurotransmitter would be relevant for the antidepressant-like effect induced by Δ^9-tetrahydrocannabinol (THC). C57BL/6N mice (n=10/group) receive THC (0.1 mg/kg) or rimonabant (10 mg/kg) injections and were exposed to the forced swim test. In separate experiments, the animals were pre-treated with vehicle, the serotonin synthesis inhibitor para-chlorophenylalanine (pCPA, 100 mg/kg) or the 5-HT1A receptor antagonist WAY-100635 (1 mg/kg). The data were analyzed by ANOVA followed by the Newman-Keuls test. Both THC and rimonabant induced antidepressant-like effects. Either pCPA or WAY-100635 inhibited the effects of THC, but not of rimonabant. None of the treatments modified locomotor activity, excluding this parameter as a possible confounding factor. In conclusion, the present data suggest that, although both THC and rimonabant induce similar behavioural changes, they seem to act via different mechanisms.

CANNABINOID CB1 RECEPTOR ACTVITY IN DISTINCT FRONTOCORTICAL SUBREGIONS DIFFERENTIALLY REGULATES COPING RESPONSES IN THE FORCED SWIM TEST

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In recent years, the endocannabinoid system has emerged as an important new target in the development of novel antidepressant drugs. However, a discrepancy exists in the literature such that antidepressant effects have been demonstrated following systemic administration of both CB1R agonists and antagonists. Microinfusion studies from our laboratory and others have revealed an antidepressant effect in the forced swim test following infusions of a CB1R agonist directly into the dentate gyrus (McLaughlin et al., 2007; *Behav Pharmacol* 18, 431-438) or ventromedial prefrontal cortex (Bambico et al., 2007; *J Neurosci* 27: 11700-11711). However, the anatomical site where CB1R antagonists exert their antidepressant effects has yet to be elucidated. With that said, the present study assessed the effects of discrete infusions of a CB1R agonist (HU-210) or antagonist (AM251) directly into the ventromedial prefrontal cortex, anterior cingulate cortex, or basolateral amygdala on behavioral responses in the Porsolt forced swim test.

Following recovery from surgical implantation of bilateral guide cannulae, a 15-min pre-exposure to the forced swim test was performed, following which animals were randomly assigned to groups each receiving three direct infusions of either a low or high dose of the CB1R antagonist AM251 (1 or 2.5 μg), a low or a high dose of the CB1R agonist HU-210 (1 or 2.5 μg) or dimethyl sulfoxide (vehicle control) over a 24-hour period. The duration of immobility, swimming, and struggling was measured during a 5-min forced swim test session.

In line with the recent report from Bambico and colleagues (2007), our results demonstrated that both a high and low dose of the CB1R agonist HU210 infused into the ventromedial prefrontal cortex decreased immobility and increased swimming in the forced swim test, indicating that this treatment evoked an antidepressant response. Interestingly, no significant modification of behavioral responses in the forced swim test were seen when AM251 was infused into the ventromedial prefrontal cortex. Alternately, infusions of AM251 into the anterior cingulate cortex resulted in a dose-dependent antidepressant response as revealed by a reduction in immobility, while no significant effects were observed when AM251 was infused directly into the basolateral amygdala. Collectively, these data argue that CB1R signaling in distinct frontocortical subregions has differential effects on emotional behavior, and that the anterior cingulate cortex may be an integral neuroanatomical site mediating the reported antidepressant effects seen following systemic administration of a CB1R antagonist.

DEVELOPMENTAL CANNABINOID EXPOSURE PERSISTENTLY ALTERS FOXP2 EXPRESSION WITHIN ZEBRA FINCH STRIATUM

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Early cannabinoid exposure is causes changes in zebra finch vocal learning. This learning is dependent upon successful progress through distinct developmental stages. Physiological changes associated with this development include changes in expression of the transcription factor, FoxP2. In addition to evidence for a role in zebra finch vocal learning, FoxP2 mutations are associated with specific language disorders in man. Cannabinoid-altered song learning and FoxP2 involvement in normal processes of vocal development led us to investigate a potential mechanistic relationship.

Following song tutoring by an adult male, groups (n = 15) of young male zebra finches were injected with vehicle or the cannabinoid agonist WIN55212-2 (WIN, 1 mg/kg IP) from 50-75 days of age (approximately adolescence) and allowed to age to young adulthood (at least 100 days) in visual isolation. Songs were recorded and groups were further divided into three groups (n = 5) to receive: no further manipulation; a vehicle injection or; 3 mg/kg of WIN. Ninety minutes following these final treatments brains were processed for immunohistochemistry using an anti-FoxP2 antibody. Immuno-positive nuclei per unit area were determined and compared across treatment groups.

In adults, acute WIN treatment resulted in significantly increased FoxP2-expressing cells within zebra finch striatum. Similar patterns of increased expression were seen following early, chronic WIN exposure, demonstrating changes that persisted from adolescence through early adulthood. Persistent changes in FoxP2 expression following early WIN exposure suggest that cannabinoid-altered vocal development involves gene expression under FoxP2 control.

IN VIVO BRAIN TYPE 1 CANNABINOID RECEPTOR AVAILABILITY IN PATIENTS WITH ANOREXIA AND BULIMIA NERVOSA

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Introduction : The endocannabinoid system is an important modulator in the regulation of food intake and energy metabolism and a possible novel target in the treatment of eating disorders. We used positron emission tomography (PET) with [^{18}F]MK-9470 to test whether *in vivo* binding of this type 1 cannabinoid receptor (CB1R) specific ligand is altered in bulimic and anorectic patients in comparison to age- and gender-matched healthy volunteers.

Methods : We investigated 17 female patients with bulimia nervosa (BN; mean age = 23.9±6.9; range 17-45 years) and 10 patients with anorexia nervosa (AN; mean age = 20.9±3.9; range 17-30 years) using [^{18}F]MK-9470 PET and T1 MPRAGE MRI. The control group consisted of 19 women (mean age = 25.2±8.5; range 18-45 years). Following coregistration to MRI data and spatial normalization, parametric standardized uptake value (SUV) images reflecting receptor availability were calculated. For regional analysis, SUV values were normalized on the individual lobal grey matter SUV. Optimized voxel-based morphometry (VBM) was performed. On the CB1R PET images, both statistical parametric mapping (SPM2; $p_{height}<0.001$, uncorrected) and predefined volume-of-interest (VOI; unpaired t-tests, $p<0.05$) analyses were performed.

Results : No significant changes in gray matter concentration were found, hence no partial volume correction was carried out. No global changes in SUV between groups were detected. Regionally, in the BN patients, relative CB1R availability was significantly increased in the left insular cortex (peak voxel value +5.0%; SPM: $p_{cluster}=2.0\ 10^{-5}$ corrected; VOI: p=0.0002) and in the left superior frontal cortex (+3.5%; SPM: $p_{cluster}=0.003$ corrected; VOI: p=0.019). Relative CB1R availability was increased bilaterally in the insular cortex of AN patients (+5.9%; SPM: $p_{cluster}<0.011$ corrected, VOI: p=0.0026). Between AN and BN patient groups, no differences in relative CB1R availability were found.

Conclusions : Regionally, CB1R availability is increased both in bulimia and anorexia nervosa in the insular cortex, a region important in the integration of interoceptive information including primary gustatory information.

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FOOD DEPRIVATION AND ANANDAMIDE ADMINISTRATION AFFECT CB1 EXPRESSION IN THE GOLDFISH BRAIN

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Studies performed in vertebrates and also in invertebrates suggest that the involvement of the endocannabinoids in the control of food intake is conserved through phylogeny.

In mammals it was reported that endocannabinoids behave as orexigenic mediators and indeed CB1 antagonists have been developed for obesity treatment. Furthermore, it has been demonstrated that fasting induces the release of brain endocannabinoids. By contrast, data on CB1 expression modifications after food deprivation are lacking, with the exception of a reported increase of CB1 receptors in rodent nodose ganglion.

Our previous investigations carried out in different bonyfish species have shown that CB1 receptors are abundantly distributed in brain regions, e.g. telencephalon and inferior lobes of the posterior hypothalamus, which control different aspects of the feeding response. In the goldfish, experimental food deprivation is followed by a significant increase of anandamide levels in the telencephalon and anandamide administration affects in a dose-dependent manner the food intake, suggesting that endocannabinoids might variously contribute to fish adaptation to food shortage.

In the present study we investigated the possible influence of fasting and refeeding on the expression of CB1 mRNA in the goldfish brain. Indeed, 24 hrs and 48 hrs food deprivations induce an increase of CB1 mRNA levels that lower after refeeding.

Moreover, we performed intraperitoneal anandamide injection experiments in order to evaluate possible changes in the expression of CB1 receptors, as well as of NPY, an important orexigenic molecule widely distributed in the goldfish brain.

Our results point to an involvement of CB1 cannabinoid receptors in the central control of bonyfish feeding response.

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ENDOCANNABINOID AND OREXIN-1 INTERACTIONS IN THE HYPOTHALAMUS: ONE POSSIBLE CLUE TO OBESITY

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Endocannabinoids and CB_1 receptors stimulate food deprivation-induced food intake by acting on hypothalamic neurons. Endocannabinoid levels change in the hypothalamus following food deprivation (Kirkham et al., *Br. J. Pharmacol*., 2002) and in rodents with defective leptin signaling (Di Marzo et al., *Nature* 2001). Orexin is produced in a distinct subset of neurons of the lateral hypothalamus (LH) projecting to all regions of the brain (Sukurai et al., *Cell* 1998). Pretreatment with subeffective doses of rimonabant attenuates the orexigenic actions of orexin-1 (Crespo et al., *Neuropharm.* 2008), whereas electrophysiological data support the inhibitory role of cannabinoids on orexinergic neurons in physiological conditions (Huang et al., *J. Neurosci.* 2007), although it remains to be clarified if the same phenomenon also occurs in obesity, especially since high neural plasticity occurs in this circuitry for adequate regulation of energy balance (Horvart & Gao, *Cell Metab*. 2005). We have investigated here endocannabinoid/orexin-1 interactions in C57Bl/6j lean mice and in mice made obese following 14 weeks of a diet-induced obesity (DIO) with 25.5% fat.

Immunohistochemical studies (immunoperoxidase, confocal and electron microscopy, single or double-staining) were performed in the LH for orexin-1 (OX-1), CB_1, the endocannabinoid biosynthesising enzymes, DAGL-α and NAPE-PLD, and vescicular GABA or glutamate transporters as markers of GABAergic or glutamatergic fibers. The immunoperoxidase staining was followed by quantitative analysis of each immunoreactive signal using a digital densitometric system (Imaging Computer System, Leica©). Endocannabinoid levels were measured by means of LC-MS.

LH orexinergic neurons exhibited DAGL-α immunoreactivity (IR) in the somatodendritic compartment and were immersed in a meshwork of CB_1-positive fibers. Increased DAGL-α , but not NAPE-PLD, was observed both in the LH and arcuate nucleus of DIO *vs.* lean mice. CB_1 IR was localized on both glutamatergic and GABAergic axon terminals surrounding orexinergic neurons. A significant increase of 2-AG, but not of AEA, levels was also found in the hypothalamus of DIO *vs*. lean mice.

The reciprocal cellular and subcellular localization of CB_1, GABA, glutamate, DAGL-α and OX-1 in the LH suggests a model of retrograde control by CB_1 over OX-1 release. DAGLα–mediated synthesis of 2-AG might be triggered by glutamatergic terminals that innervate OX-1 neurons. In DIO mice, the increase of DAGL-α and 2-AG in orexinergic neurons might cause a postsynaptic disinhibition of orexin release by 2-AG acting on presynaptic CB_1-expressing GABAergic neurons and contribute to hyperphagia. Electron microscopy observations and electrophysiological experiments are ongoing to substantiate this hypothesis.

BRAIN DELETION OF CB1 CANNABINOID RECEPTORS INDUCES RESISTANCE TO DIET-INDUCED OBESITY

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Obesity, a disease characterized by impaired energy homeostasis, is one of the main health problems in industrialized countries, creating an urgent need to generate new pharmacologic anti-obesity drugs. During the last years the endocannabinoid system (ECS) has been shown to play a central role in the regulation of food intake and energy balance. A growing number of data indicate that the ECS participates in the development of obesity both via its functions in the central nervous system and in the periphery. However, the differential involvement of the ECS functions in the brain as compared to peripheral organs has only started to be systematically investigated. In order to start dissecting the central and peripheral roles of the ECS in the development of obesity, we performed feeding experiments using complete CB_1 knock-out mice (CB_1-KO, Marsicano et al., Nature, 2002), conditional mutant mice lacking the expression of CB_1 in the forebrain principal neurons (CaMK-CB1-KO, Marsicano et al., Science 2003) and their control littermates (CB_1-WT and CaMK-CB1-WT, respectively).

Mice were fed with two different diets, pelleted standard mouse chow (SD, 18,6 KJ/g) or high fat diet (HFD, 21,3 KJ/g) for 12 weeks starting at 8 weeks of age. Body weight and food intake were monitored twice per week. Energy expenditure was measured in all groups during the 7th week of diet treatment. Feaces were collected, and their energy content was measured by bomb calorimetry. Derived factors, assimilated energy and loss energy, were calculated. In order to better characterize the residual expression of CB_1 mRNA in CaMK-CB1-KO mice, single and double *in situ* hybridization and quantitative RT-PCR were performed in the brain and peripheral organs, including organs known to be involved in metabolic processes and sympathetic ganglia. Detailed expression analyses revealed that CB1 expression was virtually absent in the hypothalami of CaMK-CB1-KO mice, but was still present both in sympathetic ganglia and in all the peripheral organs analysed.

CaMK-CB1-KO mice showed a decreased body weight as compared to controls under SD conditions. However, this difference was less pronounced than in full CB1-KO mice. Under HFD, CB1-KO mice maintained the same difference of body weight as compared to CB1-WT, whereas CaMK-CB1-KO, after a first period during which they gained some weight, showed with time a lower weight gain as compared to CaMK-CB1-KO littermates, which was clearly evident at the end of HFD period. Interestingly, daily food intake of CaMK-CB1-KO mice was not significantly different from control littermates. Bomb calorimetry data showed that these mutant mice produced faeces with higher energy content compared to their littermates under HFD, while no differences were present between genotypes under SD conditions.
Our data show that brain CB1 receptors play an important role in the control of energy balance, especially in HFD conditions. This function is likely not related to alterations in food intake, but rather to higher levels of energy dissipation. However, given the overall stronger phenotype of complete CB1-KO as compared to CaMK-CB1-KO, our data indicate that peripheral CB1 receptors substantially participate in the functions of the ECS in the control of energy balance.

CB2 RECEPTOR ANTAGONISM AS A NOVEL APPROACH IN THE MANAGEMENT OF OBESITY AND THE METABOLIC SYNDROME

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Recent data have established the crucial role of inflammation in obesity, insulin resistance and metabolic steatosis. Growing evidences indicate that the endocannabinoid system plays a major role in obesity and steatosis via CB1 receptors. However, the possible contribution of the cannabinoid receptor CB2 has not been reported. Interestingly, CB2 receptors are predominantly expressed in inflammatory cells and regulate immune and inflammatory responses. Therefore, in the present study, we investigated the role of CB2 receptors in insulin resistance and hepatic steatosis, owing to the use of CB2KO and wild type mice exposed to a high fat diet for 15 weeks.

After 15 weeks of high fat diet, body weight was significantly lower in CB2 KO mice as compared to WT mice, despite similar food intake. As expected, WT mice developed insulin resistance as assessed by elevated serum insulin levels, increased HOMA-IR and reduced insulin tolerance tests. In contrast, these parameters were significantly improved in CB2 KO animals, indicating reduced insulin resistance. Finally, whereas WT mice developed severe fatty liver, as shown by histological analysis of liver tissue sections and increased hepatic triglycerides, hepatic CB2 KO mice exhibited minimal hepatic steatosis.

Since obesity is associated with a low grade inflammation in the adipose tissue that contributes to the development of insulin resistance and NAFLD, we also investigated the consequences of CB2 antagonism on obesity induced fat inflammation. Obese WT mice exhibited marked induction of CB2 receptor expression in white epididymal adipose tissue. As expected, obese WT mice displayed increased fat inflammation , as indicated by a strong increase in the density of macrophages and a parallel induction of TNF-a and MCP-1 mRNA expressions. In contrast, high fat diet-fed CB2-/- mice showed significantly lower inductions of F4/80, TNF-a and MCP-1 mRNAs in the adipose tissue. both tissues.

In conclusion, our results unravel a novel role for CB2 in the pathogenesis of obesity, insulin resistance and hepatic steatosis, by a mechanism that may involve a proinflammatory effect of CB2 receptors in the adipose tissue. These data identify CB2 as a potential novel therapeutic target for the management of obesity, insulin resistance and NAFLD.

N-OLEOYLETHANOLAMINE-EVOKED SATIETY: A PERIPHERAL NON-ENTOURAGE ACTION

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N-Oleoylethanolamine (OEA) is a naturally occurring endocannabinoid-like compound (ECL), which increases in the small intestine in response to feeding, and decreases food intake upon exogenous administration. Although OEA appears to lack direct effects at CB_1 and CB_2 receptors, it has previously been hypothesised (along with other ECLs) to have indirect effects on cannabinoid receptors by diverting the hydrolytic activity of FAAH, thereby leading to accumulation of canonical ECLs - the "entourage effect". The aims of this investigation were, first, to determine the distribution of OEA following acute intraperitoneal (i.p) administration to identify a locus of action and, second, whether administration of OEA was able to influence levels of other ECLs in the mouse.

Using the Comprehensive Lab Animal Monitoring System (CLAMS), OEA (10 mg/kg, i.p in C57bl mice) was observed to elicit a transient decrease in food intake (vehicle 0.19 ± 0.02, OEA 0.075 ± 0.02 g; P<0.05) 0-2 hours post-injection. In a parallel experiment, four groups of four adult male C57bl mice received a single i.p injection of either vehicle (5% Tween 80, 5% polyethylene glycol, 90% sterile saline) or OEA (10 mg kg^{-1}). 30 or 60 min after injection, levels of OEA, PEA, AEA, 2LG and 2AG in brain, gut and liver tissues were quantified by LC-MS.

Basal levels of OEA in the hippocampus and hypothalamus were 844 ± 102 and 755 ± 63 pmol g^{-1}, respectively and were unaltered following OEA administration. In the liver, OEA levels were lower: 85 ± 16 pmol g^{-1}, and tended to increase following OEA administration at both 30 min and 60 min (422 ± 219; 473 ± 160 pmol g^{-1}, respectively), albeit non-significantly. OEA levels were significantly elevated in the ileum at 30 min (vehicle 0.5 ± 0.04; OEA, 14.3 ± 5.2 nmol g^{-1} of tissue; P<0.01) and at 60 min (vehicle 1.92 ± 1.10; OEA, 8.7 ± 4.9 nmol g^{-1};P<0.05) and in duodenum at 30 min (vehicle 0.2 ± 0.03; OEA 3.1 ± 1.1 nmol.g^{-1} of tissue; P<0.05) and at 60 min (vehicle 0.01 ± 0.3; OEA 2.9 ± 1.03 nmol.g^{-1} of tissue; P<0.05). In the hippocampus and hypothalamus, AEA levels were 120 ± 4 and 66 ± 5 pmol g^{-1}, respectively. In the liver, duodenum and ileum, AEA levels were lower (6 ± 1; 15 ± 2; 14 ± 0.9 pmol g^{-1}). AEA levels in all tissues were unaltered following OEA administration. Basal levels of PEA followed a similar pattern of distribution, with higher levels in the hippocampus and hypothalamus (902 ± 53 pmol/g), compared to the peripheral tissues (133-295 pmol/g). PEA levels in the brain, liver and duodenum were unchanged following OEA administration.

In summary, administration of OEA appears to alter levels of OEA in peripheral, but not central tissues, implicating the periphery as the target for OEA-evoked satiety signalling. Furthermore, we have no evidence for increased levels of other ECLs in the liver and GI tract after OEA administration, indicating that the actions of OEA are not a result of an entourage effect *in vivo*.

EFFECTS OF A HIGH-FAT DIET DURING ADOLESCENCE ON SENSITIVITY TO Δ^9-TETRAHYDOCANNABINOL

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An emerging body of evidence suggests that the consumption of a high-fat diet is associated with changes in CB_1 receptor (CB_1R) density in some areas of the brain. CB_1R density declined significantly in the striatum and hypothalamus when mice were fed a high-fat diet (36% for calories from fat) for 6 weeks. While reductions in CB_1R density were significant in both of these brain areas, the observed changes were more pronounced in the striatum than in the hypothalamus. Other research suggests that, in rats, sustained consumption (10 weeks) of a diet containing elevated levels of polyunsaturated fats is associated with significant reductions in CB_1R density in some areas of the brain (e.g., hippocampus, nucleus accumbens), but not in the hypothalamus. Given the possible relationship between high-fat diets and CB_1R density, a series of experiments was undertaken to explore how sustained consumption of a diet high in fat affects sensitivity to Δ^9-tetrahydrocannabinol (Δ^9-THC). A group of male Long-Evans rats (n = 9) was fed a high-fat diet (45% of calories from fat) from postnatal day 30 (PD30) through PD128 (14 weeks). A separate group of male Long-Evans rats (n = 8) was fed a control diet (13.5% of calories from fat) over the same developmental period. Cannabinoid activity was assessed for both groups in a triad of measures (hypothermia, catalepsy & locomotor inhibition) on PD30, PD37, PD44, PD61, PD68, & PD128. In order to obtain a full dose-effect curve in each rat, Δ^9-THC was administered in a cumulative dosing procedure (0, 3, 10, 30 & 100 mg/kg) on each of these days. When tested on PD128, rats fed a high-fat diet were found to be significantly less sensitive to the catalepsy-inducing effects of a moderate dose of Δ^9-THC (10 mg/kg) than animals fed the control diet. Despite the observed differences in catalepsy, the groups were not different from each other in a measure of gross locomotor activity. Similarly, there were no significant differences in patterns of movement (e.g., no differences in thigmotaxis) between the groups. Finally, these experiments did not reveal any inter-group differences in hypothermia. These results suggest that the sustained consumption of a high-fat diet throughout adolescence and into adulthood can affect sensitivity to some, but not all, of the pharmacological effects of Δ^9-THC. These results complement the nascent body of work suggesting that CB_1R density changes in a region-specific manner after the sustained consumption of a high-fat diet.

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HYPOPHAGIC PROPERTIES OF SYNTHETIC AND PLANT-DERIVED CANNABINOID RECEPTOR ANTAGONISTS

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Introduction: The prevalence of obesity and childhood obesity has increased over the last 25 years, with obesity being attributed to increased consumption of foods containing high levels of sugar and saturated fats, combined with a reduction in physical activity. The synthetic CB_1 receptor antagonists SR141716A (rimonabant) and AM251 are known to suppress food intake. (-)-Δ^9-tetrahydrocannabivarin (Δ^9-THCV) is a cannabinoid present in cannabis. Recent work has shown that Δ^9-THCV behaves like a CB_1 receptor antagonist both *in vitro* and *in vivo*.

Methods: A standard fasted protocol and a novel long-term home cage observation system 'Phenotyper' with free-feeding animals were used to assess the feeding behaviour of C57Bl6 mice treated with the CB_1 antagonist AM251 (10 mg/kg i.p.). The effects of pure Δ^9-THCV (3, 10, 30 mg/kg i.p.) and a Δ^9-THCV rich extract alone and in combination with cannabidiol (CBD) (10 mg/kg i.p.) were also determined in free-feeding animals. The extract was administered to give Δ^9-THCV doses of 0.48, 0.96, 1.44, 3, 10, or 30 mg/kg i.p.

Results: AM251 suppressed food intake and weight gain in both fasted and non fasted animals. The suppression of food intake was continuous following injections repeated once-daily for 4 consecutive days. Pure Δ^9-THCV induced hypophagia and weight reduction in non-fasted animals even at a dose as low as 3 mg/kg. No rebound effects were observed when treatment was discontinued with all drug groups returning to normal activity and feeding patterns on the following day. Treatment with the Δ^9-THCV-rich cannabis-extract however, failed to suppress food intake and weight gain. This is possibly due to residual Δ^9-THC present within the extract. The Δ^9-THC effect was overcome by co-administration of CBD suggesting a possible synergistic interaction between CBD and Δ^9-THCV or CBD-induced antagonism of Δ^9-THC.

Conclusion: The home cage observation system is an effective model for assessing feeding behaviour in free-feeding animals. The phytocannabinoid (Δ^9-THCV) has hypophagic properties similar to AM251 and is a potential medicine for the treatment of obesity.

SUPPRESSION BY RIMONABANT OF THE REINFORCING AND MOTIVATIONAL PROPERTIES OF A CHOCOLATE-FLAVOURED BEVERAGE IN RATS

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Introduction – Pharmacological blockade of the cannabinoid CB_1 receptor has been repeatedly reported to suppress intake of food, including highly palatable foods, in laboratory animals. The present study was designed to investigate whether treatment with the cannabinoid CB_1 receptor antagonist, rimonabant, would reduce the reinforcing and motivational properties of a highly palatable chocolate-flavoured beverage in rats trained to self-administer this beverage under an operant conditioning procedure.

Methods – Male adult non-food- and -water-deprived Wistar rats were initially trained to lever-press for the chocolate-flavoured beverage [containing 5% (w/v) chocolate powder] under the fixed ratio (FR) 10 (FR10) schedule of reinforcement for 5-s reinforcer presentations in daily sessions of 20 min. After a period of training and maintenance of the self-administration behaviour, separate groups of rats were used to test the effect of acute and repeated treatment with rimonabant (1-5.6 mg/kg, i.p.) on two schedules of reinforcement [FR10 and progressive ratio (PR)] and extinction responding.

Results – All rats rapidly acquired and steadily maintained high levels of self-administration of the chocolate-flavoured beverage under the FR10 schedule. Acute treatment with rimonabant dose-dependently suppressed self-administration of the chocolate-flavoured beverage. When rimonabant was administered repeatedly, only a modest degree of tolerance developed to its reducing effect on self-administration of the chocolate-flavoured beverage. Acute treatment with rimonabant resulted also in a dose-dependent reduction of the motivational properties of the chocolate-flavoured beverage, measured by the PR schedule of reinforcement and extinction responding procedure.

Conclusion – These results suggest that the cannabinoid CB_1 receptor is a crucial component of the neural substrate mediating the reinforcing and motivational properties of a highly palatable food such as a chocolate-flavoured beverage.

EFFECTS OF FAAH INHIBITION ON MORPHINE, COCAINE AND NICOTINE ACTIONS IN MESOLIMBIC DOPAMINE NEURONS IN THE RAT

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A large body of evidence indicates that the endocannabinoid (eCB) system is implicated in the modulation of addictive behaviour. Hence, it was demonstrated that eCB system is involved in the mechanism of action of different drugs of abuse, including nicotine, opiates and psychostimulants. For example, blockade or deletion of cannabinoid receptors type-1 (CB1) is associated with a decrease in opiates self-administration (Ledent et al., 1999), and acute administration of cocaine increases anandamide (AEA) levels in the striatum (Arnold, 2005). All the above-mentioned addictive drugs interact with the mesolimbic dopamine (DA) system, which processes many behavioural responses evoked by drugs of abuse. Furthermore, eCBs are implicated in the regulation of DA neuron synaptic functions.

For this reason, we studied the effect of enhanced levels of eCBs induced by the FAAH inhibitor URB597 on nicotine, morphine and cocaine actions on ventral tegmental area (VTA) DA neurons. We utilized extracellular single unit electrophysiological recordings in chloral hydrate anaesthetized rats. Animals were pretreated with URB597 or vehicle and then the effects of nicotine, morphine or cocaine were studied on firing rate and discharge pattern of VTA DA neurons.

Nicotine (0.2 mg/kg i.v.) stimulated the firing rate and the percent of burst firing of VTA DA neurons. When we pretreated the animals with URB597 (0.1 mg/kg i.v., 1-2 hours before recordings) we observed a complete blockade of the stimulatory effects of nicotine. Morphine also (4 mg/kg i.v.) increased the firing rate and the percent of burst firing of VTA DA neurons, but its effects were not antagonized by URB597 pretreatment. Similarly, the inhibitory effects of cocaine (1 mg/kg, i.v.) on firing rate and burst firing were not prevented by URB597.

These findings suggest that URB597 selectively blocks nicotine-induced effects, being ineffective on morphine- and cocaine-evoked excitation or inhibition, respectively, of DA neuronal activity. Thus, our data point toward a specific vulnerability of nicotinic acetylcholine receptors to the negative modulation induced by eCBs, or other fatty acid ethanolamides. The molecular mechanisms underlying these selective actions are currently under investigation.

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ALTERATIONS IN NICOTINE REWARD BY MODULATION OF ENDOCANNABINOID SYSTEM ACTIVITY

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Converging evidence suggests that the rewarding effects of nicotine, which underlie its abuse potential, can be modulated by altering the activity of the endocannabinoid system. In a recent study, we found that treatment of rats with the fatty acid amide hydrolase (FAAH) inhibitor URB597 (cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester), which blocks metabolic degradation of the endocannabinoid anandamide (AEA), impedes development of nicotine-induced conditioned place preference (CPP). In this study, we compared effects of URB597 with those of the anandamide transport inhibitor AM404 (N-(4-hydroxyphenyl) arachidonoyl-ethanolamide) and the classical cannabinoid CB1-receptor agonist, Δ^9-tetrahydrocannabinol (THC) on development of nicotine-induced conditioned place preference. Like URB597, AM404 (5mg/kg i.p.), given before nicotine conditioning sessions, blocked development of conditioned place preference induced by nicotine at a dose of 0.4mg/kg s.c.. AM404 did not potentiate effects of a low threshold dose of nicotine (0.05 mg/kg s.c.), which, alone, did not induce conditioned place preference. In contrast, THC (1mg/kg i.p.) did not affect development of conditioned place preference induced by 0.4mg/kg nicotine, but it potentiated rewarding effects of the low 0.05mg/kg dose of nicotine. When given alone, THC doses ranging from 0.1 to 3 mg/kg id not produce conditioned place preference or aversion, while AM404 produced conditioned place preference at a high 10 mg/kg dose but did not produce conditioned place preference or aversion at lower doses. Our findings suggest endogenously released anandamide can functionally antagonize nicotine reward, while exogenous administration of cannabinoid agonists may potentiate nicotine reward.

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CB1 RECEPTOR ANTAGONISTS AM251 AND SR141716A ATTENUATE COCAINE-INDUCED REDUCTION IN EXTRACELLULAR GLUTAMATE AND GABA, BUT FAIL TO ALTER COCAINE-ENHANCED EXTRACELLULAR DOPAMINE IN THE VENTRAL PALLIDUM OF RATS

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We have recently reported that blockade of cannabinoid CB1 receptors by AM251 or SR141716 inhibits cocaine self-administration under progressive-ratio reinforcement, cocaine-enhanced electrical brain-stimulation reward and cocaine-induced reinstatement of drug-seeking behavior (Xi et al., J Neurosci, 2006; Xi et al., Neuropsychopharmacology, 2007). However, the underlying mechanisms remain unclear. Neither AM251 nor SR141716 alter cocaine-enhanced extracellular dopamine (DA) in the nucleus accumbens (NAc) (Xi et al., J Neurosci, 2006; Caille and Parsons, Neuropsychopharmacology, 2006), suggesting that DA-independent mechanisms are involved. In the present study, we observed the effects of CB1 receptor antagonists on cocaine-induced changes in extracellular DA, glutamate and GABA in the ventral pallidum (VP), the major projection area of the NAc medium-spiny GABAergic neurons. We found that: 1) acute cocaine (3-10 mg/kg, i.p.) dose-dependently increased extracellular VP DA, while it decreased extracellular VP glutamate and GABA, an effect that was blocked by intra-VP perfusion of tetrodotoxin (TTX, 1 μM); 2) pretreatment with AM251 (10 mg/kg, i.p.), but not SR141716A (10 mg/kg, i.p.), attenuated cocaine-induced reduction in both VP glutamate and GABA, but failed to alter cocaine-enhanced VP DA; however, intra-VP perfusion of AM251 or SR141716 (1 mM) blocked cocaine-induced changes in glutamate and GABA, but not in DA; 3) systemic administration of AM251 or SR141716 alone failed to alter extracellular VP DA, glutamate or GABA, while intra-VP perfusion of AM251 or SR141716 lowered extracellular VP glutamate, but not DA or GABA. These findings suggest that: 1) Cocaine-induced reduction in VP glutamate and GABA may play an important role, in addition to cocaine-induced alterations in DA, in cocaine reward and addiction; 2) cocaine-induced reduction in VP glutamate and GABA are cannabinoid-CB1 receptor-dependent, while cocaine-enhanced VP DA is not. Such VP glutamate- and GABA-related mechanisms may in part underlie the antagonism by CB1 receptor antagonists of cocaine's actions in animal models related to drug addiction; 3) there is low tonic cannabinoid modulation of VP glutamate and GABA release; and 4) local AM251- or SR141716-induced reduction in VP glutamate could be related to inverse agonist properties of these cannabinoid antagonists.

DIFFERENTIAL RESPONSE TO A SELECTIVE CANNABINOID RECEPTOR ANTAGONIST IN MICE BRED FOR HIGH VOLUNTARY WHEEL-RUNNING BEHAVIOR

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Exercise is known to be a naturally rewarding behavior in human beings, and can be associated with feelings of euphoria and analgesia. Multiple lines of physiological evidence support a role for the endocannabinoid system in the perception of neurobiological rewards following prolonged exercise. We are employing a novel mammalian system to explore whether alterations in the endocannabinoid system are associated with high levels of voluntary exercise. Specifically, we are studying four replicate lines of laboratory mice that have selectively bred for high total revolutions during days 5 and 6 of a 6-day exposure to activity wheels (1.12 m circumference). As compared with four non-selected Control (C) lines, the four High Runner (HR) lines show 3-fold elevated wheel running (revolutions/day), a similar fold difference in daily home-cage activity when deprived of wheels, but little difference in open-field behavior measured in a 3-minute test in a 1-m square arena. Our general hypothesis is that the four HR lines will have evolved neurobiological mechanisms that increase the incentive salience of aerobically supported, endurance-type exercise, as compared with the four Control lines. As an initial test of this hypothesis, we injected HR and C mice with Rimonabant (SR141716), a selective cannabinoid receptor antagonist/inverse agonist, and measured locomotor response via voluntary wheel running. Experiments were conducted at night, during the time of normal peak wheel running, for both sexes. Each mouse received in a randomized order; low dose Rimonabant (0.1 mg/kg), high dose Rimonabant (3.0 mg/kg), or vehicle injection (20% DMSO, 10% Tween-80 and 70% physiological saline), over a period of 9 days. Drug response was quantified as wheel revolutions 10-70 minutes post-injection. In addition, we analyzed maximum and average speed (in revolutions per minute), and time spent running (minutes). Nested analysis of covariance for repeated measures (mixed-model ANCOVA) indicated that rimonabant decreased wheel running in all mice; however, as compared with Control lines, females from HR lines differentially decreased total wheel revolutions in the 10-70 minutes post-injection (interaction $p= 0.023$), as well as maximum ($p=0.047$) and average speed ($p=0.019$). Male control and HR mice did not differ in the wheel-running response to Rimonabant (all interaction $p>0.50$). These results suggest an association between sex, wheel-running behavior, and the endocannabinoid system. Supported by NSF IOB-0543429 to T.G.

CENTRAL AND CARDIOVASCULAR EFFECTS OF CHRONIC CANNABINOIDS IN THE RAT

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Introduction. Cannabinoids are proposed for the treatment of an increasing number of pathologies, but the side effects of their chronic administration are not well-known. In the rat, acute administration of cannabinoids induces both central and cardiovascular alterations. We studied the effects of the mixed CB1/CB2 agonist WIN 55,212 (WIN) in these systems during and after two different patterns of chronic administration which prevented the development of neuropathic signs in the rat (Costa et al, 2004; Vera et al, 2007).

Methods. Male Wistar rats received saline, vehicle or WIN (0.5 or 5 mg/kg, i.p.), either once a day for 14 days or once a week for 4 weeks. Central and cardiovascular (CV) effects were evaluated right after the first dose (acute), after the last dose of either chronic treatment (chronic) or 1-7 days after treatment (residual effect). Central effects were evaluated with the cannabinoid tetrad in conscious rats. Arterial blood pressure (BP) and heart rate (HR) were measured in anaesthetised animals, after cannulation of the right carotid artery. In addition, a dose-response curve of the effect of WIN (0.03-1 mg/kg, i.v.) in the CV parameters was obtained in all cannulated animals.

Results. WIN at 0.5 mg/kg (i.p.) did not induce any significant central or CV effect when acutely or chronically administered. Central effects: the highest dose of WIN induced antinociception, catalepsy, hypothermia and hypolocomotion. After daily WIN, tolerance had developed to catalepsy, hypothermia and hypomotility but not to analgesia. After weekly administration, tolerance had partially developed only for hypothermia. No residual central effect was detected one week after chronic treatment suspension. CV effects: although no modifications on BP or HR were observed after a single dose of WIN 5 mg/kg (i.p.), in all animals the cannabinoid induced a dose-dependent reduction in BP when administered intravenously. The slope of the dose-response curve for i.v. WIN was significantly slower in those animals which had received, acutely or daily, WIN 5 mg/kg (i.p.) compared with the corresponding animals treated with saline, vehicle or WIN at 0.5 mg/kg (i.p.), indicating the development of tolerance. Instead, the effect of the i.v. cannabinoid did not change after weekly administration of WIN at 5 mg/kg (i.p.).

Conclusion. Even though tolerance may develop to the central and cardiovascular cannabinoid alterations (at high doses and daily administration), these side effects should not be disregarded and could interfere with therapy.

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CHARACTERISATION OF THE RESPONSE PRODUCED BY AEA IN THE MURINE CAROTID ARTERY AND EFFECTS OF CANNABINOIDS ON ERK 1/2 PHOSPHORYLATION

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Anandamide (AEA) is a vasoactive endocannabinoid that acts as a partial agonist at CB_1 receptors, with low efficacy at CB_2 receptors (Pertwee 1999). There is also evidence that AEA activates other receptors including the vanilloid receptor (Randall et al.,2002) and novel unidentified sites. AEA is thought to be primarily metabolised by fatty acid amine hydrolase (FAAH), into arachidonic acid and ethanolamine, however it has been shown that COX-2, but not COX-1, can also metabolise AEA at high substrate concentrations (Kozak et al.,2002). The importance of metabolic conversion in mediating the vascular responses to AEA in the mouse has, to date, not been fully explored. Vascular smooth muscle cells are the most important cell type in the progression of atherosclerosis and restenosis, and it has previously been shown that CB_2 receptor activation can attenuate TNF-α-triggered proliferation in human smooth muscle cells (Rajesh et al.,2008). The combined aims of this present study were therefore to: (i) characterise the relaxant responses to AEA: its metabolism by FAAH, COX-1 and COX-2 and the receptor(s) mediating the AEA responses and (ii) investigate the effects of CB_1 and CB_2 agonists, and endogenous cannabinoids on mouse smooth muscle cell ERK 1/2 phosphorylation.
Using a dual bath myograph vessels were maintained in Krebs solution at 37º C and, following normalisation and sensitisation, vessels were pre-contracted with U46619 ($5x10^{-7}$M) and cumulative dose-response curves to AEA were constructed in the presence or absence of pharmacological intervention. ERK1/2 phosphorylation was measured (ELISA) in lysates from smooth muscle cells stimulated with serum. AEA (10^{-9}- $3x10^{-5}$M) produced a small relaxant response in pre-contracted artery rings, which was marginally increased in the presence of the selective FAAH inhibitor URB597 (10^{-7}M) $P>0.05$. To preclude any interference from FAAH in tissues where the endogenous activity was high, URB597 was included in all subsequent experiments. Neither the selective COX-1 inhibitor SC560 (10^{-7} M) nor the selective COX-2 inhibitor DUP 697 (10^{-7}M) had any significant effect on AEA induced relaxation ($P>0.05$). The CB_1 receptor antagonist AM251 (10^{-6}M) attenuated the response although not to levels of significance, while the CB_2 receptor antagonist AM630 (10^{-6}M) had no effect on the AEA response. Preliminary studies of ERK 1/2 phosphorylation demonstrated that both the CB_1/CB_2 agonist CP55940 (10^{-8} - `10^{-5}M) and URB597 (10^{-7}M) increased levels of ERK1/2 phosphorylation.
The results from this study show that the response produced by AEA in the carotid artery is not modulated by COX-1 or COX-2 and does not involve the CB_2 receptor. Moreover, initial studies suggest that CB receptor stimulation with a combined CB_1/CB_2 receptor agonist may modulate pathways involved in smooth muscle cell proliferation, an effect that was also observed in response to FAAH inhibition.

INFLUENCE OF ACUTE HYPERTENSION ON HAEMODYNAMIC EFFECTS OF WIN55212-2 IN CONSCIOUS RATS

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We have recently demonstrated that the endocannabinoid, anandamide causes depressor effects and more pronounced vasodilator responses in acutely hypertensive, conscious rats[1]. Here, we have examined the cardiovascular responses to the synthetic cannabinoid WIN55212-2, and the CB_1 receptor antagonist AM251, in normotensive and acutely hypertensive, conscious rats. Male Wistar rats (350-450g) were implanted with miniaturised pulsed Doppler flow probes and catheters as described previously[2]. On the day of experiment, animals received continuous i.v. infusion of either saline (0.4ml/h), or angiotensin II (AII; 500ng/kg/h) and arginine vasopressin (AVP; 50ng/kg/h). After 45min, AM251 (3mg/kg; i.v. infusion at 2ml/h for 30min) or its vehicle was administered, followed 30min later, by bolus injection (0.12ml, i.v.) of 150µg/kg WIN55212-2. Data are shown as mean±SEM.

Infusion of AII/AVP significantly ($P<0.05$, Mann-Whitney U test) increased the mean arterial blood pressure (BP; 151±2 vs 112±2 mmHg), reduced vascular conductance (VC) in renal (RVC; 41±3 vs 71±4), mesenteric (MVC; 22±2 vs 71±6) and hindquarter beds (HVC; 17±2 vs 48±5 $(kHz/mmHg)x10^3$) and reduced heart rate (HR; 236±9 vs 373±8 beats/min). AM251 alone had no consistent haemodynamic effect (data not shown). Haemodynamic responses to WIN55212-2 are shown in Table 1.

Table 1. Cardiovascular effects of 150µg/kg WIN55212-2 (n=8-9). *$P<0.05$ vs baseline within group (Friedman's test); #$P<0.05$ vs corresponding values in the vehicle group (Mann-Whitney U test)

		Vehicle			AM251		
		2	6	15 min	2	6	15 min
Saline	HR	-11±7	-32±8*	+7±8	-1±6	+22±15#	-4±10
	BP	+15±4*	+11±3*	+7±3*	+4±3#	+2±2	-1±2#
	RVC (%)	-29±5*	-19±6*	-14±6*	-7±3#	-5±3	-5±4
	MVC (%)	-44±5*	-22±5*	-18±6*	-5±8#	-2±5#	-0±4#
	HVC (%)	+55±14*	+27±7*	+20±7*	+9±4#	+15±7	+8±4
AII/ AVP	HR	+21±16*	-7±14	-7±17	+22±10	+12±16	-12±10
	BP	-11±5*	-7±3*	-8±4	-3±2#	-9±1*	-5±2*
	RVC (%)	+10±9	+26±16	+32±17	-0±4	+7±4	+6±5
	MVC (%)	-11±7	+15±9*	+16±12	-5±6	+4±7	-1±11
	HVC (%)	+187±37*	+65±21*	+52±33*	+35±10*#	+29±12*	+21±12

These data suggest that WIN55212-2 causes depressor effects and more pronounced vasodilator responses in acutely hypertensive, conscious rats. Haemodynamic responses to WIN55212-2 are either attenuated or abolished by AM251.

[1]Ho WS & Gardiner SM (2007) ICRS meeting in Saint-Sauveur, Canada; C057
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A ROLE FOR GPR55 IN MEDIATING BLOOD PRESSURE RESPONSES

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A role for cannabinoid ligands and receptors in the maintenance and control of blood pressure has been established over a number of years and has been shown to be complex. Recent work has suggested that in addition to CB1 and TRPV1 receptors there are other receptors such as GPR55 that bind to and are regulated by cannabinoid ligands. In this study we have tested the hypothesis that the novel cannabinoid receptor GPR55 functions in blood pressure regulation. Using a GPR55 selective agonist, O-1602, in spontaneously hypertensive rats we have demonstrated a dose dependent decrease in blood pressure that is revealed upon CB1 antagonism. This decrease in blood pressure was prevented by prior treatment with the selective GPR55 antagonist cannabidiol. To further investigate the specific need for CB1 antagonism for the effect of GPR55 agonism to be revealed we repeated our studies in CB1 knockout mice and wild-type siblings. O-1602 demonstrated a significant dose dependent decrease in blood pressure only in CB1 -/- mice and this effect could be blocked by cannabidiol. To unequivocally demonstrate that GPR55 was the target of O-1602 and cannabidiol in these studies we then used GPR55 -/- mice. Unlike CB1 -/- mice that show a normotensive phenotype GPR55 -/- animals demonstrate a significant hypertension. Furthermore, O-1602 was without effect with and without CB1 antagonism. In conclusion these data demonstrate that GPR55 plays a significant role in the maintenance and control of blood pressure.

DO ENDOCANNABINOIDS PROTECT AGAINST REPERFUSION-INDUCED VENTRICULAR FIBRILLATION?

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Cannabinoids (CBs) and endocannabinoids (ECs) activate CB1 and CB2 receptors and may protect hearts against ischaemia and reperfusion injury *in vitro* and against arrhythmias *in vivo*. Some of the cardioprotective effects of CB1 agonism depend on nitric oxide (NO). Reperfusion-induced (RI) ventricular fibrillation (VF) is rare in rat hearts reperfused after 60 min of regional ischaemia, in part because endogenous NO is cardioproctective. We tested whether ECs may endogenously cardioprotect against RI VF via CB1 agonism, mimicking NO.

In a blinded study, rat hearts were perfused with solution containing (in mM) NaCl 118.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.4 and KCl 3 for 10 min, then 1 μM AM251 or vehicle (DMSO 0.01%) were added. After 10 more min, hearts were subjected to 60 min regional ischaemia and 10 min reperfusion.

AM251 increased RI VF incidence from 0 to 22%. AM251 perfusion caused an immediate increase in CF (in ml/min/g) (18±1 vs. 13±1, $p < 0.05$). These differences persisted throughout the 10 min perfusion and for the first 15 min of ischaemia. AM251 increased QT90 intervals (ms) at 50 (71±4 vs. 60±2, $p < 0.05$) and 59 min (69±3 vs. 60±2, $p < 0.05$) of ischaemia and 5 min after the start of reperfusion (72±4 vs. 56±2, $p < 0.05$) and caused small increases in PR intervals (ms) at 10 min (43±1 vs. 40±1, $p < 0.05$) 40 min (47±2 vs. 41±2, $p < 0.05$) and 59 min (47±2 vs. 42±1, $p < 0.05$) into ischaemia.

In conclusion, there was an increase in reperfusion VF as a nonsignificant but noteworthy trend in AM251 treated hearts. This effect was of the same order of magnitude to that seen in the lab previously with L-NAME- an effect that required n=20 to reach statistical significance. Increased group sizes may establish whether the present drug effect is real. As CB1 agonism has been shown to relax vascular smooth muscle, the increase in CF may suggest that AM251 has effects in addition to CB1 antagonism.

CANNABIDIOL SUPPRESSES ARRHYTHMIAS AND PREVENTS TISSUE INJURY IN AN ANAESTHETISED RAT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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Myocardial ischaemia and reperfusion (MIR) injury is associated with increased production of reactive oxygen species, activation of an inflammatory response, apoptosis, generation of ventricular arrhythmias and activation of platelets. Cannabidiol (CBD) has been demonstrated to inhibit apoptosis (Iuvone *et al.,* 2004), inflammation (Lastres-Becker *et al.,* 2005), and possess antioxidant activity (Hampson *et al.,* 1998) in the central nervous system. It is therefore possible that CBD may exert cardioprotective effects in the setting of MIR injury through the aforementioned activities. The present studies were performed to test this hypothesis.

Male Sprague Dawley rats (300-400g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1} i.p.), the right jugular vein cannulated for drug and anaesthetic (3-6mg kg^{-1} i.v. as required) administration and the heart prepared for a 30 min period of coronary artery occlusion (CAO) followed by 2h of reperfusion (REP). Mean arterial blood pressure (MABP) was recorded via a carotid cannula, and heart rate (HR) calculated from a Lead I ECG recorded from subcutaneous limb leads. In control animals, a bolus dose of vehicle (saline; n=12) was administered 10 min prior to CAO, and again 10min prior to REP. In experimental group I, CBD (50µg kg^{-1}; n=10) was administered as a bolus intravenous dose 10min prior to CAO. In experimental group II, animals were administered CBD (50µg kg^{-1} i.v.; n=7) 10min prior to reperfusion. The appearance of ventricular premature beats (VPB's), ventricular tachycardia (VT), and ventricular fibrillation (VF) during the period of ischaemia and in the immediate post-reperfusion period, was monitored from the ECG. After 2hr of reperfusion, arterial blood was withdrawn for platelet aggregation studies prior to euthanasia with an overdose of anaesthetic. The hearts were then removed, perfused (via the aorta) with Evan's blue dye to delineate area at risk (AAR). The heart was sliced and stained with triphenyl tetrazolium to determine infarct size. Data are expressed as mean ± s.e.m and compared using either an ANOVA with Dunnett's post-hoc test, two-way ANOVA with Bonferroni post-hoc test, or a Student's T-test. The incidence of VT, VF, and mortality, was compared using Fisher's exact test.

CBD induced a transient decrease in MABP prior to ischaemia, significantly reduced the number of ischaemia-induced VPB's (628±71 vs. 1843±134 in controls; $p<0.001$) and attenuated the development of VF. CBD also significantly reduced infarct size when administered either prior to (28±3% of AAR; $p<0.001$) or after ischaemia (31±2%; $p<0.001$) compared to control (59±3%). Administration of CBD prior to, but not after, ischaemia also significantly attenuated collagen-induced platelet aggregation compared with the control (7±1Ω vs. 27±3Ω; $p<0.001$). This study demonstrates for the first time that CBD exerts cardioprotective effects in terms of reducing the incidence of ischaemia-induced ventricular arrhythmias, attenuating infarct size, and inhibiting platelet aggregation *ex vivo*.

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STUDIES ON THE EFFECTS OF CANNABIDIOL AND THE CB_1 AGONIST ACEA ON COLLAGEN AND ADP-INDUCED PLATELET AGGREGATION IN PORCINE WHOLE BLOOD

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We have recently shown that cannabidiol (CBD) exerts an ex vivo anti-aggregatory effect on collagen induced platelet aggregation in rats subjected to acute myocardial ischaemia and reperfusion (this meeting). While there is some data that has previously demonstrated an anti-aggregatory effect of CBD in platelet rich plasma (Formukong et al., 1989), no previous studies have explored the mechanism of this effect. The aim of these present studies was therefore to determine the ability of CBD to inhibit platelet aggregation induced by collagen and ADP in whole blood. To investigate the role of the CB_1 receptor in any actions of CBD we also undertook a comparison of the effects of CBD with the CB1 agonist ACEA and assessed the effects of the CB_1 antagonist, AM251, on the responses to CBD.

Porcine blood was collected into heparinised plastic blood tubes from the local abattoir on a daily basis. Whole blood aggregation in response to collagen (5μg/ml) and ADP (5μM) was determined, using impedence aggregometry, in the absence and presence of CBD (1-100μM), ACEA (10nM-10μM). CBD and ACEA were incubated with the blood for 10 minutes prior to the addition of stimulant. For the antagonist study, AM251 (1μM) was added to the blood 10 minutes prior to the addition of CBD (1-100μM). Responses were measured in ohms (Ω).

Over the concentration ranges tested, neither CBD nor ACEA had any significant effect on either collagen or ADP-induced platelet aggregation. AM251 alone slightly reduced the response to ADP, although this was not statistically significant. In the presence of AM251, CBD produced a concentration dependent decrease in the extent of platelet aggregation, with total abolition of aggregation at the highest concentration. Preliminary studies to determine the effects of the CB_2 antagonist AM630 in combination with CBD suggest that this does not have any effect ob platelet aggregation.

These findings suggest that CBD may exert multiple actions at the level of the platelet which counteract each other and that an antiplatelet effect is only unmasked in the presence of CB_1 receptor blockade. Studies are continuing to determine the mechanisms underlying this antiplatelet effect of CBD.

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ALTERATIONS IN GASTROINTESTINAL MOTILITY INDUCED BY CHRONIC CANNABINOIDS IN THE RAT

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Introduction. Cannabinoids, which have been used for ages, are receiving renewed attention for their therapeutic potential. However, their effects in the gastrointestinal (GI) tract upon chronic administration are not well known. We studied the effects of the mixed CB1/CB2 agonist WIN 55,212 (WIN) in GI motility during and after two different patterns of chronic administration which prevented the development of neuropathic signs in the rat (Costa et al, Br. J. Pharmacol. 2004, 141, 4–8; Vera et al, Life Sci. 2007, 81, 468–479).

Methods. Male Wistar rats received saline, vehicle or WIN (0.5 or 5 mg/kg, i.p.), either once a day for 14 days or once a week for 4 weeks. Radiological techniques were used to determine the acute (after first dose), chronic (after last dose) and residual (one week after treatment) effects of the cannabinoid on GI motility. Additional studies were made one day and/or one week after treatment to determine fine alterations in GI motility (charcoal method: GI transit) and morphology (histochemistry).

Results. Gastric emptying was delayed after WIN, but only at the highest dose. This effect did not change after daily but increased (hypersensitisation) after weekly chronic treatment. Intestinal transit was dose-dependently reduced after the first cannabinoid administration. Tolerance to this effect developed upon daily but not weekly treatment. No significant cannabinoid effect on motility (X-rays or charcoal transit) or morphology (histochemistry of the different GI regions) was detected one day or one week after either treatment.

Conclusion. Cannabinoids depress GI motility in a dose- and region-dependent fashion. Low doses of cannabinoids, beneficial in neuropathy, did not dramatically alter GI motility. High doses exerted prominent although transient depressant effects. GI acute and chronic effects of cannabinoids may interfere with efficacy of therapy and need to be taken into account. More research is needed to determine the mechanisms involved in development of tolerance and hypersensitisation to the cannabinoid GI effects.

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CANNABIDIOL ATTENUATES MOTILITY DISTURBANCES IN RATS WITH TNBS-COLITIS

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Colonic inflammation causes a reduction in smooth muscle contractility and damage to the enteric nervous system, which results in motility disturbances in inflammatory bowel disease (IBD). Marihuana is self-medicated by patients with IBD in order to reduce symptoms. Cannabidiol (CBD) has been shown to possess anti-inflammatory properties in a number of *in vivo* studies, but it is not known if this non-psychoactive cannabinoid could reduce inflammation and functional disturbances occurring in colitis. The aim of this study was to characterize the effects of CBD on inflammation and *in vitro* muscle contractility in the TNBS (trinitrobenzenesulfonic acid) model of colitis.

Colitis was evoked in male Wistar rats by intra-colonic administration of 6.7mg TNBS in 0.25ml 25% ethanol. The degree of inflammation was quantified using a macroscopic damage score (MDS, 0-13 scale) and myeloperoxidase (MPO) activity. Longitudinal muscle strips were used for the motility studies. Spontaneous activity (amplitude of low frequency contractions, ALF), contractile responses to carbachol ($1x10^{-8}$ - $3x10^{-5}$M) and to electrical field stimulation (EFS, 1-15Hz, at voltage supramaximal at 5Hz, 0.2ms pulse width) were evaluated. The amplitude of contractions was calculated per g tissue (dry weight). CBD (5, 10, 15 and 20 mg/kg) was administered *i.p.* in a short-term dosing regimen (0.5hour before and 24 and 48 hours after TNBS administration) and animals (n=4-11 per group) were sacrificed 72 hours after colitis-induction.

In preliminary studies with TNBS treated animals all the contractile responses of muscle strips were significantly depressed, whereas MDS and MPO activity increased. CBD treatment dose-dependently reduced TNBS-induced increases in neutrophil infiltration ($P<0.05$ for CBD 20mg/kg MPO: 2.7±0.4 v. vehicle: 6.0±0.8 ng/mg protein). MDS was lowered by CBD 10mg/kg, but this failed to be statistically significant. ALF and contractility to carbachol ($1x10^{-6}$ – $3x10^{-5}$M) were significantly increased by CBD at 10mg/kg (ALF 67.1±11.3 v. vehicle: 36.9±4.8), the dose-response relationship of this cannabinoid showing a bell-shaped pattern. The EFS evoked contractions remained unchanged.
This study demonstrates that CBD attenuates motility disturbances in rat in the TNBS acute colitis model by improving the contractility of colonic smooth muscle. In addition, CBD reduces neutrophil infiltration to the site of inflammation *in vivo*. The enhancement in muscle contractility by CBD does not correlate with its inhibitory effect on neutrophil influx, suggesting involvement of other cell types.

Acknowledgements: The authors would like to thank GW Pharmaceuticals for CBD used in this study

EFFECTS OF COMBINED TREATMENT WITH Δ^9-TETRAHYDROCANNABINOL AND CANNABIDIOL IN RATS WITH TNBS-COLITIS

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Cannabidiol (CBD) has been shown to interact with Δ^9-tetrahydrocannabinol (THC) in behavioural studies, but it is not known if these cannabinoids interact *in vivo* in inflammatory disorders. We have used the TNBS (trinitrobenzenesulfonic acid)-model of colitis to characterize the effects of THC alone and in combination with CBD on inflammation and *in vitro* motility and secretory parameters.
Colitis was evoked in male Wistar rats by intra-colonic administration of 6.7mg TNBS in 0.25ml 25% ethanol. The degree of inflammation was quantified using a macroscopic damage score (MDS, 0-13 scale) and myeloperoxidase (MPO) activity. Using the Ussing chamber technique, secretory responses evoked by veratridine (a nerve stimulant, $1x10^{-5}$M) were assessed and expressed as % response to carbachol (1x10-5M). Longitudinal muscle strips were used for the motility studies and contractile responses to carbachol (1x10-8 - 3x10-5M) were evaluated. The dose of CBD (10mg/kg) was chosen on the basis of previous studies. THC (5, 10 and 20 mg/kg) and THC (5 and 10mg/kg), with CBD (T5+C and T10+C respectively) were administered i.p., once daily starting 0.5hour before TNBS administration and animals (n=6-11 per group) were sacrificed 72 hours after colitis induction.
In preliminary studies with TNBS treated animals all the contractile and secretory responses were significantly depressed, whereas MDS and MPO activity increased. CBD treatment produced a clear reduction in MDS, but this failed to be statistically significant. CBD also reduced MPO in a dose dependent manner, but at the chosen dose, 10mg/kg, the effect was not significant. CBD increased secretory and contractile responses ($P<0.05$). The MDS was lowered by THC 10mg/kg, the dose-response relationship of this cannabinoid showing a bell-shaped pattern (V: 6.0±0.5, THC5: 6.5±0.8, THC10: 2.7±1.0, THC20: 4.7±0.7). THC at 10 and 20mg/kg reduced TNBS-induced neutrophil infiltration (MPO ng/mg protein: V: 3.4±0.4, THC5: 4.3±0.4, THC10: 1.8±0.1, THC20: 2.1±0.2). Combined treatment T5+C resulted in reduction of MDS and MPO (4.2±0.5, 2.4±0.3, respectively, $P<0.05$ v. THC5). In the T10+C group CBD did not significantly affect these parameters (MDS: 3.5±0.6, MPO: 2.6±0.6). THC alone did not affect secretory responses to nerve stimulation, but the responses in the T10+C group were significantly enhanced (62.8±8.8, $P<0.05$ v. THC10, 30.3±7.5 and vehicle, 24.4±8.2). Although responses to carbachol were not significantly increased by THC they were by T10+C ($P<0.05$ v. vehicle).
In conclusion, both THC and CBD have beneficial effects in the TNBS model of colitis but their combination does not act synergistically.

Acknowledgements: The authors would like to thank GW Pharmaceuticals for CBD used in this study

EFFECT OF CANNABIDIOL ON SEPSIS-INDUCED MOTILITY DISTURBANCES IN MICE: INVOLVEMENT OF CB1 RECEPTORS AND FATTY ACID AMIDE HYDROLASE

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Sepsis is an inflammatory condition that is associated with reduced propulsive gastrointestinal motility (ileus). A therapeutic option to treat sepsis is to promote intestinal propulsion preventing bacterial stasis, overgrowth and translocation. Recent evidence suggests that anti-oxidants improve sepsis-induced ileus. Cannabidiol, a non-psychotropic component of Cannabis sativa, exerts strong anti-oxidant and anti-inflammatory effects without binding to cannabinoid CB1 or CB2 receptors. Cannabidiol also regulates the activity of fatty acid amide hydrolase (FAAH) which is the main enzyme involved in endocannabinoid breakdown and which modulates gastrointestinal motility. Because of the therapeutic potential of cannabidiol in several pathologies, we investigated its effect on sepsis-induced ileus and on cannabinoid receptor and FAAH expression in the mouse intestine. Sepsis was induced by treating mice with lipopolysaccharides for 18 h. Sepsis led to a decrease in gastric emptying and intestinal transit. Cannabidiol further reduced gastrointestinal motility in septic mice but did not affect gastrointestinal motility in control mice. A low concentration of the CB1 antagonist AM251 did not affect gastrointestinal motility in control mice but reversed the effect of cannabidiol in septic mice. Sepsis was associated with a selective upregulation of intestinal CB1 receptors without affecting CB2 receptor expression and with increased FAAH expression. The increase in FAAH expression was completely reversed by cannabidiol but not affected by AM251. Our results show that sepsis leads to an imbalance of the endocannabinoid system in the mouse intestine. Despite its proven anti-oxidant and anti-inflammatory properties, cannabidiol may be of limited use for the treatment of sepsis-induced ileus.

METHANANDAMIDE REDUCES CARBACHOL-INDUCED UTERINE CONTRACTIONS

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Introduction

There considerable data that show that the endogenous cannabinoids Anandamide (AEA) and 2-arachidonyl glycerol (2-AG) play a role in the regulation of smooth muscle contractility of the lung, small intestine, vas deferens, and vascular. To datae, there is very little evidence for there role in the regulation of uterine smooth muscle contractility and tone. Here, we examine the effects of pre-incubation of meth-anandamide on carbachol-induced contractions in rat uterine strips from the stage of estrus. \

Methods

Three- to four-month old virgin female Sprague Dawley rats were maintained on a 12:12 light:dark cycle, and provided with food and water ad libitum. Vaginal smears were monitored daily to determine estrous cycle stage. Rats were decapitated and uterine horns were rapidly removed, trimmed from surrounding tissue and fat, and opened longitudinally. Strips of longitudinal uterine smooth muscle (1 cm in length) were prepared and mounted in tissue baths containing 10ml of physiologic buffer that of the following composition (mM): NaCl, 118; KCl, 5; CaCl2, 2; NaHCO3, 25 and glucose, 11 (pH = 7.4) and 10 µM indomethacin. The preparations were bubbled continuously with 95% O2: 5% CO2 and warmed to 37°C. Mechanical responses were recorded isometrically by means of a pressure transducer-A/D converter combined with a computer analysis program by Biopac (Goleta, CA) that allowed for online and offline analysis of the waveforms. The tissue was immersed in buffer and equilibrated for 1 hour (with changes in bath fluid every 15 minutes) under a resting tension of 0.5g. After the equilibration period, the preparation was challenged with 10nM, 100nM, and 500nM carbachol washing between each challenge and resting for 5 minutes to generate a dose-response curve. The tissue was then incubated for 30 minutes with vehicle (DMSO) or methanandamide (10µM) and then challenged again with 10nM, 100nM, and 500nM carbachol.

Results and Discussion

Incubation with meth-anandamide markedly reduced the level of carbachol-induced contractions at both 10 and 100nM carbachol. No differences were seen in with DMSO alone. There was no difference at 500nM carbachol in either treatment group. There was also a marked reduction in the number of spontaneous uterine contractions after methanandamide. These data demonstrate cannabinoid involvement in the regulation of uterine smooth muscle contractions.

LONG TERM USE OF HU-210 ADVERSELY AFFECTS SPERM PERFORMANCE IN RATS BY MODULATING THE ENDOCANNABINOID SYSTEM

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Cannabis extracts (marijuana, hashish) have been used for medical and recreational purposes for many centuries, and have constantly evoked tremendous interest and controversy within the public domain and medical research. Discovered in 1988 at the Hebrew University, HU-210 is a synthetic cannabinoid ~800 times more potent than the natural Δ^9-tetrahydrocannabinol (THC, the active principle of *Cannabis*), and with an extended duration of action. It is well-known that HU-210 is a potential analgesic, that has been implicated in preventing inflammation, cognitive impairment and loss of neuronal markers in Alzheimer's disease. This means that HU-210 could potentially be used as a drug; hence, it is very important to understand its action on different tissues and body organs.
The aim of the present study was to evaluate whether HU-210 injection in male rats had any effect on weight of male reproductive organs and sperm performance in adult life, possibly through an unbalance of the endocannabinoid system.

Material and method
Twelve week Lister hooded male rats (Harlan, UK) were housed with free access to water and food on 12:12h day/night cycles in a pathogen free animal facility. All experiments were performed under UK Home Office regulations. Animals were injected daily (5 days a week) with HU210 (50 μgkg^{-1}) (Tocris Cookson, Bristol, UK) in a vehicle of Tween 80 or with Tween 80 alone (controls). Drug treated (n=6) and control (n=6) animals were sacrificed after acute treatment (4-6h) or 2, 4, or 7 weeks of treatment by inhalation of CO_2, followed by cervical dislocation. Body weight was recorded and both the testis, epididymis, vas differentia, seminal vescicles, ventral prostate, were dissected out and weighed. The contents and fluid from caudal epididymis and vas were squeezed in the M199 medium at 37˚C and total sperm concentration determined using the haemocytometer and expressed as $10^6/ml^{-1}$. Sperm motility was assessed as percentage motile sperm. Differences between control and test groups were analyzed using independent t tests. $P < 0.05$ was considered significant. In sperm cells, the binding to type-1 (CB1) cannabinoid receptors, and the activity of FAAH were assayed through standard procedures.

Results
Rats treated for 7, 4 and 2 weeks with HU-210 showed a significant reduction ($p<0.001$) of body weight compared with controls, and in fact during the dissection rats treated with HU-210 showed a remarkable reduction in fat compared with the controls. However, treatment with HU-210 had no significant effect on the weight of the various reproductive organs analyzed. Total sperm motility and count from epididymi showed a significant reduction after 2, 4 and 7 weeks of treatment compared with controls ($p<0.025$), without marked differences after acute treatment.
Significant effects of HU-210 were found also in CB1 binding and FAAH activity of sperm cells after 2 and 4 weeks of treatment, compared with controls ($p<0.05$), whereas no differences between treated and untreated animals were observed after acute (2 hours) or 7 weeks of treatment.

Conclusion
Long term use of HU-210 adversely affects spermatogenesis and sperm function in rats, by unbalancing the endocannabinoid system.

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PGE_2 GLYCEROL ESTER INHIBITS THE FORMATION OF HUMAN OSTEOCLASTS IN VITRO

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PGE_2 glycerol ester (PGE_2-G) is a novel prostanoid generated by the oxygenation of the endocannabinoid 2-AG by cyclooxygenase 2 (COX-2). There is evidence to suggest that 2-AG is a natural substrate for COX-2, the major product being PGE_2 glycerol ester (PGE_2-G). Recent reports have demonstrated that PGE_2-G triggered calcium mobilization via the IP3 and PKC pathway in the RAW264.7 mouse macrophage cell line (Nirodi *et al.*, 2004). The aim of this study was to investigate the actions of PGE_2-G on the differentiation and function of human osteoclasts, which are the multinucleated cells responsible for bone resorption and are derived from the differentiation and fusion of cells of the monocyte/macrophage lineage.

Human osteoclasts were generated in vitro from RANKL-stimulated M-CSF-dependent peripheral blood monocytes. PGE_2-G (10 nM to 10 μM) was found to dose-dependently inhibit the formation of $\alpha v\beta 3$-positive multinucleated osteoclasts by up to 60%. Although this inhibitory effect was also observed with PGE_2, the metabolic product of PGE_2-G, preliminary data suggests that the inhibition of osteoclast formation by PGE_2 is more susceptible than PGE_2-G to antagonism by the specific EP4 antagonist, GW627368X. Studies on the effect of PGE_2-G on osteoclast function showed that treatment with PGE_2-G (10 nM – 1 μM) caused a trend towards an increase in the area of resorption when the cells were cultured on dentine discs, although the number of osteoclasts was not affected. The ERK pathway is known to play a crucial role in the survival and differentiation of osteoclasts. PGE_2-G caused a 50% decrease in phosphorylated ERK1/2 levels after 10 min, compared to vehicle-treated cells. PGE_2-G was also able to inhibit the stimulatory effect of TNFα on ERK phosphorylation by 40 %, although phosphorylation of p38 was not altered by PGE_2-G treatment.

These data suggest that PGE_2-G, a COX-2 metabolite of 2-AG, has complex effects on the formation and perhaps function of human osteoclasts in vitro and could play a role in modulating bone resorption in vivo.

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CANNABINOID CB_2 RECEPTOR IS ESSENTIAL FOR MESENCHYMAL STEM CELL VIABILITY

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Adult mesenchymal stem cells (MSCs) are a multipotent population of stem cells that can differentiate along the osteogenic, chondrogenic and adipogenic lineages. Recently, the skeleton has been identified as a major endocannabinoid target through both the CB_1 and CB_2 cannabinoid receptors (Idris *et al*., 2005; Bab 2007). The aim of this study was to examine the role of the CB_2 receptor in the regulation of MSC viability.

MSCs were isolated from the bone marrow of adult Wistar rats and expanded in culture. MSCs were treated with CB_2 agonist, JWH015 (10nM, 100nM and 1µM) alone or in the presence of the CB_2 antagonist, AM630 (0.001-100µM) for 24h and colorimetric TUNEL assay was performed to detect the percentage of apoptotic cells. JWH015 alone showed no significant apoptosis compared to the control cells whereas AM630 (10 and 100 µM) significantly increased the percentage of apoptosis; thus in control conditions 9.08% of cells were apoptotic and this was significantly increased to 23.08% and 42.33% apoptotic cells by 10 and 100 µM respectively ($p<0.001$, ANOVA, n=3, Newman-Keuls Multiple Comparison test) and this was prevented by JWH015. This study further elucidated the signalling pathways involved in AM630- induced apoptosis. Thus, treatment of MSCs with AM630 (1-100µM) for 1- 24h demonstrated a significant increase in expression of the stress activated protein kinase, phospho-JNK (c-Jun N-terminal kinase) and the pro-apoptotic cysteine protease, caspase 3, in a dose-dependent and time-dependent manner. Western immunoblot further confirmed a significant increase in expression of phospho-JNK3 when MSCs were treated with AM630 (10 µM) for 24h. Furthermore, the AM630 induced increase in phospho-JNK and caspase 3 expression were abrogated by the D-JNK inhibitor 1(1 µM) and caspase 3 inhibitor (Ac-DEVD-CMK, 1 µM). When MSCs were treated with AM630 (10 µM) for 24h a significant expression of phospho c-Jun in the nucleus was observed providing evidence of a role for c- Jun in the apoptotic cascade.

This study demonstrates that inhibition of the CB_2 receptor leads to MSC apoptosis and suggests that tonic activation of the CB_2 receptor is necessary to maintain survival of MSCs. This provides evidence of another cellular target for cannabinoid receptors which may be necessary for the maintenance of bone health.

Idris AI, van't Hof RJ, Greig IR, Ridge SA, Baker D, Ross RA, Ralston SH. (2005) *Nat Med*. 11(7):774-9.

Bab IA. (2007) *Ann N Y Acad Sci*. 1116:414-22

COMPREHENSIVE CHARACTERISATION OF CB2 RECEPTOR PROTEIN LEVELS BY FLOW CYTOMETRY IN PERIPHERAL BLOOD IMMUNE CELLS FROM HEALTHY HUMAN VOLUNTEERS

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Introduction It is widely accepted from mRNA studies that the CB2 receptor gene is expressed by most cell types of the rodent and human immune system. However, the abundance of CB2 protein in human immune cells is currently not well characterised. We have conducted a detailed analysis of CB2 protein levels using human immune cells from whole blood (healthy donors).

Aim The primary objective of this study was to determine the relative expression levels of CB2 receptor protein across each of the major immune cell types in blood from healthy human volunteers. We have used immune cells from fresh whole blood and PBMC preparations to conduct live-cell flow cytometry to measure the abundance of CB2 receptor protein and to phenotypically identify CB2 positive cells.

Results Flow cytometry was conducted using PBMC and whole blood live-labelled with an N-terminal anti-CB2 antibody (ABR) in conjunction with antibodies directed at a selection of immune cell markers (including CD3, CD4 and CD8 for T-lymphocytes, CD14 for monocytes, CD20 for B-lymphocytes, CD69, CD83 and the viability stain 7AAD) to phenotype cells. The majority of T-lymphocytes (CD4+CD3+ or CD8+CD3+) expressed low levels of cell surface CB2. Most B-lymphocytes and monocytes expressed moderate levels of cell surface CB2. Interestingly, the highest levels of cell surface CB2 were detected on CD8+ cells, which lacked CD3 suggesting a phenotype consistent with NK cells rather than T-lymphocytes. The absence of CD69 and CD83 expression by lymphocytes and monocytes respectively demonstrates that these cells were not activated, consistent with the samples coming from healthy donors.

Conclusions This method is ideal for the quantification of CB2 receptor levels in living human immune cells and for the phenotypic identification of CB2 positive cells. CB2 has been implicated in the immuno-modulation of various immune functions and it has been reported that CB2 levels can change rapidly under various circumstances. It is therefore of critical importance to be able to accurately determine the cell types that express the receptor under basal conditions and subsequently how expression levels change following activation of the immune system.

R(+)-METHANANDAMIDE INDUCED SECRETION OF IL6 ON PROSTATE CANCER PC-3 CELLS THROUGH PI3K/AKT PATHWAY

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Cannabinoids can regulate the cytokine network, promoting not only inflammatory responses but also, regulating cell growth. One of the most important cytokine in prostate cancer is IL-6, which seems to be implicated in apoptosis decrease, invasive capacity increase and angiogenesis. In this work we examined the effect of R(+)-Methanandamide (MET) and JWH-015 on IL-6 secretion by androgen-independent prostate cancer PC-3 cells.

We have observed that MET and JWH-015 induced a dose-dependent IL-6 secretion increase. Pre-incubation with SR2, a specific CB2-R antagonist, partially blocked cannabinoid-induced cytokine secretion, indicating a CB2 receptor-dependent effect. In addition, MET induced Akt activation which was confirmed by the blockage of the IL-6 secretion in presence of the PI3K inhibitor LY294002. The IL-6 secreted by PC-3 cells didn’t have any effect on PC-3 cells viability. However, it was able to activate STAT3, a downstream effector of this cytokine, in LNCaP and HepG2 cells. We therefore analyzed the expression of STAT3 protein in PC-3 cells and we corroborated that this transcription factor was not expressed on PC-3 cells. These results suggested that PI3K/Akt pathway is involved in IL-6 secretion, which is also dependent of CB2 receptor.

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INFLAMMATORY PATHWAYS IN MACROPHAGES MODULATED BY CANNABINOIDS

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Obesity is characterized by a chronic low grade inflammatory state of the adipose tissue. Evidence is accumulating that within the adipose tissue macrophages are responsible for this chronic state by producing pro-inflammatory cytokines in response to adipocytes released factors. CB2 receptors are highly expressed within immune cells, including macrophages and it has been shown that inflammatory responses can be modulated by targeting the CB2 receptor mediated pathway. We investigate whether the chronic low grade inflammatory state within the obese adipose tissue can be modulated via the CB2 receptor mediated pathway. As a first step we used a systematic approach to characterize which inflammatory pathways in macrophages can be modulated by (endo)cannabinoids. Methods: RAW 264,7 were cultured and activated using varying concentrations of LPS or LPS/IFN-γ. U937 were treated with PMA and activated with LPS. Cytokine release was tested by using ELISA. Nitric oxide release was evaluated by measuring nitrite using Griess reagents at 24 and 48 hours. Results: In the innate (LPS) activated murine macrophage cannabinoids inhibited NO release. The potent synthetic cannabinoids HU210 and CP55,940 and the CB2 specific GP1a evoked an inhibition of NO release in LPS (1µg/ml) activated RAW264,7. For HU210 a dose-response relationship was observed with thresholds at 100 nM. In contrast, cannabinoids did not modulate TNFα and IL10 cytokine secretion. A large number of (endo)cannabinoids tested at several time points failed to elicit any reduction or increase in the pro-inflammatory cytokine TNFα and the anti-inflammatory IL10 respectively. Similar effects on cytokine secretion were observed for the human U937 macrophage. Preliminary experiments showed that in the classical activated macrophage (LPS and IFN-γ) CP55,940 had a pronounced effect on the cell viability of RAW 264,7. Conclusions: Our results show that in the innate activated murine macrophage cannabinoids exert an inhibitory effect on NO release but not on cytokine secretion of TNFα and IL10. Additionally to literature studies performed on NO, results indicate that HU210 is a more potent inhibitor than CP55,940. Results will be further used to study potential modulatory effects of cannabinoids in relation to obesity induced inflammation in adipocyte tissue.

MECHANISM OF CANNABINOID MODULATION OF MICROGLIAL CYTOKINE GENE EXPRESSION

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Cannabinoids have been shown to modulate the immune system *in vitro* and *in vivo*. A major area of interest is how cannabinoids impact the brain, considering that a variety of neuropathies or brain disorders, such as AIDS dementia, Parkinson's disease, Multiple Sclerosis and Alzheimer's disease, are associated with hyperinflammatory responses. Microglia, the resident macrophages of the brain, have been implicated as a major cell type responsible for the elicitation of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α. *In vitro* experiments have demonstrated that the partial exogenous cannabinoid agonist THC and the potent synthetic exogenous cannabinoid agonist CP55940 downregulate the robust production of pro-inflammatory cytokines elicited in response to bacterial lipopolysaccharide (LPS) at the mRNA level. These observations suggest that cannabinoids have the potential to be therapeutic agents for ablating chronic brain inflammation. Thus, the goal of this study was to define the mechanisms in which cannabinoids modulate cytokine gene expression in microglia. We focused our studies on the universal transcription factor NFκB, that can be comprised of several different subunits: p65 (Rel A), Rel B, c-Rel, p50, p52; however the classical NFκB protein involved in immune regulation and induction of pro-inflammatory cytokine genes is the p65/p50 heterodimer. For our *in-vitro* studies, we employed the murine microglia-like cell line BV-2 to perform Electrophoretic Mobility Shift Assays (EMSA), NFκB Reporter Activity assays and Western blot analyses, in order to assess the effects of THC and CP55940 on the universal transcription factor NFκB as it relates to binding interactions to its cognate promoter binding site, transcriptional activity, its regulation within the cytoplasm, and subsequent transport into the nucleus. Results indicated that binding and transcriptional activity of NFκB in LPS-induced BV-2 cells were downregulated by both THC and CP55940 in a concentration-related fashion. Furthermore, Western blot analyses demonstrated that cannabinoids affected the regulation of NFκB in the cytoplasm by modulating the degradation of its inhibitor protein, IκBα, following phosphorylation. Collectively, these results suggest that these cannabinoids suppress microglial pro-inflammatory cytokine gene expression in microglia at the promoter transcriptional activation level.

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EFFECTS OF Δ-9 THC PRE-TREATMENT ON ACUTE SYSTEMIC CANDIDIASIS AND ACQUIRED IMMUNE RESPONSE IN MICE

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The immunomodulatory activity of Cannabinoids has been well established in various *in vivo* infectious models. Δ-9 THC has been shown to facilitate an immune shift from Th1 to Th2 in response to antigenic challenge with bacteria (Klein, 2000) and viruses (Friedman, 2003), demonstrated by lowered serum levels of interferon-gamma (IFN-γ), interleukin-2 (IL-2) and increased levels of IL-4. Furthermore, Δ-9 THC has been shown to modulate the acquired memory immune response to a sub-lethal pathogenic challenge demonstrated by increased morbidity upon subsequent lethal pathogenic challenge (Klein, 2000). To date, similar studies have not been carried out using fungal pathogens. The aim of our present study is to investigate the effects of Δ-9 THC pre-treatment on cytokine profile in response to acute systemic infection with C.albicans. Eight-week old Female Swiss-Webster mice were given a dose of 16mg/kg Δ-9 THC i.p. or vehicle control 18-hours prior to i.v. administration of C.albicans (1x107cells) or vehicle control. Mice were euthanized by cardiac puncture under anesthesia; blood and spleens were collected and analyzed for cytokine levels by ELISA and Luminex multiplex flow cytometry assay.

Preliminary studies have shown pre-treatment with 16mg/kg Δ-9 THC i.p. to modulate levels of IL-6, IL-12p70 & IL-10 in response to C. albicans challenge. Further research will clarify to what extent these changes depend on dose and time of data collection.

We will also investigate the role Δ-9 THC plays in acquired immunity. For this purpose, eight-week old Female Swiss-Webster mice will be treated with 16mg/kg Δ-9 THC i.p. 18-hours prior to administration of a sub-lethal i.v. dose of C. albicans (2-3x106cells) or vehicle control. Three weeks later, the mice will be challenged with a lethal dose of C. albicans (6-8x106 cells) and assessed for survival. Due to the previously demonstrated ability of Δ-9 THC to cause a Th1 to Th2 immune shift (Klein, 2000), we expect that pre-treatment with Δ-9 THC will interfere with the ability of mice sublethaly challenged to develop an acquired memory immune response. These outcomes will be reported at the meeting.

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EXPOSURE OF JURKAT CELLS TO CB2-AGONISTS INHIBITS THE T CELL RECEPTOR-ACTIVATED SIGNALING CASCADE

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In addition to neuronal effects of cannabinoids, they potently modulate immune functions. Several reports suggested immunosuppressive effects of these drugs, which may, in part, be explained by inhibition of the functions of activated T cells. It was reported that cannabinoids inhibit the induction of IL-2 from activated T lymphocytes and the activity of the transcription factors, which are involved in the induction of IL-2. However, there is little information on the mechanisms leading to these inhibitory effects and on the molecular modules, via which T cells and cannabinoids communicate. Using Western blot analysis, effects of cannabinoids on the T cell receptor-mediated activation of the proteins Lck, ZAP-70, LAT and MAPK, which are involved in transducing the activatory signals from the T cell receptor to the nucleus, were studied. T cell receptor activation was performed using CD3/28 mAbs. Cannabinoid-mediated changes in the intracellular cAMP concentration of Jurkat cells were assayed via competitive cAMP-ELISA. Here we report that incubation of Jurkat cells with THC (500 nM) for 2 h up to 24 h caused a dramatic inhibition of the CD3/28 mAbs-induced activation of Lck, ZAP-70, LAT and MAPK. In contrast, shorter incubation of the cells with THC had no effect on the T cell signaling cascade. The inhibitory effects of THC on the T cell signaling cascade after 2h incubation can be blocked by the CB2 specific antagonist AM630 (500 nM), but not by the CB1 specific antagonist AM251 (500 nM). This indicates that these effects are mediated via CB2 receptors, which was reconfirmed by experiments using the specific CB2 agonist JWH015 (250 nM). It is known that T cell receptor-mediated activation is regulated by cAMP. Although short-time (less than 1 h) incubation of Jurkat cells with THC and the CB2 agonist JWH015 caused a decrease in the forskolin-induced levels of cAMP, we observed a significant, between two- and five-fold increase in cAMP levels, when cells were incubated between 2 h and 24 h. It is accepted that cAMP dependent activation of PKA leads to a robust activation of Lck, which tonically represses the initiation of the T cell receptor-induced signaling cascade. Our data demonstrate for the first time mechanisms how cannabinoids interfere with the T cell receptor-activated signaling cascade and suggest that the inhibitory effects of the drugs are transduced via superactivation of the adenylate cyclase/cAMP system. These results help to understand immunosuppressive effects of cannabinoids.

CANNABINOID INFLUENCE ON PRIMARY HUMAN MONOCYTE ACTIVATION AND ISOLATION OF HUMAN MICROGLIA

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Introduction: Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system. Previous clinical studies by our group suggest that cannabinoids may slow progressive MS. Relevant animal models have associated microglial activation with disease severity, but have shown that stimulation of microglial cannabinoid-2 receptors (CB_2R) may down-regulate microglial activation and neuroinflammation. We hypothesise that cannabinoid dysregulation and chronic microglial activation contributes to neuronal loss and disability in progressive MS. CB_2R agonists may ameliorate this. We have developed assays of cytokine production in primary human monocytes following stimulation by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) and will apply these to human microglia to determine cannabinoid influence on activation state *in vitro*.

Materials & Methods: Primary human monocytes were isolated from healthy volunteers using iodinated density gradient media (NycoPrep and OptiPrep). Cell purity was established by flow cytometry using CD14. CB_2R expression was determined by immunofluorescence, flow cytometry and Western blot. Monocytes were stimulated with LPS (100 ng/ml to 10 µg/ml) and IFN-γ (10 ng/ml). TNF-α production was measured by ELISPOT. The assay was validated using methylprednisolone and repeated with THC and AM630. Cell viability was assessed by the MTS assay. Protein lysate from stimulated monocytes was analysed for changes in CB_2R expression. We received ethical approval to use tissue obtained from patients undergoing surgery for brain tumours at our local neurosurgical centre. Macroscopically normal brain was dissociated by mechanical disruption and enzymatic digestion. Microglia were purified by differential adherence.

Results: Monocytes obtained by density gradient centrifugation were of >90% purity as assessed by CD14 expression. Methylprednisolone and THC reduced TNF-α production measured by ELISPOT in a dose-dependent manner with minimal effects on cell viability. AM630 prevented THC-induced reduction in TNF-α production when THC was less than 10 µg/ml. Preliminary data from Western blots indicate that CB_2R is expressed at low or undetectable levels in unstimulated monocytes, but increases in response to LPS stimulation and is maximal in combination with IFN-γ. CB_2R has not yet been detected at the cell surface of unstimulated monocytes using flow cytometry. Viable cultures of primary microglia have been established using tissue obtained during surgery to resect brain tumours.

Conclusion: ELISPOT is a valid technique for evaluating cannabinoid-induced changes in TNF-α production in stimulated primary human monocytes. We have confirmed that up-regulation of CB_2R expression in these cells is related to their activation state. Viable primary human microglia have been isolated using previously published protocols.

DIFFERENTIAL EFFECTS OF THC AND CBD ON THE NFkB PATHWAY IN ACTIVATED MICROGLIAL CELLS

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Although the anti-inflammatory activity of cannabinoids has been well established in various experimental systems, little is known about the mechanisms underlying these effects. In the present studies we have been investigating the signaling pathways underlying the anti-inflammatory effects of Δ^{9}-tetrahydrocannabinol (THC) and of the non-psychoactive cannabinoid, cannabidiol (CBD) in microglial cells.
Using lipopolysaccharide (LPS, a bacterial endotoxin) to activate BV-2, a murine microglial cell line, we found that LPS induced the production (qRT-PCR) and secretion (ELISA) of proinflammatory cytokines including interleukin-1beta (IL-1beta) and IL-6. The production and secretion of these cytokines were reduced by THC and by CBD. We found, however, that THC and CBD act through different intracellular mechanisms.
NFkB is a primary modulator of the inflammatory response. It is known that LPS leads to the degradation of IL-1 receptor kinase (IRAK-1) and of IkB (inhibitor of NFkB) proteins and via this pathway it activates NFkB dependent transcription. We found that CBD, but less so THC, reversed the LPS-induced IRAK-1 and IkB degradation (Western blot analysis). In agreement with the effects of THC and CBD on IRAK-1 and IkB we found that the LPS induced up-regulation of NFkB dependent gene expression was significantly attenuated by CBD pretreatment while the effect of THC was much less pronounced. However, contrary to the effect on NFkB, both cannabinoids decreased to a similar extent the activation of MAP kinases in the LPS-stimulated microglial cells.
In conclusion, although both THC and CBD exert inhibitory effects on the production of inflammatory cytokines in microglial cells, their activity may involve different intracellular pathways.

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N-ARACHIDONOYL GLYCINE ELEVATES THE PRODUCTION OF TUMOR NECROSIS FACTOR ALPHA IN ACTIVATED RAW264.7 CELLS

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N-Arachidonoyl glycine (NAGly), an endogenous ligand of GPR18, appears to be involved in the modulation of pain and inflammation (Kohno et al., 2006; Huang et al., 2001). It was first shown to attenuate pain responses in the second phase of formalin-induced pain behavior in rats (Huang et al., 2001). More recently it was found to reduce pain sensitivity in rat models of inflammatory and neuropathic pain (Succar et al., 2007; Vuong et al., 2007). It also reduced phorbol ester-induced ear edema in mice (Burstein et al., 2007). While such studies have shown that NAGly mediates inflammation and pain sensitivity, the cellular and molecular bases for these actions are not well understood. Here, we examined the effect of NAGly on the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) in RAW264.7 cells, a model of murine macrophage cells.

Method: TNF-α release in murine macrophage-like RAW264.7 cells was measured using an enzyme-linked immunosorbent assay. Cells were seeded at 100,000 cells/well in a 24 well plate, 20 hours prior to drug treatment. They were then co-treated with lipopolysaccharide (10ng/ml) and drugs for 1 hour at 37°C, following which TNF-α levels were tested in their media.

Results: NAGly potently increased TNF-α production in activated RAW264.7 cells in a concentration-dependent manner with an EC_{50} of 70.5nM. The maximal effect of NAGly was observed at 1μM, at which it caused a >2.5-fold increase in TNF-α release when compared to the vehicle (DMSO) treatment. This effect was absent in cells treated with glycine or arachidonic acid, the products of NAGly hydrolysis.

Conclusion: NAGly potentiates TNF-α release in murine macrophage-like RAW264.7 cells. This effect does not appear to be mediated by its hydrolytic products. These results suggest that NAGly may play a role in activating immune responses through the production of the pro-inflammatory cytokine TNF-α.

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N-ARACHIDONOYL GLYCINE MODULATES BV-2 MOUSE MICROGLIAL CELL MIGRATION

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Microglial cells are the resident macrophage of the CNS. During neuroinflammation activated microglia migrate along concentration gradients to sites of inflammation where they aggravate local cell damage. Many chemotactic ligands act upon $G_{i/o}$ G-protein-coupled receptors (GPCRs) to initiate transduction of their pro-migratory signal. AEA, 2-AG and other cannabinoids are known to act as powerful modulators of immune cell migration via CB_1, CB_2 and novel cannabinoid receptors. *N*-arachidonoyl glycine (NAGly) is an endogenous arachidonoyl amide and analogue of AEA that activates the $G_{i/o}$ GPCR, GPR18, at nanomolar concentrations. This study was directed at investigation of the modulation of BV-2 mouse microglial cell migration by *N*-arachidonoyl glycine.

BV-2 cells were cultured in DMEM with 10% FBS and 1% P/S. BV-2 cells were harvested and then re-suspended at a concentration of $1x10^6$ $cellsml^{-1}$ in PBS containing $CaCl_2$ and $MgCl_2$. *In vitro* cell migration assays were performed using a modified 96-well Boyden chamber. Incubation lasted 3 hours in a 5% CO_2 atmosphere at 37°C. Following incubation, the migrated cells on the 10µm pore filter were stained using a Diff-Quik stain set. Each well was counted in ten non-overlapping fields (x40) using a light microscope.

BV-2 migration was significantly induced in a concentration-dependent manner by 0.1nM, 1nM, 10nM, 100nM, 1µM and 10µM NAGly, the response being 91.3 ± 9.1%, 123.4 ± 7.3%, 183.7 ± 13.7%, 270.0 ± 22.7%, 446.5 ± 46.0%, 586.0 ± 66.1% of the response to 1µM fMLP respectively; n = 4. Checkerboard analysis indicated that the migration produced by NAGly is primarily due to chemotaxis, but also includes an element of chemokinesis. The migration of BV-2 cells to 1µM NAGly was unaffected by 100nM or 1µM concentrations of SR141716A. However, in the presence of 100nM SR144528, BV-2 migration in response to NAGly was significantly attenuated. Palmitoyl glycine (PALGly), an endogenous analogue of NAGly, failed to induce significant BV-2 cell migration over a concentration range of 0.1nM to 10µM. Likewise, the FAAH metabolites of NAGly, arachidonic acid and glycine (0.1nM to 10µM) also failed to induce significant migration. Consistent with this, URB597 (100nM) similarly had no significant effect on the ability of NAGly to induced BV-2 cell migration.

These data indicate that *N*-arachidonoyl glycine acts as a potent pro-migratory signalling molecule toward BV-2 mouse microglial cells.

EFFECTS OF 2-ARACHIDONYLGLYCEROL ON TH1 AND TH2 CYTOKINE GENE REGULATION IN MACHROPHAGE-LIKE HL60 CELLS

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Macrophages are involved in early immune response. Human promyelocytic leukemic HL60 cells have high peripheral cannabinoid receptor expression, and can be differentiated into macrophage-like cells by phorbol 12-myristate 13-acetate (PMA). By examining the expression levels of 84 genes of Th1 and Th2 cytokines (Human Th1-Th2-Th3 RT Profiler™ PCR Array from Superarray), we investigated the effects of 2- arachidonylglycerol (2-AG) on this cytokine mRNA profile in PMA-differentiated HL60 cells. More specifically, the effect of 2-AG on lipopolysaccharide (LPS)-treated cells was determined. The expression of 84 genes was assayed one and two hours after the cells were treated with DMSO (0.05%), LPS (10 mg/L), 2-AG (1uM), or LPS+2-AG (10 mg/L and 1uM, respectively). Significance in gene expression between treatment groups and the DMSO control was declared in those groups in which a three-fold expression difference and a Student's *t* test p value < 0.05 was observed. To determine the effect of 2AG, the gene expression of LPS + 2AG group was compared to that of LPS alone.

In comparison to DMSO, no differences were observed in the expression of the 84 genes after one hour treatment with LPS, 2-AG, or LPS+2-AG. However, after two hours, compared to the DMSO control, a total of seven genes (IL23A, CSF2, FASL6, IL6, IL10, IL12B and TNF) were differentially regulated by LPS. Of the seven, all were up-regulated by LPS except for FASL6. A single gene (IL7, a CD4+ cell marker) was up-regulated about four-fold in the 2-AG group. Interestingly, among the seven genes differentially regulated by LPS, only the level of IL-10 expression was inhibited by 2-AG ($p < 0.05$) when 2-AG was added in combination with LPS. IL10 is involved in B and T-cell activation and the Th2 immune response. Therefore, 2-AG when added in combination with LPS inhibits the Th2 cytokine IL10 in PMA-differentiated HL60 cells.

We are currently investigating the role of 2-AG in the LPS-mediated response in PMA-differentiated HL60 cells at other time points (4 and 8 hour post treatment).

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ROLE OF PALMITOYLETHANOLAMIDE ON MODULATION OF THE MAST CELL-T CELL INTERACTIONS. AN IN VITRO STUDY

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Mast cells are important resident effector cells in allergic and inflammatory responses. Upon stimulation, mast cells are able to synthesize and release a vast array of bioactive mediators that facilitate the development of allergic inflammation. Moreover, mast cells have been found to have immunoregulatory roles by interacting with cells of the adaptative immune system and to present exogenous antigen to T cells. Morphologic and biochemical studies carried out in inflamed allergic tissues, have shown co-localization and bidirectional interactions between mast cells and T cells. It has been well demonstrated that the interaction is not only mediated by soluble factors released from both cells, but it also involves the binding of cell surface molecules. This last pathway of activation results in the expression and release of different proinflammatory cytokines such as TNF-alpha, MIP-2, IL-4, IL-6 and IL-8.
Based on the capability of palmitoylethanolamide (PEA) to act mainly as an anti-inflammatory and immunoregolatory agent through the down-regulation of mediators release from mast cells, our study focused on the possibility that this cannabimimetic compound, could be able to affect the interaction between these two important cell types mediating inflammatory processes.
As a first step, we analyzed supernatants obtained from co-culturing HMC-1 (human mast cell line) and activated Jurkat T cell line treated with different concentrations of PEA (nM range) or its vehicle, for released IL-8 by ELISA. We found that PEA was able to decrease the amount of released IL-8 (~ 45%) in a concentration-independent manner. To verify whether PEA was able to interact per se with mast cells or T cells activity, we performed experiments in which HMC-1 or activated Jurkat T cells were treated with PEA before carrying out co-cultures. We found no differences in IL-8 release when supernatants from pre-treated HMC-1 co-cultured with activated Jurkat T cells were analyzed, but, surprisingly, a decrease by 75% was observed in supernatants obtained from pre-treated Jurkat T cells and HMC-1. The CB2 receptor, a still under discussion PEA binding site, seems not to be involved in PEA effects as demonstrated by the inefficacy of SR144528 antagonist in reverting the IL-8 inhibition.

In order to be sure that PEA was not a toxic agent, we also performed MTT test in which the same concentrations of PEA were used. We found no alterations of viability induced by PEA at any concentration tested.

These results suggest that PEA can effectively modulate the mast cells-T cell interaction, corroborating its valuable anti-inflammatory efficacy.

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ANALYSIS OF CB_2 CANNABINOID RECEPTOR EXPRESSION IN THE NERVOUS SYSTEM

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Munro et al. (1993; Nature 365, 61) first investigated CB_2 cannabinoid receptor expression using radiolabelled oligonucleotide probes and found that CB_2 mRNA was present in the marginal zone of spleen white pulp but was not detectable in the brain. Immunocytochemical evidence of neuronal CB_2 expression in the brain has been reported (Van Sickle et al. 2005, Science 310, 329; Gong et al. 2006, Brain Res. 1071, 10), but more extensive use of tissue from CB_2-knockout mice is needed to determine the system-wide specificity of antibodies to CB_2. Zhang et al. (2003; Eur. J. Neurosci. 17, 2750) have reported that in a rat model of neuropathic pain there is induction of CB_2 expression in the spinal cord that coincides with the appearance of activated microglia.

Here we have used high resolution mRNA in situ hybridisation methods employing DIG-labelled RNA probes to investigate basal expression of CB_2 in the rat central nervous system (CNS) and induction of CB_2 expression in the rat spinal nerve ligation model of neuropathic pain.

Basal expression of CB_2 in the CNS was analysed by using the relatively low-level expression of CB_1 mRNA in hippocampal pyramidal cells to determine the end point for experiments and by using the robust expression of CB_2 in the spleen as a positive control test for the CB_2 anti-sense probes employed. CB_2 mRNA expression was not detected in any region of the brain and spinal cord, consistent with the original findings of Munro et al. To investigate induction of microglial CB_2 expression in the rat spinal nerve ligation model of neuropathy, the spinal cords of rats that developed allodynia were analysed 7 or 14 days after injury in comparison with cords from sham-operated animals that did not develop allodynia. Up-regulation of spinal OX-42 expression on the side ipsilateral to ligation was observed in spinal nerve ligated animals, indicative of microglial activation. However, CB_2 expression was not detected in microglia or in other cell types in the spinal cords of spinal nerve ligated or sham-operated animals. These data indicate that induction of CB_2 expression in the rat spinal nerve ligation model of neuropathic pain reported by Zhang et al. (2003) is not readily reproducible.

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CHARACTERIZATION OF A NOVEL CB_2 RECEPTOR SELECTIVE LIGAND, A-836339, IN *IN VITRO* PHARMACOLOGICAL ASSAYS AND *IN VIVO* ANIMAL MODELS

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Introduction: Recent findings in cannabinoid research have provided evidence demonstrating that selective activation of the CB_2 receptor by cannabinoid ligands produces anti-hyperalgesic and anti-allodynic effects in preclinical animal models of nociceptive and neuropathic pain. However, current tool compounds, although used extensively in cannabinoid research, such as AM1241, JWH-015 and JWH-133, either display modest selectivity or lack full agonist efficacy in their *in vitro* pharmacological profiles. Here we report the identification of A-836339 (2,2,3,3-tetramethyl-cyclopropanecarboxylic acid [3-(2-methoxy-ethyl)-4,5-dimethyl-3H-thiazol-(2Z)-ylidene]-amide) as a novel CB_2 agonist and its characterization in *in vitro* pharmacological assays and in *in vivo* preclinical models of pain.

Methods: A-836339 was evaluated for affinity, agonist efficacy and selectivity for the human and rat cannabinoid receptors, and was additionally evaluated in multiple rodent models of inflammatory, post-operative, and neuropathic pain.

Results: In radioligand binding assays, A-836339 displayed high affinities at the human and rat CB_2 receptors (K_i = 0.4 and 0.8 nM, respectively) and significant selectivity over the CB_1 receptor (425- and 189-fold) of the respective species. Similarly, in *in vitro* functional assays, high potencies, full agonist efficacies, and selectivity at the CB_2 receptor were observed. In the Complete Freund's Adjuvant (CFA) model of inflammatory pain, A-836339 fully reversed CFA-induced decrease in paw withdrawal latency with an ED_{50} of 1.8 μmol/kg, i.p. The anti-hyperalgesic effects of A-836339 were fully blocked by an antagonist selective at the CB_2 (SR144528), but not the CB_1 (SR141716A) receptor. Unlike AM1241, the anti-hyperalgesic properties of A-836339 were independent of the opioid system, as the μ-opioid receptor antagonist, naloxone, failed to block its effects. A-836339 was also efficacious in neuropathic pain (spinal nerve ligation, SNL and chronic constriction injury CCI of the sciatic nerve, CCI) and post-operative pain models in addition to capsaicin-induced secondary mechanical hyperalgesia model. Furthermore, no tolerance was developed to the anti-allodynic effects of A-836339 following repeated dosing in the CCI model.

Conclusion: A-836339 is a selective CB_2 ligand exhibiting full agonist efficacy in *in vitro* functional assays. A-836339 exhibits broad-spectrum analgesic efficacy across multiple models of nociceptive and neuropathic pain, effects that are selectively blocked by CB_2 antagonists, but are unaffected by either CB_1-selective or μ-opioid receptor antagonists. These data demonstrate that A-836339 can be a useful tool for studying the role of CB_2 receptor agonists in modulation of pathologic pain.

THE DISCOVERY OF NOVEL HETEROCYCLIC CANNABINOID RECEPTOR AGONISTS FOR THE TREATMENT OF PAIN

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A novel CB_1 receptor agonist lead compound was identified from in-house compound library by a high-throughput screening approach. The initial screen resulted in a novel confirmed hit with poor water solubility. Hit and lead optimisation led to the identification of a potent water soluble indole-3-carboxamide derivative. This compound demonstrated effective levels of analgesia within a range of *in vivo* models with a short duration of action following intravenous administration. As the compound was rapidly metabolised in the presence of human liver microsomes, in an endeavour to identify a longer acting compound, a number of approaches were undertaken. Identification of the metabolically unstable part of the molecule, followed by a rational optimisation study afforded a metabolically more stable CB_1 agonist which showed a longer half life in a human liver microsomal assay. This compound was also active in a number of *in vivo* animal pain models with a longer duration of action compared to the lead indole-3-carboxamide derivative.

SELECTIVE ACTIVATION OF CANNABINOID CB_2 RECEPTORS SUPPRESSES PAINFUL PERIPHERAL NEUROPATHY INDUCED BY THE CHEMOTHERAPEUTIC AGENT PACLITAXEL IN RATS

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Activation of cannabinoid CB_2 receptors suppresses neuropathic pain induced by traumatic nerve injury. The present studies were conducted to evaluate the efficacy of cannabinoid CB_2 receptor activation in suppressing painful peripheral neuropathy evoked by chemotherapeutic treatment with the anti-tumor agent paclitaxel. Rats received paclitaxel (2 mg/kg i.p. per day) on four alternate days to induce mechanical hypersensitivity (tactile allodynia). Tactile allodynia was defined as a lowering of the threshold for paw withdrawal to stimulation of the plantar hindpaw surface with an electronic von Frey stimulator. Tactile allodynia developed in paclitaxel-treated animals relative to groups receiving the cremophor vehicle at the same times. Two structurally distinct cannabinoid CB_2 agonists — R,S-AM1241 and AM1714— dose-dependently suppressed established paclitaxel-evoked tactile allodynia following systemic administration. Pretreatment with the CB_2 antagonist SR144528 but not the CB_1 antagonist SR141716 blocked the anti-allodynic effects of both R,S- AM1241 and AM1714. Moreover, R-AM1241 was more effective than S-AM1241 in suppressing paclitaxel-evoked tactile allodynia, consistent with mediation by CB_2. Administration of either the CB_1 or CB_2 antagonist alone failed to alter paclitaxel-evoked mechanical hypersensitivity. Moreover, neither R,S-AM1241 nor AM1714 altered paw withdrawal thresholds in rats that received the cremophor vehicle in lieu of paclitaxel. These data suggest that cannabinoid CB_2 agonists can suppress established neuropathic pain evoked by paclitaxel treatment without inducing antinociception. Our data suggest that cannabinoid CB_2 receptor subtypes may be important therapeutic targets for the treatment of chemotherapeutic neuropathy.

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IN VIVO CHARACTERIZATION OF AM-2389, A POTENT CB1R SELECTIVE AGONIST

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A medical use of Δ^9-THC, the major psychoactive constituent in marijuana, has been to alleviate pain in people suffering from arthritis, AIDS, cancer, skeletal disorders and other life altering pain disorders. However, controlling the binding affinity of Δ^9-THC and its synthetic derivatives to the desired receptors has been challenging, thereby producing adverse effects. Synthesized ligands with increased selectivity will lead to improved derivatives that target the receptor(s) of interest. A novel cannabinoid agonist has proven to be more potent than the well documented Δ^9-THC. AM-2389 shows great affinity and selectively for the CB1 (ki = 0.16 nM) versus the CB2 (ki = 15.8 nM) receptor. Characterization of this ligand *in vivo* used the following behavioral assays; drug discrimination, locomotor activity, body temperature, inverted screen, catalepsy, and paw thermal stimulation. Male ICR mice (n=6 per group) were used for all assays except drug discrimination, where male Sprague Dawley rats (n=8-12) were used. AM-2389 showed clear psychotropic activity in drug discrimination at doses considerably lower than those of Δ^9-THC (range: 85 to 125 fold potency difference). There was a significant decrease in locomotor activity and body temperature upon administration of AM-2389 compared to Δ^9-THC and vehicle groups. In addition, AM-2389 reduced locomotor activity for a longer duration than Δ^9-THC but with a later onset of effect. Inverted screen test measured the latency for the mice to climb over to the reverse side of the flipping apparatus. Vehicle and Δ^9-THC groups moved to the reverse side of the screen in less time than the AM-2389 groups. AM-2389 caused a significant increase in latency for the inverted screen test. Catalepsy measured the latency for mice to remove their paws from an elevated bar. Mice administered AM-2389 showed a greater trend towards increased latency compared to Δ^9-THC and vehicle groups. Analgesia was measured using a paw thermal stimulation apparatus. AM-2389 administered mice were less responsive to pain as indicated by increased paw withdrawal latencies compared to Δ^9-THC and vehicle treated groups. There was an increased latency in pain response upon administration of AM-2389 even at low doses. It was found that AM-2389 produced central effects and analgesic responses at a greater potency compared to Δ^9-THC. Furthermore, AM-2389 had a slower onset and a longer duration of action than Δ^9-THC.

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THE EFFECTS OF THC FROM MARINOL® IN LABORATORY MICE

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Δ^9-tetrahydrocannabinol (THC) is a Schedule I substance according to the US Drug Enforcement Agency (DEA). The pharmaceutical Marinol® contains dronabinol (synthetic THC) in sesame oil and is a Schedule III drug. To examine the effects of THC from Marinol as a source for THC for laboratory research, experiments were and are being conducted on the time course, relative potency, and efficacy of injected Marinol in mice. These data will be compared to previously published data on the time course and ED_{50} values of the effects of pure THC in a variety of behavioral and physiological measurements.

To prepare the THC for injection, the sesame oil containing THC was removed from the Marinol capsule and mixed with 1 part ethanol, 1 part emulphor and 7 parts water. For comparison, a vehicle was prepared with equal parts sesame oil (generously donated by the manufacturer that supplies the sesame oil for the production of Marinol), ethanol and emulphor and 7 parts water. Mice were tested for effects in spontaneous activity, rectal temperature and antinociception assays after i.p. injection of various doses of THC from Marinol or vehicle. Spontaneous activity was measured using a Limelight video tracking system to determine total distance moved and average velocity in an open field during 10 minute test periods at various times after drug injection. Changes in rectal temperature were determined by taking one measurement before and several after various times following an injection. Antinociception was determined by measuring latency to withdraw the tail from a hot water bath set to 53 degrees (tail flick test), and latency to jump or lick a hind paw after being placed on a hot plate set to 56 degrees (hot plate test).

Results comparing 10 mg/kg pure THC and 10 mg/kg THC from Marinol indicated that the effects of Marinol were blunted or delayed as this dose of Marinol had very little effect, while pure THC produced 50% MPE in the tail flick test and a 4.5 degree decrease in body temperature. Assays of 50 mg/kg THC from Marinol gave results that were more similar to those observed for 10 mg/kg pure THC: a 5 degree drop in temperature but only 15% MPE in the tail flick test. Effects with the 50 mg/kg dose of Marinol showed a longer duration of action than 10 mg/kg pure THC. In the hot plate test, 50 mg/kg Marinol reached a maximum %MPE 90 minutes after injection. Preliminary results in spontaneous activity assays showed that 50 mg/kg Marinol produced decreases compared to vehicle starting about 45 minutes after injection.

These results show that Marinol exhibits cannabinoid behavioral effects similar to what has previously been published for THC, though the pharmacokinetics may be different. This indicates that Marinol may be used effectively as a source for THC in laboratory experiments, making it a viable alternative to obtaining a Schedule I license to conduct research with THC. Further studies will be performed to more completely characterize the time course and dose-response for THC from Marinol.

EFFECT OF CANNABIDIOL DOSE IN CFA-INDUCED MONO-ARTHRITIC RAT MODEL

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Cannabidiol, a non-psychoactive cannabinoid, has been examined for its therapeutic potential for neuropathic pain, cancer pain, multiple sclerosis and inflammation. Transdermal delivery of cannabidiol can alleviate potential side effects and avoid first pass metabolism that are often associated with oral dosing. Transdermal delivery allows for a more controlled drug delivery rate and in the case of pain control, transdermal delivery would allow for treatment of specific target sites. For this study, cannabidiol gel was evaluated in the mono-arthritic rat model.

Treatment with cannabidiol gel was initiated on day four or seven after complete Freund's adjuvant (CFA)-induction in the rear leg of Sprague-Dawley rats (260-280 mg). Hair from the dorsal side was removed the day prior to gel dosing with clippers to accommodate the 3 different areas (3.5 cm^2, 17.5 cm^2, and 35.0 cm^2) required for the appropriate dose (0.62 mg, 3.1 mg, and 6.2 mg, respectively). Cannabidiol gel was applied daily for four consecutive days. After the fourth day of dosing and behavioral testing (Hargreave's paw withdrawl latency (PWL) test), animals were sacrificed and blood and tissue samples were harvested. Plasma samples harvested from the blood were extracted and analyzed for cannabidiol levels with LC/MS.

With the 6.2 mg cannabidiol gel dose, improvement was seen with CFA-induced mono-arthritic rats compared to CFA-induced mono-arthritic rats receiving placebo gel. However with 3.1 mg and 0.62 mg cannabidiol gel dose, no significant differences were seen with the behavioral measurements between the treated rats and rats receiving placebo gel. Plasma cannabidiol levels displayed a linear correlation (slope=1.0, R^2=0.999) with the dose of cannabidiol administered topically to the rats (4.3 ± 2.6 ng/mL, 18.8 ± 2.8 ng/mL, and 34.6 ± 11.0 ng/mL for the corresponding areas of 3.5 cm^2, 17.5 cm^2, and 35.0 cm^2, respectively). Cannabidiol gel may be a viable option for the treatment of pain associated with arthritis.

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LOCAL ADMINISTRATION OF PALMITOYLETHANOLAMIDE REDUCES CHRONIC GRANULOMA-ASSOCIATED PAIN IN RATS

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Chronic inflammation, as granuloma is often associated to pain. Palmitoylethanolamide (PEA) has shown great efficacy in the treatment of pain and inflammation. The anti-inflammatory and analgesic effects of PEA are hypothesized to be mediated, at least in part, by mast cell down-modulation. Mast cells are immune competent cells strategically located around nerves and blood vessels in tissues directly interfacing with external environment to perform critical protective and homeostatic functions. Mast cell mediators, including those acting to nerve fibers, released at early stage of the inflammatory process, drive the inflammatory reaction to chronicity as it happens in λ-carrageenin-induced granulomatous tissue formation.

In this study we investigated the effect of PEA on granuloma formation induced by λ-carrageenin-soaked sponge implant in male Wistar rats.

Our results demonstrate that local administration of PEA significantly decreases weight and new nerves formation in granulomatous tissue, evaluated both by histological analysis and nerve growth factor (NGF) protein expression. The results indicate that PEA, given locally, may represent a potential therapeutic tool in controlling chronic inflammation and it could be proposed for the treatment of all those diseases accompanied by hyperalgesia.

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GROUP I METABOTROPIC GLUTAMATE RECEPTORS MODULATE ENDOCANNABINOID-MEDIATED STRESS-INDUCED ANALGESIA AT SPINAL AND SUPRASPINAL LEVELS THROUGH THE PHOSPOLIPASE C/ DIACYLGLYCEROL PATHWAY

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In previous work we showed that a nonopioid form of stress-induced analgesia (SIA) is mediated by mobilization of endocannabinoids such as 2-arachidonylglycerol (2-AG) in the midbrain periaqueductal gray (Hohmann et al. (2005) *Nature* 435: 1108-1112) and lumbar spinal cord (Suplita II (2006) *Neuropharmacology* 50: 372-379). However, the mechanisms underlying the formation of 2-AG *in vivo* remain unknown. The present studies used a pharmacological approach to further examine the role of 2-AG in SIA at supraspinal and spinal levels. 2-AG is synthesized *in vitro* through the consecutive activation of two distinct enzymes– phospholipase C (PLC) and diacylglycerol lipase (DGL). First, the 2-AG precursor diacylglycerol (DAG) is formed from PLC-mediated hydrolysis of membrane phospholipid precursors. DAG is subsequently hydrolyzed by DGL to generate 2-AG. We examined whether activation of group I metabotropic glutamate receptors (mGluRs), which are positively coupled to PLC, would enhance SIA at supraspinal and spinal levels. Both 2-AG and anandamide are mobilized in the midbrain PAG following exposure to footshock stress. By contrast, 2-AG but not anandamide is mobilized in the lumbar spinal cord. Site-specific administration of the group I mGluR agonist DHPG into either the dorsolateral PAG (dPAG) or lumbar spinal cord enhanced SIA through a CB_1-dependent mechanism. We also evaluated whether pharmacological inhibitors of DGL would suppress SIA. Microinjection of the DGL inhibitors tetrahydrolipstatin (THL) or RHC80267 into the dPAG suppressed SIA. By contrast, off-site intracranial microinjection of either DGL inhibitor failed to suppress SIA. THL also suppressed SIA following intrathecal injection. The enhancement of SIA induced by activation of group I mGluRs was also dependent upon DGL. The DGL inhibitor THL blocked the enhancement of SIA induced by DHPG following intracranial or intrathecal injection. Our results are consistent with the hypothesis that exposure to an environmental stressor stimulates biosynthesis of 2-AG through the PLC/DGL pathway at spinal and supraspinal levels to induce SIA. Our data further suggest that this process may be initiated by activation of group I mGluRs. Our findings collectively suggest that postsynaptic mGluR mechanisms regulate endocannabinoid signaling in the PAG and spinal cord to induce SIA (Supported by DA021644, DA022478 and DA014022 to A.G.H.).

EFFECT OF *N*-ARACHIDOYLSEROTONIN (AA-5-HT), A FAAH INHIBITOR WITH ANTAGONISTIC ACTIVITY AT VANILLOID TRPV1 RECEPTOR, IN AN ANIMAL MODEL OF ACUTE INFLAMMATION

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Endocannabinoids exert potent analgesic and anti-inflammatory effects. Therefore the endocannabinoid system (receptors and endocannabinoid degrading enzymes) could represent a potential target for the development of compounds with therapeutic use against pain and inflammation. One possible strategy could be the employment of hybrids targeting both receptors and enzymes. Based on this, we investigated whether AA-5-HT, a dual FAAH/TRPV1 blocker, was able to produce anti-inflammatory and anti-hyperalgesic effects in an animal model of acute inflammation, induced in mice by an intraplantar injection of carrageenan, and we compared its effects with those evoked by a selective FAAH inhibitor (URB597) or a selective TRPV1 antagonist (capsazepine). AA-5-HT was administered i.p. at different doses (0.1-1-5 mg/kg, all devoid of psychoactivity) 30 min before carrageenan and the anti-inflammatory and anti-hyperalgesic efficacy was assessed 2 and 3 hours after flogogen injection, respectively. AA-5-HT significantly decreased both edema and thermal hyperalgesia in a dose dependent manner with the highest dose (5 mg/kg) producing a complete inhibition. To explore AA-5-HT mechanism of action, we employed different receptor ligands: olvanil, a TRPV1 receptor agonist, rimonabant, a selective CB1 receptor antagonist and SR144528, a CB2 receptor antagonist, administered 15 min before AA-5-HT. The results obtained show that only the TRPV1 receptor is involved in the anti-inflammatory effect of AA-5HT, while both CB1 and TRPV1 receptors are involved in the anti-hyperalgesic effect. We suggest that AA-5-HT can act directly by blocking TRPV1 receptors and/or indirectly by enhancing AEA that activates CB1 receptor and inhibits pain and inflammation. Thus, AA-5-HT strongly reduced both signs of acute inflammation through its dual activity as FAAH inhibitor and TRPV1 antagonist, without exerting unwanted effects. On the contrary, the specific FAAH inhibitor URB597 (3 mg/kg), in the same animal model, reduced edema but was unable to counteract thermal hyperalgesia. In addition, when administered to inflamed mice, the TRPV1 receptor antagonist, capsazepine, exhibited only a partial effect on both edema and thermal hyperalgesia at a high dose (10 mg/kg).In conclusion, the use of a compound with a dual target, such as AA-5HT, seems to be more effective than single-target compounds in terms of efficacy and toxicity.

INHIBITION OF FAAH PRODUCES PPAR-α MEDIATED ANALGESIA IN A RAT MODEL OF INFLAMMATORY PAIN

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Inflammatory pain behaviour can be attenuated following a peripheral pre-administration of the FAAH inhibitor URB597, an effect which is accompanied by increased levels of endocannabinoids in the inflamed paw (Jhaveri *et al.,* ICRS 2007 symposium). In addition to the clear role of cannabinoid receptors, there is increasing evidence for a role of peroxisome proliferator-activated receptors (PPARs) in the inhibitory effects of endocannabinoids and endocannabinoid-related compounds. The aims of this study were to investigate the neuronal mechanisms underlying the analgesic effects of URB597 by determining the effects of URB597 on receptive field expansion of spinal neurones in a model of inflammatory pain and to characterise the receptors mediating these effects.

Extracellular recordings of wide dynamic range dorsal horn (WDR) neurones (laminae V and VI) were made in anaesthetised (1.5% isoflurane in 66% N_2O/33% O_2) male Sprague Dawley rats (250-300g). Peripheral receptive fields of WDR neurones on the hind-paw were mapped using innocuous (8g) and noxious (26g) mechanical stimuli (von Frey monofilaments) before and after intraplantar injection of carrageenan (100µl 2% in saline). Effects of intraplantar pre-administration (30 min prior) of URB597 (25µg/50µl) or vehicle (50µl 3% Tween80 in saline) on carrageenan (2%) -induced expansion of peripheral receptive fields were determined. The contributions of cannabinoid CB_1 receptors and PPAR-α receptors to the effects of URB597 were investigated using the CB_1 receptor selective antagonist AM251 (30µg/50µl) and PPAR-α selective antagonist GW6471 (30µg/50µl) respectively, both of which were co-administered with URB597.

Intraplantar administration of carrageenan produced a robust expansion of peripheral receptive fields of WDR neurones in vehicle treated rats (278±78% and 245±65% of pre-drug area for 8g and 26g stimuli, respectively). This inflammation-evoked expansion of peripheral receptive fields of WDR neurones was significantly attenuated following pre-administration of URB597 (25µg/50µl; 106±18% and 107±8% of pre-drug area for 8g and 26g stimuli, respectively). Inhibitory effects of URB597 were significantly blocked following co-administration of URB597 with the PPAR-α antagonist GW6471 (30µg/50µl; 269±44% and 256±43% of pre-drug area for 8g and 26g stimuli, respectively), but not the CB_1 receptor antagonist AM251 (30µg/50µl; 131±16% and 132±18% of pre-drug area for 8g and 26g stimuli, respectively).

These data suggest that the analgesic effects of URB597 in models of inflammation arise as a result of inhibition of peripheral and central sensitisation of WDR neurones. Previously we have shown that intraplantar URB597 increases in levels of 2-AG and AEA in the carrageenan-inflamed hind paw. Since effects of URB597 were blocked by a PPAR-α antagonist and not a CB_1 receptor antagonist, our data suggest a role for PPAR-α in mediating analgesic effects of FAAH inhibition on receptive field expansion.

EFFECTS OF CHRONIC ADMINISTRATION OF URB597 IN THE CARRAGEENAN MODEL OF INFLAMMATORY PAIN

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Anandamide (AEA), oleoylethanolamide (OEA) and palmitoyl ethanolamide (PEA) are primarily metabolised by fatty acid amide hydrolase (FAAH). Systemic FAAH-inhibitors attenuate inflammatory pain (Jayamanne et al 2006). ECs are also substrates for cycloxygenase-2 (COX-2) (Kozak *et al.* 2004), which can metabolise AEA to prostaglandin ethanolamides (prostamides). Prostamides have been detected when FAAH is knocked out and mice are challenged with AEA (Weber et al, 2004). Thus, when FAAH is inhibited, COX-2 may be the main enzyme responsible for the metabolism of AEA. The aim of our study was to investigate the effects of chronic dosing with the FAAH inhibitor URB597 on nociceptive behaviour and levels of ECs and prostamides in a model of inflammatory pain.

Adult male Sprague-Dawley rats (240-260g) were dosed daily with 0.3 mg/kg (ip) URB597 or vehicle (saline + Tween 80) for 4 days. Weight bearing on the hindpaw was measured with an Incapacitance Tester (Linton Instrumentation, U.K.). On the day of behavioural testing, rats received a final dose of URB597 30 minutes prior to intraplantar injection of 2% carrageenan or saline (100µl). Weight bearing on the left (injected) and right hindpaw was assessed 30 min. prior to injection of carrageenan/saline and at 90, 150 and 210 min. post-injection. Spinal cord and paw tissue were collected for LCMS/MS analysis of endocannabinoids and prostamides. Weight bearing data were analysed using a two-way ANOVA and spectrometric data were analysed with Mann-Whitney non-parametric test.

Intraplantar injection of carrageenan significantly increased the difference in weight bearing between ipsilateral and contralateral hindpaws 2-3 hr post-injection, compared to saline injection ($p<0.001$). Levels of AEA, OEA and PEA were significantly decreased in the vehicle-treated carrageenan-inflamed hindpaw, compared to contralateral paw (Table 1). FAAH activity in the liver was substantially decreased ($p<0.005$) in the chronic URB treated group, compared to vehicle group. Chronic URB597 treatment did not attenuate the carrageenan induced changes in weight bearing, but delayed the onset of carrageenan-induced hyperalgesia. Chronic URB597 did not alter levels of AEA, OEA and PEA in the carrageenan inflamed hindpaw compared to the vehicle-treated carrageenan group. In the spinal cord, URB597 significantly increased levels of AEA and PEA in the contralateral spinal cord, compared to the vehicle-treated group (Table 1). Levels of 2AG were unaltered by URB597 and prostamides were not detected in any of the groups.

Table 1: Endocannabinoid levels in the hindpaw and spinal cord. Values are means ± SEM of concentrations of compounds in wet tissue (n = 6), Carr=carrageenan; URB=URB597. *$p<0.05$, **$p<0.005$ compared to Veh Carra ipsi , $^{\$}$ $p<0.05$ compared to Veh Carra Contra.

	Hindpaw AEA (pmol/g)	Spinal AEA (pmol/g)	Hindpaw PEA (nmol/g)	Spinal PEA (nmol/g)
Veh Carra contra	8.34±1.76 *	16.84±3.13	3.81±0.86 *	0.24 ±0.09
Veh Carra ipsi	4.061±1.04	18.22±5.97	1.66±0.35	0.45±0.15
URB Carra contra	9.18±1.70 **	58.91±18.44 *$^{\$}$	3.09±0.43 **	0.71±0.19 $^{\$}$
URB Carra ipsi	2.12±0.30	26.15±7.53	1.69±0.16	0.56±0.18

Thus, although FAAH was inhibited by chronic treatment with URB597, this did not alter levels of ECs in the hindpaw of rats. These data suggest that in the presence of FAAH inhibition, ECs are metabolised by other pathways in the periphery. We were, however, unable to measure prostamides in the hindpaw, suggesting a minor role of COX2 in this effect. The differential effect of URB597 treatment on levels of ECs in the hindpaw and spinal cord suggest there is a tissue specific role of FAAH at these key sites involved in nociceptive processing.

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CB2 RECEPTORS STIMULATE LIVER REGENERATION FOLLOWING ACUTE LIVER INJURY

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The liver has a remarkable ability to regenerate in response to resection or liver injury. We have recently demonstrated the central role of the endocannabinoid system in the regulation of fibrogenesis during chronic liver diseases that associate profibrogenic effects of CB1 receptor and antifibrogenic effects of CB2 receptor (Teixeira-Clerc, 2006 ; Julien, 2005). In this study, we investigated the role of CB2 receptor during liver regeneration, in a model of acute hepatitis induced by a single injection of carbon tetrachloride (CCl4), characterized by parenchymal necrosis and liver inflammation, followed by hepatic regeneration from hepatocytes. Hepatic CB2 receptor expression was markedly induced following CCl4-induced liver injury. In $CB2^{-/-}$ mice, acute CCl_4 administration was associated with increased liver injury and reduced hepatocyte proliferation as compared to WT mice, suggesting that CB2 receptor stimulates liver regeneration following acute liver injury. Accordingly, treatment of WT mice with a single dose of CB2 specific agonist, JWH-133, accelerated liver regeneration in response to CCl4 exposure. Analysis of the signaling pathways of CB2-mediated liver regeneration was analyzed in CB2-/- mice and demonstrated that impaired regeneration was associated with a reduction in the expression of IL6, a cytokine with antiapoptotic and mitogenic properties that play a central role in the liver regeneration process, and with the blockade of its signalling pathways, ERK and STAT-3. Taken together, our data unravel the essential role of CB2 receptors in the activation of hepatic regeneration.

INHIBITION OF SPASTICITY USING VSN16

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Whilst spasticity can be controlled by stimulation of central CB_1 cannabinoid receptors (CB_1R) in the central nervous system (CNS), medical cannabis will also invariably cause concomitant stimulation of CB_1R in brain regions causing the psychoactive effects. Therefore, one route to increase the therapeutic window of cannabinoids may be to target peripheral CB_1R between the CNS and muscle and avoid stimulation of CB_1R in the brain. A series of chemicals were synthesized (Hoi, PM et al. 2007. Br J Pharmacol. 152:751) and one compound (VSN16. 3-(5-dimethyl-carbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide) was found to be water soluble and largely excluded from the CNS. The i*n vitro* activity of VSN16 was blocked by the addition of CB_1R antagonists, SR141617A and AM251 and i*n vivo* it was demonstrated that VSN16 inhibited spasticity in an experimental autoimmune encephalomyelitis model of multiple sclerosis. Surprisingly however, VSN16 failed to bind or signal via CB_1R or CB_2R and some in vitro functions could be inhibited by 0-1918, the abnormal cannabidiol receptor/GPR55 antagonist, demonstrating that VSN16 acts at a novel cannabinoid-related target.

SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY CANNABINOIDS IN HEPATIC CELL

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Hepatocellular carcinoma is one of the major diseases with a very poor prognosis, being the fifth cause of cancer mortality and the third most common cause of cancer related death. Cannabinoids have been well demonstrated that they can affect cell proliferation and cell cycle control in many cancer types. In hepatic cancer it has been shown a role of cannabinoids in the cell viability. These lipid ligands can exert their effects through cannabinoids receptors or in an independent way. In this work, we have investigated the signaling pathways of some cannabinoids in the human hepatoma cell line, Hep G2.

First, we have explored the potential utility of cannabinoids as antitumoral agents in Hep G2 cells, which express cannabinoid receptors CB1 and CB2. We have analyzed the effect of JWH-015 on the cell survival. Then, we have studied the role of the cannabinoid receptors and the signalling pathways activated by convectional techniques as western blot using antibodies appropriated. We have observed using MTT and flow citometry experiments that JWH-015 decreased cell viability, which was reversed with the CB2 antagonist SR144528. These results indicated a role of CB2 receptor in the action mechanism of JWH-015. Then, we have been examined the role of JWH-015 in the activation of the PI3K/Akt and the MAPK signalling pathways. Others mechanisms will be studied to find the action of JWH-015 in Hep G2 cells.
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EXPRESSION OF CANNABINOID RECEPTORS TYPE 1 AND TYPE 2 IN NON HODGKIN'S LYMPHOMA: GROWTH INHIBITION BY RECEPTOR ACTIVATION

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Endogenous and synthetic cannabinoids exert anti-proliferative and pro-apoptotic effects in various types of cancer and in mantle cell lymphoma (MCL). In this study we evaluated the expression of cannabinoid receptors type 1 and type 2 (CB1 and CB2) in non-Hodgkin lymphomas of B cell type (n=62). A majority of the lymphomas expressed higher mRNA levels of CB1 and/or CB2 as compared to reactive lymphoid tissue. With the exception of MCL, which uniformly overexpresses both CB1 and CB2, the levels of cannabinoid receptors within other lymphoma entities was highly variable, ranging from 0.1 – 224 times the expression in reactive lymph nodes. Low levels of the splice variant CB1a, previously shown to have a different affinity for cannabinoids than CB1, were detected in 44% of the lymphomas, while CB1b expression was not detected. In functional studies using MCL-, Burkitt lymphoma (BL)-, chronic lymphatic leukemia (CLL)- and plasma cell leukemia cell lines, the stable anandamide analogue R(+)-methanandamide (R(+)-MA) induced cell death in only in MCL and CLL cells, which overexpressed both cannabinoid receptors, but not in BL. *In vivo* treatment with R(+)-MA caused a significant reduction of tumor size and mitotic index in mice xenografted with human MCL. Together, our results suggest that therapies using cannabinoid receptor ligands will have efficiency in reducing tumor burden in malignant lymphoma overexpressing CB1 and CB2.

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 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 as well as to study the physiological role of CB1 in aRMS.

First, we demonstrated that cannabinoid receptor agonist treatment of aRMS cells with either HU210, THC, or Met-F-AEA was able to specifically reduce the viability of CB1-positive aRMS cells (Rh4) compared to CB1-negative eRMS cells (RD) and non-cancer control cells (MRC-5). The viability reducing effect of HU210 is mediated by the activation of CB1, as addition of a CB1 specific antagonist (AM251) was able to reverse this effect. Caspase-3 assays and PARP-cleavage analysis indicated that the reduced viability upon cannabinoid treatment is caused by apoptosis. Upstream of the apoptotic cascade, treatment of aRMS cells with HU210, THC, or Met-F-AEA leads to a fast dephosphorylation of AKT, which is followed by an upregulation of the pro-apoptotic protein p8 (candidate of metastasis). Further characterization of signalling components involved in this apoptotic effect will help to elucidate the antitumoral effect of cannabinoids in aRMS. Additionally, xenograft experiments are ongoing to validate the results obtained in vitro. Hence, we conclude that cannabinoid receptor agonists might have a therapeutic potential in aRMS and therefore should be considered as effective agents for the future treatment of aRMS.

AEA INDUCES TUMOR CELL DEATH IN A COX-2 DEPENDENT MANNER

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Non-melanoma skin cancer is the most prevalent cancer in the United States with greater than 1 million new cases identified each year. These tumors and other epithelial tumors (eg., breast, colon and lung) overexpress cyclooxygenase-2 (Cox-2). Cyclooxygenases are enzymes that utilize arachidonic acid as substrate to synthesize prostaglandins and prostaglandins are involved in tumor promotion and carcinogenesis. Several investigators have recently determined that endocannabinoids are also substrates for Cox-2 producing prostaglandin ethanolamide - derivatives. The goal of this research is to determine if endocannabinoids can be developed as novel agents to prevent skin cancer. In this study, the JWF2 squamous cell carcinoma cell line was exposed to anandamide (AEA) in increasing concentrations and a dose-dependent induction in cell death was found. To determine the mechanism of AEA-induced cell death we measured cleavage of the well-known apoptosis marker, PARP and found that apoptosis was also induced in a dose-dependent manner. Because AEA is a substrate for Cox-2 we measured the production of E-, F- and D-type prostaglandins that occurred in cells treated with AEA and found an increase in each of the prostaglandins. To clarify the contribution of each prostaglandin to AEA-induced cell death we treated cells with commercial prostaglandin preparations. Both D-series and F-series prostaglandins induced cell death in JWF2 keratinocytes. Next, we investigated the role of Cox-2 in AEA-induced D-type prostaglandin production by exposing cells to a selective Cox-2 inhibitor and found that the production of D-type prostaglandins required Cox-2 activity. Finally, we measured the induction of apoptosis in a non-tumorigenic keratinocyte cell line that expresses low basal levels of Cox-2. Cell treatment with AEA up to a final concentration of 120uM was unable to produce cell death in these cells. These data suggest that AEA selectively induces cell death in squamous cell carcinoma cells due to the overexpression of Cox-2 and the resulting conversion of AEA to cytotoxic prostaglandins.

OPIATE SPARING EFFECTS OF CANNABINOID IN CRPS PATIENTS

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INTRODUCTION: To demonstrate the efficacy and opiate sparing effect of cannabinoid in the management of severe CRPS patients requiring high dose opiates.

METHODS: We conducted retrospective analysis of five CRPS patients requiring high dose opiate whom received cannabinoid resulting in significant reduction of pain intensity and opiates.

RESULTS: Pt, #1- 47yo.female with CRPS II following L5S1 discectomy. Unable to weight bear on her right leg, with severe allodynia. Medication, June/2004: Oxycodone 30mg, Topamax 200mg, Zyprexa 7.5mg, Amitriptyline 75mg, Neurontin 400mg. Pain level@ 10/10. January 2005 started on Nabilone 10mg/day and Topamax 200mg/day. July 2007, Amitriptyline 25mg/day opiates and Neurontin discontinued. Able to weight bear fully and function at home. Pain level 6/10.

Pt, #2 – 41yo.female with CRPS II following left arm brachial plexus injury. She has neuropathic pain 10/10, constipation, nausea, and vomiting. Medication:Fentanyl patch 125mg 72 hour, Gabapentin 200mg, Wellbutrin 200mg and Trimipramine 150mg. October 2006 started on Nabilone 10mg/day. July 2007: Gabapentin and Fentanyl patch discontinued. Pain level 6/10, no constipation or nausea.

Pt, #3 – 21yo. female with CRPSI following right knee arthroscopy. Unable to weight bear on right leg, severe swelling to her right calf. Pain level 10/10. Medication: Morphine 240mg/day. December 2006 started on Nabilone 6mg/day. June 2007, able to weight bear fully with reduced swelling. Pain level 4/10. Walking daily. Morphine 160mg/day. Enrolled in a hair dressing course.

Pt, #4 – 49yo.female with CRPS I in all extremities following MVA, June 2000. Burning pain and cold sensation throughout the whole body, with allodynia. Thyroid function normal. Pain level 10/10. Medications: Codeine 240mg, Topamax 100mg, Prozac 20mg, and Olanzapine 7.5mg. July 2005 started Nabilone 1mg. October 2005 pain level 4/10. Codeine discontinued. She felt warm and allodynia was reduced.

Pt, #5 – 40yo.female with CRPS II post soft tissue injury to her right arm October 2003. She had swelling, severe burning pain, allodynia and autonomic changes of the right arm and hand. Pain level 10/10. Medications: Morphine 90mg, Effexor 150mg, Trileptal 1500mg/day. September 2007placed on Nabilone 3mg/day. December 2007, off Morphine, sleeping well, working part time. February 2008 working full time. Pain level 4/10.

CONCLUSIONS: Cannabinoid seems efficacious in the management of severe CRPS patients, resulted in significant reduction of pain intensity (40%-60%), near complete opiate elimination and functional improvement.

NABILONE FOR FIBROMYAGLIA: ASSESSING SLEEP AND PAIN BENEFITS

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Introduction

Fibromyalgia (FM) is characterized by: widespread pain, fatigue, depression, migraine, IBS and disturbed sleep, often with excessive alpha rhythm intrusion. Treatments vary from antidepressants, opiates, anticonvulsants, cannabinoids. Central sensitization may contribute to the pain augmentation of FM. Nabilone (*Cesamet*), oral synthetic analogue of TetraHydroCannabinol has previous reports of: synergistic effects with endogenous pain inhibitory mechanisms (Redmond, *et al*, 2007); improved pain and QOL parameters in a controlled study of 40 FM patients (Skrabek, Galimova, 2007); modest pain and sleep benefits in a small controlled study of 11 chronic pain patients (Chung, *et al,* 2007).

Method

96 FM patients attended this single FM treatment clinic. Most patients had moderate to severe FM, as reflected by high pain Numerical Rating Scale (NRS) levels (10 being the most severe pain, 0 = nil pain), plus above average scoring on Fibromyalgia Impact Questionnaires (FIQ), with 70 –100 scores reflecting severe functional hardships. First follow up was 4-6 weeks from baseline in an uncontrolled observational, "real world" trial, with no interruption of any regularly used medications.

Results (mean scores)

Subjective Findings	N = total 96	Baseline: NRS	FIQ	Follow Up: NRS	FIQ
good pain reduction	5	8.2	82.5	7.3	64.6
fair pain reduction	15	7.3	77.6	6.9	71.1
sleep improvement only	25	5.2	8.3	5.1	6.6
only FIQ #16 results for sleep group: sleep; 0 = awoke well rested, 10 = awoke very tired					
discontinued re: side-effects	43				
discontinued re: no benefits	8				

Most common side effects resulting in discontinuation: dizziness, somnolence, feeling "altered". Initiating a slower titration using ½ the lowest available dose, could possibly ameliorate some intolerable side-effects, which contributed to the 44.5% patient drop out.

Discussion

Sleep disturbance causes substantial distress to FM patients. This observational trail of Nabilone for treating moderate to severe FM showed initial sleep improvement in 26% of the sample. The sleep cohort's NRS pain scores did not change from baseline, plus reflected more moderate FM pain when compared to the pain reduction cohort. This could be a factor in the sleep vs. pain benefits of Nabilone treatment for FM patients. The 5 patients reporting good pain relief scored a 22% reduction in FIQ. They were also very grateful (to the investigator) for the improvement in function. Dosing adjustments and longer follow-up may also impact on pain and sleep results.

SATIVEX (THC:CBD) TO TREAT FIBROMYALGIA PAIN: COMPARING AUTUMN & SPRINGTIME TRIALS

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Introduction

The Fibromyalgia (FM) Syndrome is an enigma, defined by: widespread pain, fatigue, sleep and mood disorders. Pain reduction treatments are limited. Recently revealed is the similarity of the endocannabinoid anandamide to TetraHydroCannabinol (THC) from inhaled plus some pharmaceutical preparations. The novel buccal spray of *Sativex* (THC/Cannabidiol) has Canadian approval for severe Multiple Sclerosis and cancer pain and shows some amelioration of FM pain in two matched cohorts during uncontrolled, observational trials, perhaps slightly better during Springtime vs. Autumn.

Method

67 FM patients began *Sativex* – Fall/06, 53 began Spring/07, with results cutoff dates in early winter & early summer, to isolate seasonal variation. Assessments: pain via VAS (Visual Analogue Scale); pain, everyday function, sleep and mood via FIQ (Fibromyalgia Impact Questionnaire). Patients continued all other medications, without dose alterations.

Results

Cooler Weather Cohort (CWC): 32/67 patients (47.8 %) reported pain reduction from baseline to Week-4. Follow up to Week-8, N = 22/32. 17/22 reporting pain reduction.
Warmer weather cohort (WWC): 30/53 patients (56.6%) reported similar positive benefits from baseline to Week-4. Follow up toWeek-8, N = 17/30. 15/17 pain reduction.

	FIQ SCORES		**VAS SCORES**	
	Cool Cohort	**Warm Cohort**	**Cool Cohort**	**Warm Cohort**
Baseline mean:	74.8 (100/33.1)	74.7 (98.2/30.4)	6.9	7.3
Week-4 mean:	58.9 (88.3/28.5)	59.1 (91.2/8.6)	5.8	5.8
Week-8 mean:	58.3 (81.6/28.2)	52.1 (77.9/9.0)	5.5	5.6

Discontinuing *Sativex*: **CWC**: 21 due to side-effects (S-E): drowsiness, dizziness, altered feeling, oral lesions; Insufficient Benefits (I-B) = 14. **WWC**: S-E = 15; I-B = 8.

Discussion

Sativex for FM: two cohorts, each with similar symptomatology and adequate patient enrollment, both yielding very similar initial benefits, especially for function. Drop-outs for both cohorts were still high around 50%. Longer follow-up and controlled design should provide long-term accuracy and reliability results. Warmer temperatures and longer daylight hours may answer: 1) the 15.5% pain benefit edge in # of patients in spring vs. autumn; 2) a trend to steadily improving FIQ and VAS scores found only in the WWC. Perhaps seasons of the year need to be considered in future FM trial designs?

FOLLOW-UP SURVEY OF PATIENTS PRESCRIBED CANNABIS BASED MEDICINAL EXTRACT (CBME) FOR CHRONIC PAIN AND SPASTICITY – PATTERNS OF USE

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Introduction

Patients at our hospital have participated in clinical trials of pharmaceutical grade oromucosal CBME (Sativex - Δ^9-Tetrahydrocannabinol 2.7mg+Cannabidiol 2.5mg/0.1ml spray actuation) since May 2000. Many have continued to use CBME on a named-patient-basis beyond or outside of formal clinical trials. This survey aimed to determine the patterns of drug usage that had emerged during long term use.

Methods

A postal questionnaire-based survey of 72 subjects who had used CBME for between 7years and 6 months was conducted. Information on the frequency of use, the route of administration, dosage, dosage variability, use of other medicines and the main ongoing benefits was sought.

Results

55 (76%) patients responded and 48 agreed to participate. 33 had Multiple Sclerosis(MS), 2 had diabetic neuropathy and 13 had chronic pain due to other causes (neuropathy, nerve injury or trauma). The mean total number of sprays per day was 8.3 ± 4.2 (mean ± sd). Only 6 subjects were taking more than 12 sprays per day and the maximum was 18. There was no relationship between the number of sprays/day and duration of use of CBME.12 subjects take their CBME only once/day, 8 at night; 30 patients between 2-4x/day; 7 patients used it >4x/day. MS patients tended to take doses at each end of the day (6-10 am and 8-10 pm).The main sites for spraying CBME was sublingual (81%), buccal (17%), swallowed with food (2%). 10% admitted using multiple routes.

CBME has an unpleasant taste and can cause stinging. Of 42 subjects, 15 swallowed CBME in <1 minute after application, 17 subjects from 1-5 minutes and 10 for > 5 minutes. . There was no relationship to total daily dose.

Of 50 patients, 25 had kept their dose the same, 10 had increased (9 because of worsening symptoms) and 13 had reduced their dose. Pain, insomnia and spasticity were the main symptoms alleviated. 36 subjects reported more than one main symptom that is improved by CBME use. Other symptoms alleviated were bladder and bowel control, stress and anxiety, cramps, tingling and spasm.

Conclusions

Patients have been encouraged to optimise their use of CBME (dose, time etc.) and to adjust their other medications accordingly. Patients have done this effectively without intensive physician supervision. Ongoing benefits are commonly multiple in patients with MS and Chronic Pain.

FOLLOW-UP SURVEY OF PATIENTS PRESCRIBED CANNABIS BASED MEDICINAL EXTRACT (CBME) FOR CHRONIC PAIN AND SPASTICITY – CHANGES IN CONCOMITANT MEDICATION

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Introduction

Patients at our hospital have participated in clinical trials of Sativex oromucosal spray (Δ^9-Tetrahydrocannabinol 2.7mg + Cannabidiol 2.5mg/spray actuation) since 2000. Many have continued to use the drug on a named-patient basis beyond or outside of formal clinical trials. This survey aimed to determine the changes in concomitant medication use by patients occurring during long term use of Sativex.

Methods

A postal questionnaire-based survey of 72 subjects who had used CBME for between 7years and 6 months (mean 3.4 years) was conducted. Information was sought regarding changes to all concomitant medications since initiation of treatment with Sativex, along with information as to why these changes had occurred.

Results

55 (76%) responded and 49 agreed to participate. Of these participants, 33 had multiple sclerosis (MS), 6 had chronic back pain, 2 had diabetic neuropathy and 8 had chronic pain due to other causes (e.g. OA, nerve injury or trauma). Participants were aged 30-76 years (mean 55.7). Overall 14 patients had remained on the same num ber of medications, 20 had increased and 15 had decreased overall. Of analgesic medications 13 patients stopped taking one or more and 5 patients identified Sativex as being better. One patient stopped taking 5 different opioids. 6 patients reported a reduction in dose of analgesic medications taken, 4 stating that they no longer needed such a high dose. 13 started new analgesics and 2 patients had their dose increased. 5 patients with MS (mean Sativex use=2.9 years) stopped anti-spasmodic medications, 2 reduced their dose but 5 (mean Sativex use=5.5 years) commenced treatment with this class of drug. One patient stopped anti-convulsive medication due to Sativex, 3 reduced their dose but 7 were started on this class of drug. 2 patients stopped their Tricyclic Anti-depressants, 3 reduced their dose and 2 commenced treatment. Analyses of changes in other types of medications (benzodiazepines, bladder drugs, NSAIDs, other antidepressants) were also undertaken.

Conclusions

Almost all patients using Sativex have been through all conventional options for symptom control. For many their disease remained progressive requiring combinations of medication. In this heterogeneous group no specific patterns emerged. Patients optimised their medication and some clearly found more benefit in the long term use of Sativex than from other types of medication.

COMPREHENSIVE ADVERSE EVENT PROFILE OF SATIVEX

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Introduction: Sativex® is an oromucosal cannabis-based spray combining a CB_1 partial agonist (THC) with a cannabinoid system modulator (CBD), minor cannabinoids and terpenoids plus ethanol and propylene glycol excipients and peppermint flavoring. It was approved with conditions by Health Canada in April 2005 for prescription for symptomatic relief of neuropathic pain in multiple sclerosis, and in August 2007, for treatment of cancer pain unresponsive to optimized opioid therapy. Sativex is a highly standardized pharmaceutical product derived from two *Cannabis sativa* chemovars combining Tetranabinex (THC extract) and Nabidiolex (CBD extract) in a 1:1 ratio. Each 100 µL pump-action oromucosal Sativex spray actuation provides 2.7 mg of THC and 2.5 mg of CBD. Sativex effects commence in 15-40 minutes, an interval that permits symptomatic dose titration. Current controversy attends the risks and benefits of cannabinoid medicines, prompting this examination of adverse event profiles for Sativex employed adjunctively in randomized clinical trials (RCT) in intractable pain and multiple sclerosis (MS) symptoms, and corresponding safety-extension (SAFEX) studies.

Methods: Developmental Core Safety Information from 11 Phase III RCTs of Sativex (N=921) vs. placebo (N=853) and 2 SAFEX studies in 662 subjects taking Sativex for one year or more were examined, representing approximately 880 patient-years of experience. AEs are presented as the percentage classified as having a plausible causal relationship to medication.

Results: Patients most often attained individualized stable dosage within 7-10 days that provided therapeutic relief without undue psychotropic effects (mean 9.4 Sativex sprays per day). Dizziness was the most common AE in RCT (32.1% Sativex/9% placebo) and SAFEX studies (27.6%), but was an early and mostly a transient complaint rarely leading to discontinuation. Other CNS complaints all occurred with less than 10% incidence. In the GI area, the most common complaints were nausea (11.7% Sativex/5.7% placebo, 12.8% SAFEX), dry mouth (8% Sativex/2.6% placebo, 8.3% SAFEX), oral pain (3.4% Sativex/3% placebo, 7.7% SAFEX) and diarrhea (3.3% Sativex/1.3% placebo, 11% SAFEX). In the general category, the only complaint exceeding 10% incidence was fatigue (12.1% Sativex/6.3% placebo, 10% SAFEX). All psychiatric AE affected 5% or less, as did other AE such as vertigo, blurred vision, falls, etc. Application site AEs included dysgeusia (5.2% Sativex/1.5% placebo, 8% SAFEX) and oral pain (3.4% Sativex/3% placebo, 7.7% SAFEX). Abrupt cessation of Sativex administration was associated with recrudescence of disease complaints without consistent withdrawal-type symptoms.

Conclusion: Extensive patient experience with Sativex supports that it has an acute and long-term safety profile well within acceptable standards for prescription medicines for similar indications.

ENCAPSULATION OF CANNABIDIOL INTO POLY-ε-CAPROLACTONE MICROSPHERES AND NANOPARTICLES

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Introduction

Cannabinoids are chemical compounds practically insoluble in water with high octanol/water partition coefficients and pKa. They are also photolabile molecules susceptible to oxidation, but stable to heat (up to 20ºC) in the absence of air. Their low aqueous solubility makes necessary the use of non-aqueous solvents or dispersing agents, and also their bioavailability is low and erratic. Moreover, at low concentrations they tend to adsorb to plastic and glass, making their handling during experimental work a difficult task. Their encapsulation in multiparticulate systems such as microspheres and nanoparticles might overcome some of the drawbacks mentioned above.

Materials and Methods

Nanoparticles and microspheres were prepared by the nanoprecipitation method and the emulsion-solvent evaporation method, respectively.

Cannabidiol (CBD) was chosen as model drug because its structure, molecular weight and partition coefficient are similar to Δ^9-tetrahidrocannabinol´s. Furthermore, as CBD has no psychoactive properties its use in research is not under control, which facilitates its importation.

Poly-ε-caprolactone (PCL), a biodegradable, biocompatible, FDA-approved aliphatic polyester that degrades slowly was chosen to elaborate these delivery systems. The advantages of PCL include its high permeability to small drug molecules and its failure to generate an acidic environment during degradation as compared to polylactides and glycolides.

During manufacturing process CBD was dissolved into PCL. This originated matrix systems in which the drug was molecularly dispersed into the polymer. Cannabinoid disposition into these polymeric systems determines drug release rate.

Results

Nano- and microparticles showed high entrapment efficiency (≥95%), small and reproducible sizes, around 85 nm and 23 µm, respectively. Scanning electron microscopy showed that spherical, non-aggregated, non-porous and smooth-surfaced microparticles were obtained. DSC studies confirmed that CBD was effectively dissolved into the polymer matrix.

Conclusion

CBD is easily incorporated into PCL nano- and microparticles. These formulations are compatible with different administration routes and might represent an alternative to improve the drawbacks associated to the use of free drug.

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FORMULATION AND CHARACTERIZATION OF BIODEGRADABLE POLY-ε-CAPROLACTONE MICROPARTICLES FOR CANNABIDIOL ADMINISTRATION

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Introduction
The low aqueous solubility and sticky tarlike nature of most cannabinoids combined with their slow onset of action and significant first pass metabolism upon oral administration pose significant challenges in developing these compounds for medicinal use. Therefore, the aim of the present work was to develop and characterize a new biodegradable delivery system for cannabinoid administration. Cannabidiol (CBD) was chosen as model drug to assess the project´s viability not only due to its similar structure to THC and its easy importation, but also because it has therapeutic potential (e.g. anti-inflammatory and neuroprotective properties).

Materials and Methods
CBD-loaded poly-ε-caprolactone (PCL) microparticles were prepared using the emulsion-solvent evaporation method. The microparticles were characterized in terms of morphology, particle size distribution, drug loading and encapsulation efficiency, and *in vitro* drug release. Finally, CBD blood and brain levels were monitored after a single subcutaneous injection of a suspension of the microspheres to ICR mice.

Results
The formulation yielded a powdered product that contained a known amount of CBD and was easily handled. Scanning electron microscopy showed that the microparticles were spherical in shape. The size of the microparticles was 22.77±11.83 µm. The highest drug load obtained was 16 mg CBD/100 mg microparticles. Increasing the concentration of CBD further caused the microparticles to form aggregates. The encapsulation efficiency was above 96% in all cases. *In vitro* release studies revealed that the encapsulated drug was released over 10 days. *In vivo* pharmacokinetic studies showed that drug levels were still detected in blood and brain five days after a subcutaneous infusion of a single dose of the CBD microparticles. In contrast, no detectable drug was found in either blood or brain 48 h after a subcutaneous injection of a high dose of CBD (50 mg/kg) in a vehicle consisting of emulphor, ethanol, and saline in a ratio of 1:1:18 ($t_{1/2\beta\ blood}$=13.35h; $t_{1/2\ brain}$=7.11h).

Conclusion
The results of the present study suggest that the use of microencapsulation is a simple and useful method to overcome the low aqueous solubility of cannabinoids, facilitating their handling and dosing. Moreover, the observation that stable drug levels were detected in blood and brain for five days suggests that this approach possess utility for the long-term administration of cannabinoids to treat chronic conditions.

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PK/PD MODELING OF HUMAN CANNABINOID (CB)-SYSTEMS USING A THC CHALLENGE-TEST

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CB agonists and antagonists are in development for various indications, while little is known about the (patho)physiology of human CB systems. Studies of the effects of CB agonists in humans can provide valuable information about the role of these systems in health and disease. This study aimed to develop pharmacokinetic/ pharmacodynamic (PK/PD) models for the effects of the CB-agonist THC.

Twelve healthy male occasional cannabis users participated in this double blind, placebo-controlled, cross-over study. Rising doses of purified THC (2, 4, 6 and 8 mg) or placebo were inhaled at 90-minute intervals. Frequent plasma THC samples were obtained. PD measurements were: item ‘alertness’ from Bond&Lader visual analogue scales (VAS), item ‘feeling high’ and composite factors ‘internal’ and ‘external perception’ from Bowdle VAS, body sway, and heart rate. PK and PK/PD analyses were performed using nonlinear mixed effect modeling (NONMEM).

A two-compartment model best described the PK of THC. Relative bioavailability fractions were implemented for each dose within a subject, allowing the estimation of intra-individual variability in absorption. A four-compartment model simultaneously described the PK of THC and two major metabolites. Both models revealed Michaelis-Menten elimination for THC. PK/PD-relationships for ‘feeling high’, ‘external perception’, body sway and heart rate were best described by an Emax model, whereas ‘alertness’ could be described by a linear model. ‘Internal perception’ showed maximal effects above 2 mg. THC effects lagged behind plasma concentrations, revealing hysteresis which indicated a slow equilibrium between blood and effect compartment. Equilibration half-lives varied from 7.68 min for heart rate to 39.2-84.8 min for different CNS parameters.

In conclusion, different THC effects could be described adequately but with inherently different PK/PD models. Some effects were maximal at low THC levels (‘internal perception’) while others did not attain maximal effects (‘alertness’). Different equilibration half-lives for heart rate and CNS effects also suggested different CB-responsive systems. This study shows that PK/PD models can be used to describe and quantify different CB-responsive physiological systems. The PK/PD-properties of a THC-challenge provide a valuable research tool to investigate the characteristics of CB systems in health and disease, and to develop new drugs that act on these systems.

CANNABIS USE IN OLDER ADULTS

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Introduction

The rates of occasional use of cannabis are much higher in young people compared to adults largely due to the fact that the majority of young people are experimenters. They may try it for a few times but eventually stop. However, results from a recent Ontario adult population survey, show a significant increase in cannabis use among adults aged 30 years and older. The purpose of the present study is to present trends in cannabis use among Ontario adults from 1977 to 2005 and to examine the characteristics of aging adult cannabis users.

Methods

Data on self-reported cannabis use were obtained from 20 repeated cross-sectional surveys conducted with Ontarians aged 18 and older, by the Addiction Research Foundation from 1977 through 1998 and by the Centre for Addiction and Mental Health from 1999 through 2005. The five surveys conducted between 1977 and 1989 were based on face-to-face interviews, and the 15 surveys conducted between 1991 and 2005 were based on Computer Assisted Telephone Interviewing, using random digit dialing methods, and two-stage probability selection. The sample sizes varied between 1026 and 2776 and response rates between 58% and 69%.

Results

Overall, 42.2% of Ontario adults reported lifetime use in 2005, and 14.4% reported past 12 month cannabis use. This current rate is significantly higher than the estimate reported in 1977 (8.1%). Increases were noted among men (from 11.2% to 18.8%); women (from 4.5% to 10.3%) as well as among all age groups, except those aged 50 and older. The most significant finding was the aging of cannabis users. In 1977, 81.8% of cannabis users were aged 18-29 years of age compared to 53.7% in 2005. Also, the proportion of past year cannabis users aged 30 to 49 years of age increased from 15.4% to 39.9% in 2005. Further demographic characteristics and frequency of use among older adult cannabis users in 2005 will also be reported.

Conclusions

A significant proportion of adults appear to be continuing their cannabis use as they age. Because of the nature of the questions it was not possible to determine the motivation for continued use. Future studies should address this. This may also signal an emerging public health problem, because chronic use of cannabis has been associated with a number of chronic diseases. Physicians should ask patients of all ages about their cannabis use.

NEW CANNABINOIDS FROM HIGH POTENCY *CANNABIS SATIVA*

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The family Cannabaceae is currently recognized as containing only one genus, namely *Cannabis*, with only one highly variable species: *Cannabis sativa* L. The chemotypes of *C. sativa* L. can be divided into drug type (marijuana) with high Δ^9-THC content, reaching over 20% and low CBD content, intermediate type with variable amounts of both Δ^9-THC and CBD, and fiber type (hemp) with very low Δ^9-THC (<0.3%) and higher CBD content.

The chemistry of cannabis is very diverse with compounds from a wide range of chemical classes, e.g. mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids. The best-known and most specific class is the C_{21} terpenophenolics, the cannabinoids, with (-)-Δ^9-*trans*-(6a*R*,10a*R*)-tetrahydrocannabinol (Δ^9-THC) being the most abundant psychologically active constituent.

The medicinal properties of cannabis have been much debated from scientific and political points of view and the subject has lost and gained interest over the years. After the discovery of the primary active constituent in marijuana (Δ^9-THC) in 1964, various clinical trials were undertaken that let to its approval as antiemetic for cancer patients receiving chemotherapy and as an appetite stimulant for AIDS patients with wasting syndrome. The discovery of the endocannabinoid system in 1988, including the CB1 and CB2 receptors, added to the much renewed interest in the cannabinoids.

The availability of high potency marijuana on the illicit market with unprecedented Δ^9-THC concentrations (>20% by dry weight) prompted us to investigate the phytochemistry of these varieties. We herein report the isolation and structure elucidation (using various spectroscopic techniques) of thirty three new cannabinoids from a high potency cannabis variety. These compounds belong to the following subclasses: Δ^9-THC (12), Δ^8-THC (3), CBG (6), CBC (2), CBN (2), cannabichromanone (4) and miscellaneous (4). The *in-vitro* antimicrobial, antiprotozoal, antioxidant and CB_1 receptor binding activities of the isolates have been evaluated.

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VARIATION IN Δ^9-THC AND OTHER CANNABINOIDS CONTENT IN FIELD GROWN *CANNABIS SATIVA* L. DURING DIFFERENT STAGES OF GROWTH

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The *Cannabis* plant has acquired much interest over the last few years, not only because of the problems associated with its abuse, but also because of the therapeutic potential of many of its components. The chemistry of the plant is very complicated but the focus has been on its cannabinoids content. At the National Center for Natural Products Research (NCNPR) at the University of Mississippi, *Cannabis* is being cultivated for research purposes under contract with the National Institute on Drug Abuse (NIDA). Materials with different cannabinoids content are required for research purposes. Understanding the seasonal variation in the cannabinoids content is important in selecting the appropriate time of harvest of such materials.

In this presentation, plants of *Cannabis sativa* L. were grown from seeds in the medicinal plant garden at the University of Mississippi, USA. Each seedling was tagged with a unique barcode to construct an accurate inventory of plants and to ensure the identity of each plant for further research. At the onset of flowering, all the male plants were removed from the field to avoid cross-pollinations and only female plants were kept for further cultivation. Among these plants, randomly selected few healthy female plants from different plots were periodically analyzed for their cannabinoids content (Δ^9-THC, THCV, CBD, CBC, CBG and CBN) through the different stages of growth and development (from seedling to harvest) using GC/FID. Based on variety differences and chromatographic analysis, plants were selected from four different groups i.e. plants having very high THC (VH, >12%), high THC (H, ~8-12%), intermediate THC (INT, ~5-8%) and low THC (L, <5%).

In general, THC content increased with plant age up to a highest level during budding stage where the THC content reached a plateau for about a week before the plants were harvested. The changes in the concentration of other cannabinoids followed a similar pattern in some cases but show more variability depending on the variety. The results of this work were critical in selecting specific cultivars for future propagation depending on the chemical profile of interest.

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SOME PHARMACOLOGICAL EFFECTS OF FIVE LITTLE-INVESTIGATED PLANT CANNABINOIDS

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This investigation was performed with the plant cannabinoids cannabigerol (CBG), cannabigerolic acid (CBGA), cannabichromene (CBCh), Δ^{9}-tetrahydrocannabivarin acid (THCVA) and the propyl homologue of cannabidiol, cannabidivarin (CBDV), each of which had been extracted from cannabis and then purified. It was directed at establishing whether at 10 μM or less any of these compounds displace [^{3}H]CP55940 from specific binding sites on brain membranes, stimulate or inhibit [^{35}S]GTPγS binding to such membranes, and/or behave as cannabinoid receptor antagonists as measured by an ability to oppose CP55940-induced stimulation of [^{35}S]GTPγS binding to brain membranes.

Our experiments were carried out with adult male MF1 mouse whole brain membranes using [^{3}H]CP55940 and [^{35}S]GTPγS binding assays as described by Thomas *et al.* (2007). CBG, CBGA, CBCh, THCVA and CBDV were obtained from GW Pharmaceuticals and were all dissolved in DMSO.

CBG, CBCh, CBDV and THCVA each inhibited the specific binding of [^{3}H]CP55940 in a concentration-related manner with K_i values of 440, 1175, 4398 and 10190 nM respectively. In contrast, CBGA, even at its apparent maximal concentration (ca 10 μM), produced only partial (22%) displacement of [^{3}H]CP55940. At or above 100 nM, CBG, CBGA and CBDV significantly inhibited [^{35}S]GTPγS binding to brain membranes. At 10 μM, CBG, CBDV and CBCh all induced significant parallel dextral shifts in the log concentration-response curve of CP55940, the apparent K_B values of these compounds being 48, 150 and 152 nM respectively. For CBG and CBDV these values rise to 736 and 1125 nM respectively when their inhibitory effect on [^{35}S]GTPγS binding is taken into account as described previously for cannabidiol (Thomas *et al.*, 2007). That this inhibitory effect alone accounts for the ability of CBG and CBDV to antagonize CP55940 is unlikely as CBGA did not antagonize CP55940 at 10 μM but did inhibit [^{35}S]GTPγS binding. THCVA (10 μM) also failed to antagonize CP55940 in this assay. In contrast to the other plant cannabinoids we investigated, CBG produced a concentration-dependent stimulation of [^{35}S]GTPγS binding when administered at concentrations well below the concentration (10 μM) at which it inhibited [^{35}S]GTPγS binding. Its EC_{50} for this agonism is 0.2 nM and its E_{max} 16%, well below that of CP55940 (79%). Further experiments are now underway to identify the mechanisms underlying these effects of CBG, CBGA, CBCh and CBDV on [^{35}S]GTPγS binding.

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EXPOSURE OF CULTURED HIPPOCAMPAL NEURONES TO CANNABIDIOL DOES NOT INDUCE CELL DEATH BUT INHIBITS SYNAPTIC ACTIVITY

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Cannabidiol (CBD) is a putatively non-psychoactive component of cannabis which has been of recent interest due to its possible therapeutic potential in a number of disorders, including cancer, diabetes and schizophrenia. It is currently used in combination with another component of cannabis, **Δ^9-**tetrahydrocannabinol (THC), for the treatment of the painful symptoms of multiple sclerosis. Previously, CBD administration has been shown to result in an increase in intracellular calcium concentration in cultured hippocampal neurones (Drysdale, *et al*, *Neuropharmacology* (2006) **50**:621-31), however the effect of CBD on synaptic transmission and cell death is as yet unknown.

Whole cell patch clamp experiments were performed in the current clamp mode on cultured hippocampal neurones (12 – 16 days in vitro) using WinEDR (J. Dempster, University of Strathclyde, UK). All drugs were applied via the perfusate with changes in firing frequency being analysed using WinEDR and expressed as mean ± S.E.M. To validate the neurotoxicity of CBD, cultured hippocampal neurones (6-8 days in vitro) were treated with drugs and then incubated with propidium iodide (PI) overnight. Cells were imaged and fluorescence measured using ImageJ (NIH,). All data is expressed as mean ± S.E.M. using glutamate (1mM) as a standard.

Application of CBD (0.1µM) was without effect however application of both 1 and 10µM significantly decreased firing frequency by 66 ± 17% ($P<0.05$, n=4) and 91 ± 5 % ($P<0.001$, n=4) respectively. Application of WIN 55,212-2 (0.1µM) and THC (10µM), full and partial agonists respectively at CB1 receptors, both reduced firing frequencies in accordance with previous studies. Furthermore, exposure of cultured hippocampal neurones to CBD (0.1 -10µM, 60 mins) and THC (10µM, 60 mins) did not induce neurotoxicity.

The data presented here indicates that CBD inhibits neuronal firing to a similar extent to that previously shown for WIN 55,212-2 and THC. However, the mechanisms underlying this CBD-induced inhibition are still under investigation. In contrast to previous work on cortical cultures where THC was shown to be neurotoxic (Campbell, *Neuropharmacology* (2001) **40**:702-9), CBD and THC did not induce neuronal death. Studies are ongoing to evaluate potential neuroprotective effects of CBD in assays of apoptosis and necrosis. These data may have implications regarding the use of CBD for therapeutic use.

THE EFFECT OF Δ^9-TETRAHYDROCANNABINOL ON POST TRANSLATIONAL MODIFIERS OF THE PRO-APOPTOTIC TUMOUR SUPPRESSOR PROTEIN p53

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We have previously reported that the predominant psychoactive phytocannabinoid, Δ^9-Tetrahydrocannabinol (Δ^9-THC), induces apoptosis in cerebral cortical neurones *in vitro* and *in vivo*[1,2]. This apoptotic pathway involves the post translational activation of tumour suppressor protein, p53 and the release of pro-apoptotic protease, cathepsin-D from the lysosomes[1,3]. The biological activity of p53 can be modified by the post translational addition of certain proteins *e.g.,* Murine Double minute 2 (Mdm2) and Small Ubiquitin-like MOdifyer 1 (SUMO-1). p53 acts as a transcription factor for Mdm2, thus when activated p53 transcriptionally upregulates Mdm2 protein expression. Due to the ability of Mdm2 to inhibit p53 activity, a negative feedback loop is formed which provides a tight regulatory mechanism that controls the function of p53. The SUMO-1 protein is related to ubiquitin, however, conjugation with SUMO-1 does not target a protein for degradation, as is the case with conjugation with ubiquitin. The post translational modification of p53 with SUMO-1 has been reported to have an effect on the activity, stability and intracellular location of p53[4].

To investigate the effect of Δ^9-THC on the p53 interacting proteins, Mdm2 and SUMO-1 protein expression was assessed in cultured cerebral cortical neurones by western immunoblot and immunocytochemistry. The effect of Δ^9-THC on the level of SUMO-1-conjugated p53 was determined by co-immunoprecipitation and immunocytochemistry.

Δ^9-THC induced an increase in the expression of the p53-responsive protein, Mdm2, after 5 minutes exposure to Δ^9-THC, which coincided with the Δ^9-THC-induced increase in phosphorylated p53 (serine 15). Furthermore, Δ^9-THC induced the shorter Mdm2 protein (p76Mdm2) which does not contain a p53 binding site and therefore cannot inhibit p53. Δ^9-THC significantly increased the level of unconjugated SUMO-1 protein in the cytosol in a time-dependent manner with a maximal response observed after 15 minutes Δ^9-THC treatment. Δ^9-THC also induced the removal of SUMO-1 from p53 as shown by co-immunoprecipitation and immunocytochemistry. The CB_1 receptor antagonist, AM251, prevented the Δ^9-THC-induced deconjugation of p53 and SUMO-1.

In conclusion, the ability of Δ^9-THC to induce changes in Mdm2 provides additional evidence that the p53 pathway is pertinent in Δ^9-THC signalling in the brain. Furthermore, the observation that Δ^9-THC has an impact on the SUMO regulatory system is exciting considering the system has been implicated in the pathogenesis of several neurodegenerative diseases and is also pertinent in synaptic function[5].

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THE EFFECT OF Δ^9-TETRAHYDROCANNABINOL ON NEURAL CELL FATE IN THE ADOLESCENT RAT CEREBRAL CORTEX

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Cannabis is the most widely used drug of abuse in modern western society, and its use is prevalent amongst adolescents. *Cannabis sativa's* main psychoactive component, Δ^9-tetrahydrocannabinol (Δ^9-THC), can exert its effects by binding to the cannabinoid receptors, CB_1 and CB_2, with the CB_1 receptor being the predominant receptor found within the central nervous system. During adolescence the brain undergoes developmental changes and exposure to cannabis during this period has been linked with an increased risk of psychosis[1]. We have previously reported that in neonatal brain tissue, Δ^9- tetrahydrocannabinol causes cell death within the frontal cerebral cortex[2]. This cell death pathway involves the induction of caspase-3 activity and DNA fragmentation. However, such extensive cell death was not seen in adult tissue[2]. The effect of Δ^9-THC on the induction of apoptotic markers in the adolescent rat brain was examined in this study.

The current study focussed on the impact of Δ^9-THC on the viability of neurones following exposure of adolescent rats to a single subcutaneous injection of Δ^9-THC (1 mg/Kg). We report here that the acute administration of 1mg/kg Δ^9-THC induces cell death in adolescent rats (31 days old), similar to that previously observed in the neonatal rat brain[2]. Caspase-3, a marker of apoptotic cell death, is significantly increased in adolescent rats exposed to Δ^9-THC compared to control animals. DNA fragmentation and active caspase-3 was assessed by dTUNEL staining and immunocytochemistry.

In summary, the study demonstrates that the adolescent period of brain development in vulnerable to the apoptotic effects of Δ^9-THC and this pathway may be involved in the deleterious effect of cannabis exposure use during this critical phase of brain development.

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DIFFERENTIAL EFFECT OF TETRAHYDROCANNABINOL ON APOPTOTIC MARKERS IN THE NEONATAL AND ADULT RAT CEREBRAL CORTEX

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Cannabinoids have the proclivity to influence neural cell fate and possess both neuroprotective and neurotoxic properties. Λ^9-Tetrahydrocannabinol (THC) induces apoptosis in cultured cortical neurones isolated from neonatal rats via signaling involving p53, c-Jun N-Terminal kinase (JNK) and caspase-3 [1, 2]. In contrast to these findings in neonatal cortical cells, there is limited evidence suggesting that THC is toxic to adult neurones. This study was aimed at determining if post-natal development of the rat cortex influenced the ability of THC to activate components of the THC-induced apoptotic cascade in the rat cortex *in vivo*.

Neonatal (4 day-old) and adult (3-4 month-old) Wistar rats were anaesthetized by intraperitoneal injection of urethane (1.5g/kg). The THC group received subcutaneous injections of THC (1, 10 and 30mg/kg) in vehicle and the control group received injections of vehicle alone (5% absolute alcohol, 5% Cremophor EL and 90% saline). Three hours later rats were killed humanely. JNK expression was assessed by western immunoblotting and fluorescence microscopy. Caspase-3 activity was measured using a fluorogenic assay and Bax/phospho-Bcl-2 expression was assessed by western immunoblotting.

THC (10mg/kg) enhanced phospho-JNK2 expression in the neonatal, but not the adult, cortex, as determined by western immunoblotting ($p<0.01$, Student's t-test). Use of the fluorogenic assay revealed that caspase-3 activity was 6.7±0.9 nmole AFC/mg/min in vehicle-treated neonatal animals and this was increased to 10.5±1.2 and 12.4±1.0 nmole AFC/mg/min in neonatal rats exposed to THC at concentrations of 10mg/kg and 30mg/kg respectively ($p<0.05$ and $p<0.01$, ANOVA). This pattern was absent in adult rats. Unusually, western blot analysis revealed a THC (10mg/kg)-induced increase in pro-apoptotic phospho-Bcl ($p<0.05$, Student's t-test) and Bax expression in the adult, but not the neonatal, rat cortex.

These results suggest that the neurotoxic profile of THC varies between the neonatal and adult rat cerebral cortex and demonstrate the potential damaging effects of cannabis on the neonatal brain.

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ACTION MECHANISM OF Δ^9-TETRAHYDROCANNABINOL TO POTENTIATE BARBITURATE- OR BENZODIAZEPINE-INDUCED SLEEP IN MICE

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We previously reported synergistic effects of a major component of marijuana, Δ^9-tetrahydrocannabinol (THC), with sedative-hypnotics such as barbiturates and benzodiazepines[1)]. It is well known that CNS effects of THC, *i.e*., hypothermia, immobility, and catalepsy are mediated through CB_1 receptor, since the CNS effects of the cannabinoid are inhibited by pretreatment of CB_1 receptor antagonists, SR141716A, but not a CB_2 receptor antagonist, SR144528. We presented that prolonging effects of THC on pentobarbital- and/or diazepam-induced sleep in mice were inhibited by CB_1 receptor antagonists, but not CB_2 receptor antagonist, SR144528[2)]. Barbiturates and benzodiazepines acting sites are located on the $GABA_A$ receptor complex consisting of the chloride ionophore. Furthermore, CB_1 receptor is known to couple with G protein. In the present study, interaction of CB_1 receptor with $GABA_A$ receptor was examined.
THC (10 mg/kg, i.v.) significantly potentiated the diazepam (60 mg/kg, i.p.)-induced sleep as compare to the control sleeping time. The treatment of a benzodiazepine receptor antagonist, Ro15-1788, (60 mg/kg, i.v.) 10 min before the THC and diazepam challenge) did not cause inhibition of the THC-potentiated diazepam-induced sleep in mice, while SR141716A significantly attenuated THC synergism with diazepam. The synaptic membrane fraction from mouse brain was incubated with 0.7 nM of [^{3}H]flunitrazepam in 50 mM of Tris-citrate (pH 7.1) in the presence or absence of THC, SR141716A or SR144528 (1 nM-1uM). Although flunitrazepam inhibited specific [^{3}H]flunitrazepam binding to the synaptic membrane, THC and the both antagonists did not inhibit the specific binding. CP-55940, a CB_1 agonist, concentration-dependently reduced forskolin-induced cAMP level through human CB_1 receptor, while flunitrazepam and pentobarbital did not affect the cAMP level induced by forskolin. In conclusion, THC and the cannabinoid receptor agonists did not affect $GABA_A$ receptor binding site. Moreover, $GABA_A$ agonists/antagonists did not affect CB_1 receptor function. The results suggest that the possible interaction site of THC synergism with benzodiazepine and barbiturates might be a downstream from the CB_1 receptor, and different from $GABA_A$ receptor binding site.
Acknowledgements: SR141716A and SR144528 were gifted from Sanofi-Synthelabo Recherche, (Martpellier Cedex, France). Funded by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, and Technology of Japan 2005-2009.

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THE TOXICOLOGY OF THCV

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Introduction: The potential benefits of CB-1 receptor antagonism in the area of obesity and the metabolic syndrome is well established. However, at present, there remains doubt as to whether inverse agonism or neutral antagonism is preferable in this setting. It remains entirely possible that a small amount of a partial CB-1 agonist may in fact be beneficial in the presence of a CB-1 antagonist.

GW Pharma Ltd have bred proprietary strains of *Cannabis sativa L.* containing high levels of a neutral CB-1 antagonist, Δ^{9}-tetrahydrocannabinol (THCV), and product containing Botanical Drug Substances (BDSs) derived from such strains are now in clinical development.

Method: THCV BDS has undergone the necessary testing in order to allow the product to enter clinical studies in Europe. Genotoxicology studies in standard regulatory models of genotoxicity have been undertaken, alongside acute toxicity, teratogenicity and cardiotoxicity testing in newer models of toxicity (*Danio rerio:* zebrafish). In addition, repeat dose toxicology studies have been undertaken in rodents and dogs at doses of 2, 10 and 25mg/kg/day administered orally by gavage.

Results: THCV BDS showed no genotoxicity at clinically relevant doses in standard *in vitro* genotoxicity models (bacterial mutation assay or mammalian cell mutation assay) or standard *in vivo* models (rat micronucleus assay).

Previous toxicity testing in zebrafish revealed no acute toxicity, teratogenicity and cardiotoxicity at clinically relevant doses.

Repeat dose testing of THCV BDS in rodent and dogs revealed no significant toxicity, with toxicokinetic sampling indicating good tolerability despite high level exposure to THCV and other cannabinoids present in the THCV BDS. In either species no significant organ toxicity was apparent after 4 weeks treatment and no treatment-related macroscopic or microscopic findings were observed at any dose.

Conclusion: The potential toxicity of THCV BDS is low. At doses and exposures many times in excess of anticipated clinical doses, THCV BDS was well tolerated and induced few significant effects of toxicological importance. Hence THCV BDS has a sufficiently low toxicity profile to undergo repeat dose administration in humans.

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