24TH ANNUAL

SYMPOSIUM

OF THE

INTERNATIONAL CANNABINOID RESEARCH SOCIETY

BAVENO

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JUNE 28 - JULY 3, 2014

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PROGRAMME AND ABSTRACTS

Symposium Programming by Cortical Systematics LLC

International Cannabinoid Research Society
Research Triangle Park, NC
USA

ISBN: 978-0-9892885-1-4

These abstracts may be cited in the scientific literature as follows:

Author(s), Abstract Title (2014) 24th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Research Triangle Park, NC, USA, Page #.

Funding for this conference was made possible in part by grant 2R13DA016280 from the National Institute on Drug Abuse. The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention by trade names, commercial practices, or organizations imply endorsement by the U.S. Government.

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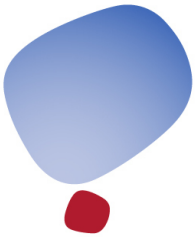

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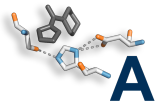

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Registration: June 28th, 2014 (16.00 – 18.00)

Welcome Reception: 18.30 – 20.00

Day 1
Sunday, June 29th

7.00	**Breakfast**		
8.15	Welcome and Opening Remarks		
Oral Session 1. Cannabinoids and Pathophysiology *Chairs*: Steve Alexander and Mauro Maccarrone			
8.30	Fabio Arturo Iannotti, Enrico Mazzarella, Ester Pagano, Elisabetta Gazzero, Raffaele Capasso and Vincenzo Di Marzo	STUDY OF THE EXPRESSION PROFILE AND PHARMACOLOGICAL ROLE OF THE CB1 RECEPTOR IN DUCHENNE MUSCULAR DYSTROPHY (DMD) MUSCLES: A NEW OPPORTUNITY TO REINFORCE MUSCLE REPAIR AND LOCOMOTOR ACTIVITY	1
8.45	Giulia Donvito and Barbara Costa	PALMITOYLETHANOLAMIDE IN A RAT MODEL OF OSTEOARTHRITIS: ITS ANTI-INFLAMMATORY AND ANTINOCICEPTIVE EFFECTIVENESS IN COMPARISON WITH NIMESULIDE AND ACETAMINOPHEN	2
9.00	Marie-Chantal Larose, Caroline Turcotte, Cyril Martin, Véronique Provost, Michel Laviolette and Nicolas Flamand	MECHANISMS OF HUMAN EOSINOPHIL MIGRATION INDUCED BY THE ENDOCANNABINOID 2-ARACHIDONOYL-GLYCEROL	3

9.15	Irina Bronova, Katalin Erdelyi, Alex Makriyannis, Pal Pacher and Evgeny Berdyshev	PERIPHERAL TARGETING OF CB1 CANNABINOID RECEPTORS PROTECTS FROM RADIATION-INDUCED PULMONARY FIBROSIS	4
9.30	Daniel J. Hermanson, Shu Xu, Naoko Brown, Jeffrey Reese, Sachin Patel and Lawrence J. Marnett	ENDOCANNABINOID AUGMENTATION BY SUBSTRATE-SELECTIVE COX-2 INHIBITORS: MECHANISM AND *IN VIVO* PROBE DEVELOPMENT	5
9.45	Laura Jimenez-Sanchez, Ruth Pazos, Hector Lafuente, Lorena Barata, Maria Ceprian, Martin Santos, Francisco Jose Alvarez and Jose Martinez-Orgado	ROLE OF 5HT1A RECEPTORS ON THE NEUROPROTECTIVE AND NEUROBEHAVIORAL EFFECTS OF CANNABIDIOL IN HYPOXIC-ISCHEMIC NEWBORN PIGS	6
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10.30	Shimon Ben-Shabat, Shiela Hauzner, Mor Cohen and Elie Beit-Yannai	PROTECTIVE ROLE OF THE CANNABINOID RECEPTOR SYSTEM IN AN *IN VITRO* MODEL OF AGE-RELATED MACULAR DEGENERATION (AMD)	7
10.45	Ariana Foinquinos, Katharina Schimmel, Jan Fiedler, Thomas Thum and Sandor Batkai	THE CANNABINOID-1 RECEPTOR IN CARDIAC FIBROSIS	8
11.00	Kevin Wilhelmsen, Samira Khakpour, Alphonso Tran and Judith Hellman	THE ENDOCANNABINOID N-ARACHIDONOYL DOPAMINE (NADA) AND WIN55,212-2 MODULATE THE INFLAMMATORY ACTIVATION OF HUMAN ENDOTHELIAL CELLS	9
11.15	Tamás Bíró, Attila Oláh, Dóra Bodnár, Lídia Ambrus, Attila G. Szöllősi, Nikolett Vasas, Judit Szabó-Papp, Ralf Paus, Michael Soeberdt and Christoph Abels	NOVEL INHIBITORS OF FATTY ACID AMIDE HYDROLASE EXERT REMARKABLE ANTI-INFLAMMATORY EFFECTS BOTH *IN VITRO* IN HUMAN KERATINOCYTES AND *IN VIVO* IN NC/TND MICE	10

11.30	Saja Baraghithy, Reem Smoum, Malka Attar-Namdar, Raphael Mechoulam and Itai Bab	STIMULATION OF BONE MASS BY A NOVEL METHYLATED OLEOYL SERINE DERIVATIVE	11
12.00	**LUNCH**		
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15.00	**PRESIDENTIAL PLENARY SPEAKER** *CANNABINOIDS REVISITED?* *NEW TARGETS, CHEMISTRY AND PLANT SOURCES* **GIOVANNI APPENDINO, PH.D.** Professor of Organic Chemistry, Università del Piemonte Orientale Department of Pharmaceutical Sciences, Novara, Italy		
16.00	**COFFEE BREAK**		
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16.30	Jenny Wilkerson, Sudeshna Ghosh, Ku-Lung Hsu, Benjamin F. Cravatt and Aron H. Lichtman	DIACYLGLYCEROL LIPASE BETA: NEW EVIDENCE FOR INFLAMMATORY AND NEUROPATHIC PAIN RELIEF IN MICE	12

16.45	Richard A. Slivicki, Liting Deng, Pushkar M. Kulkarni, Maria Cascio, Roger G. Pertwee, Ganesh A. Thakur and Andrea G. Hohmann	POSITIVE ALLOSTERIC MODULATION OF CB1 WITH GAT211 SUPPRESSES PACLITAXEL-INDUCED NEUROPATHIC PAIN WHILE BYPASSING UNWANTED SIDE EFFECTS OF CB1 RECEPTOR ACTIVATION	13
17.00	Miren-Josune Canduela, Juan-Luis Mendizabal-Zubiaga, Amanda Sierra, Naiara Royo, Sara Peñasco, Leire Reguero, Nagore Puente and Pedro Grandes	ANATOMY AND FUNCTIONAL ROLE OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 IN THE DENTATE GYRUS OF A KAINATE-INDUCED SEIZURES MOUSE MODEL	14
17.15	Heather B Bradshaw, Emma Lieshman, Jordyn M Stuart and Mark Connor	OVEREXPRESSION OF TRPV1 IN HEK CELLS DRIVES DRAMATIC CHANGES IN BASAL ENDOCANNABINOIDS AND RELATED LIPIDS WHICH ARE POTENTIATED WITH STIMULATION BY CAPSAICIN	15
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17.45	Maria Morena, Andrea Peloso, Viviana Trezza, Matthew N. Hill and Patrizia Campolongo	LEARNING UNDER STRESS DIFFERENTIALLY AFFECTS CANNABINOID MODULATION OF SPATIAL MEMORY RETRIEVAL IN RATS	17
18.00	Piray Atsak, Daniela Hauer, Patrizia Campolongo, Gustav Schelling, Raquel V Fornari and Benno Roozendaal	THE ROLE OF ENDOCANNABINOID SIGNALING IN MEDIATING EFFECTS OF SEVERAL STRESS SYSTEMS IN MEMORY CONSOLIDATION	18

18.15	Andrea Martella, Rosa Maria Sepe, Cristoforo Silvestri, Oliana Carnevali, Paolo Sordino and Vincenzo Di Marzo	FUNCTIONAL CHARACTERIZATION OF THE ENDOCANNABINOID SYSTEM DURING ZEBRAFISH (DANIO RERIO) EMBRYONIC DEVELOPMENT	19
18.30	Emma Leishman, Ben Cornett, Ken Mackie and Heather B Bradshaw	EFFECTS OF DELETIONS IN FAAH ON THE ENDOCANNABINOID AND WIDE-RANGING-RELATED LIPIDOME IN EIGHT REGIONS OF THE MOUSE BRAIN	20
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19.00	Christopher J. Fowler, Jenny Häggström, Mariateresa Cipriano and Peter Hammarsten	IDENTIFYING POTENTIAL UPSTREAM REGULATORS OF THE ENDOCANNABINOID SYSTEM IN PROSTATE CANCER	22
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Monday, June 30th

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8.45	Nilushi Karunaratne, Paul J White, Stewart Fabb, Meritxell Canals, Mark Connor and Daniel T Malone	SIGNALLING PROFILE OF CRIP1a: G-PROTEIN ACTIVATION AND SIGNAL TRANSDUCTION	24
9.00	Etienne Hebert-Chatelain, Tifany Desprez, Edgar Soria, Luigi Bellocchio, Anna Delamarre, Arnau Busquets-Garcia, Laurie Robin, Nagore Puente, Jean-William Dupuy, Uzaskun Elezgarai, Rodrigue Rossignol, Federico Massa, Pedro Grandes, Giovanni Bénard and Giovanni Marsicano	MITOCHONDRIAL CANNABINOID RECEPTORS MEDIATE SPECIFIC EFFECTS OF CANNABINOIDS VIA SOLUBLE ADENYLYL CYCLASE (sAC)	25
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9.30	Alipi V. Naydenov, Marja Sepers, Katie Swinney, Lynn Raymond, Richard Palmiter and Nephi Stella	GENETIC RESCUE OF CB1 RECEPTORS ON MEDIUM SPINY NEURONS PREVENTS LOSS OF EXCITATORY STRIATAL SYNAPSES BUT NOT MOTOR PHENOTYPE IN R6/2 MICE	27
9.45	Martin Kaczocha, Matthew W. Elmes, William T. Berger, KwanNok Leung, Iwao Ojima and Dale G. Deutsch	FATTY ACID BINDING PROTEINS (FABPS) ARE INTRACELLULAR CARRIERS FOR Δ^9-TETRAHYDROCANNABINOL (THC) AND CANNABIDIOL (CBD)	28
10.00	Michelle Glass, Courtney M Breen, Erin Cawston, and Mark Connor	FUNCTIONAL SELECTIVITY IN CB1R PHARMACODYNAMICS	29
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11.00	Zheng-Xiong Xi, Pretal Muldoon, Xiao-Fei Wang, Guo-Hua Bi, M. Imad Damaj, Aron H. Lichtman, Roger G. Pertwee and Eliot L. Gardner	EFFECTS OF Δ^8-TETRAHYDROCANNABIVARIN (Δ^8-THCV) ON APPETITIVE EFFECTS OF COCAINE AND NICOTINE IN RODENTS	31
11.15	Ryan Vandrey, Edward J Cone, John M. Mitchell, Evan S. Herrmann, George E. Bigelow, Charles LoDico and Ron Flegel	EFFECT OF ROOM VENTILATION ON THE PHARMACODYNAMIC AND PHARMACOKINETIC EFFECTS OF SECONDHAND CANNABIS SMOKE EXPOSURE	32

11.30	Miriam Schneider, Chris M. Friemel, Laura Bindila and Beat Lutz	ADVERSE PEER-EXPERIENCES THROUGHOUT ADOLESCENCE IN MALE RATS PERSISTENTLY ALTER ETHANOL INTAKE AND ENDOCANNABINOID SIGNALING IN LATER LIFE	33
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14.30	Jordyn Stuart, Samuel D. Banister, Courtney Breen, Michelle Glass, Michael Kassiou and Mark Connor	ACTIVITY OF SYNTHETIC CANNABINOID DRUGS OF ABUSE AT G PROTEIN COUPLED CB RECEPTORS AND CANNABINOID-SENSITIVE ION CHANNEL	37

14.45	Ziva D. Cooper, Richard W. Foltin and Margaret Haney	EFFECTS OF CANNABIS ON THE SUBJECTIVE-EFFECT RATINGS AND PHARMACOKINETICS OF SMOKED COCAINE	38
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16.00	Rangan Maitra, Alan Fulp, Herbert Seltzman, Yanan Zhang and Timothy Fennell	*IN VIVO* EVALUATION OF THE PERIPHERALLY SELECTIVE CB1 RECEPTOR ANTAGONIST RTI-13329-2 IN A MOUSE MODEL OF DIET-INDUCED WEIGHT GAIN	41
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16.30	Emma Leishman, Ben Cornett, Ken Mackie and Heather B Bradshaw	NAPE-PLD DELETION VIA AN ALTERNATIVE MECHANISM DRIVES SIGNIFICANT DECREASES IN AEA IN THE MOUSE BRAIN	43
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17.45	Luciana Leo, Suellen Almeida-Corrêa, Claudio Canetti, Olavo Amaral, Fernando Bozza and Fabricio Pamplona	ENDOGENOUS CB1 ALLOSTERIC ENHANCER BALANCES AGE-RELATED COGNITIVE ALTERATIONS	46
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18.15	Arnau Busquets-Garcia, Maria Gomis-González, Laura Cutando, Raj K. Srivastava, Antonio Ortega-Álvaro, Luigi Bellochio, Giovanni Marsicano, Beat Lutz, Rafael Maldonado and Andrés Ozaita	A PERIPHERAL ENDOCANNABINOID MECHANISM FOR STRESS-INDUCED AMNESIA	48
18.30	Erica Zamberletti, Marina Gabaglio, Pamela Prini, Tiziana Rubino and Daniela Parolaro	PERSISTENT MICROGLIA ACTIVATION WITHIN THE PREFRONTAL CORTEX CONTRIBUTES TO THE DEVELOPMENT OF THE DEPRESSIVE/PSYCHOTIC-LIKE PHENOTYPE INDUCED BY ADOLESCENT THC EXPOSURE IN RATS	49
18.45	Michelle Sexton and Dominic Corva	A MEDICAL ETHNOGRAPHIC REPORT OF CANNABIS USE IN PEDIATRIC INTRACTABLE EPILEPSY (IE) PATIENTS	50
19.00	DINNER - ON YOUR OWN		

Day 3
Tuesday, July 1st

7.00	**Breakfast**		
NIDA Symposium **"TRP Channels: The only TR(i)P you can have on cannabinoids?"** *Chair*: Vincenzo Di Marzo			
8.15	**Pedro Grandes** Medicine and Dentistry Basque Country University Leioa, Spain	ANATOMY AND FUNCTIONAL ROLE OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 IN THE DENTATE GYRUS OF A KAINATE-INDUCED SEIZURES MOUSE MODEL	N1
8.45	**Thomas Voets** Laboratory of Ion Channel Research (LICR) Leuven, Belgium	MODULATION OF TEMPERATURE-SENSITIVE TRP CHANNELS	N2
9.15	**Vincenzo Di Marzo** Endocannabinoid Research Group Institute of Biomolecular Chemistry Consiglio Nazionale delle Ricerche Pozzuoli, Italy	INTERACTIONS BETWEEN THE "ENDOCANNABINOIDOME" AND THE "TRPOME": A NEW "OME" FOR PHYTOCANNABINOIDS AND LIPID MEDIATORS?	N3
9.45	**Coffee Break**		

Oral Session 9. Cannabinoid Genetics

Chairs: Jahan Marcu and Roger Pertwee

10.15	Kevin J. McKernan, Amir Zare, Lei Zhang, Jessica Spangler, Vasisht Tadigotla, Ted Foss, Christine Stanley, Melissa Pulliam, Robert W. Pulliam and Richard G. Boles	EXOME SEQUENCING OF FAMILIAL ATRIAL FIBRILLATION INFORMS POSITIVE TREATMENT WITH CANNABIDIOL	51
10.30	Mauro Maccarrone, Andrea Di Francesco, Bernardo Dell´Osso, Daniela Galimberti, A. Carlo Altamura and Claudio D´Addario	EPIGENETIC REGULATION OF THE ENDOCANNABINOID SYSTEM IN HUMAN PSYCHIATRIC DISORDERS	52
10.45	J.M. van Gool, I. Heitland, L. Groenink and J.M.P. Baas	GENETIC DIFFERENCES IN THE CB1/CNR1 GENE MODULATE FEAR EXTINCTION IN HUMANS	53
11.00	John M. McPartland and Geoffrey W. Guy	A QUESTION OF RANK: USING DNA BARCODES TO CLASSIFY CANNABIS SATIVA AND CANNABIS INDICA	54
11.15	**Special ICRS Speaker** Optogenetic approaches to studying reward and substance abuse disorders **Antonello Bonci, Ph.D.** National Institutes of Health		

12.15	LUNCH	
13.15 - 15.00	POSTER SESSION 2	P2
15.00 -	OUTING	

Notes:

Day 4
Wednesday, July 2nd

7.00	**Breakfast**		
8.15 Due to scheduling conflict	Luigia Cristino, Giovanna Morello, Roberta Imperatore, Fabiana Piscitelli, Letizia Palomba and Vincenzo Di Marzo	OREXIN/ENDOCANNABINOID/ LEPTIN INTERACTION AFFECTS HYPOTHALAMIC TAU PHOSPHORILATION BY GLYCOGEN SYNTHASE KINASE-3BETA ACTIVATION	55
Oral Session 10. Focus on CB2 *Chairs:* Josée Guindon and Andreas Zimmer			
8.30	Anne-Caroline Schmöle, Ramona Göhrs, Önder Albayram, Daniele Bano, Pierluigi Nicotera, Judith Alferink and Andreas Zimmer	CANNABINOID RECEPTOR 2 DEFICIENCY ALTERS NEUROINFLAMMATION IN AN ALZHEIMER'S DISEASE MOUSE MODEL	56
8.45	Ming Gao, Zhengxiong Xi and Jie Wu	SELECTIVE ACTIVATION OF CB2Rs ELIMINATES VTA DOPAMINE NEURONAL BURSTING FIRING IN RODENTS	57
9.00	Liting Deng, Josée Guindon, Benjamin L. Cornett, Alexandros Makriyannis, Ken Mackie and Andrea G. Hohmann	CHRONIC CANNABINOID CB2 AGONIST REVERSES PACLITAXEL NEUROPATHY WITHOUT TOLERANCE, CB1-MEDIATED WITHDRAWAL OR SIDE EFFECTS	58
9.15	Josée Guindon, Liting Deng, Emma Leishman, Heather B. Bradshaw and Andrea G. Hohmann	THE COMBINATION OF AMITRIPTYLINE WITH FAAH OR MGL INHIBITORS IN CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY IS CB2 MEDIATED	59
9.30	Pritesh Kumar and Zhao-Hui Song	TAMOXIFEN IS AN ALLOSTERIC MODULATOR OF THE CB2 CANNABINOID RECEPTOR	60

9.45	Shaojuan Zhang, Pin Shao, Ningyang Jia, Qin Tong, Xiang-qun Xie, Christina Bagia, Jelena M. Janjic, Ying Ding and Mingfeng Bai	TARGETING CB2 RECEPTOR AS A NEW PHOTOTHERAPY APPROACH	61
10.00	**COFFEE BREAK**		
ORAL SESSION 10 (CONT). FOCUS ON CB2 *CHAIRS*: JOSÉE GUINDON AND ANDREAS ZIMMER			
10.30	Vanessa Petrucci, Andrea Chicca, Juan Manuel Viveros-Paredes and Jürg Gertsch	PEPTIDE ENDOCANNABINOIDS (PEPCANS) ARE PAMs OF CB2 RECEPTORS AND INVOLVED IN THE INNATE IMMUNE RESPONSE	62
10.45	Eef L. Theunissen, Pim Heckman, Elizabeth B. de Sousa Fernandes Perna, Kim P. C. Kuypers, Anke Sambeth, Arjan Blokland, Jos Prickaerts, Stefan W. Toennes and Johannes G. Ramaekers	REVERSING THC-INDUCED IMPAIRMENT OF VERBAL MEMORY IN HEALTHY HUMANS	63
11.00	Slava Rom, Holly Dykstra, Nancy Reichenbach, Viviana Zuluaga-Ramirez and Yuri Persidsky	SELECTIVE STIMULATION OF CANNABIOID TYPE 2 RECEPTORS (CB2) IN MONOCYTES PREVENTS THEIR ENGAGEMENT OF BRAIN ENDOTHELIUM PROTECTING BLOOD BRAIN BARRIER	64
11.15	Jenny L. Wiley, Brian F. Thomas, Hongfang Yang and Anu Mahadevan	CB2 AGONISTS: HOW SELECTIVE ARE THEY?	65

11.30	Mario van der Stelt, Partha Mukhopadhyay, Marc Baggelaar, Zongxian Cao, Katalin Erdelyi, Filomena Fezza, Bogna Ignatowska-Jankowska, Marc Ruben, Resat Cinnar, George Kunos, Aron Lichtman, Mauro Maccarrone and Pal Pacher	PERIPHERALLY RESTRICTED, SELECTIVE CANNABINOID CB2 RECEPTOR AGONIST LEI-101 PREVENTS CISPLATIN-INDUCED NEPHROPATHY	66
11.45	Jean-Michel Adam, Christian M. Apfel, Stefanie Bendels, Caterina Bissantz, Jürgen Fingerle, Ivan Formentini, Jürgen Funk, Uwe Grether, Sabine Grüner, Atsushi Kimbara, Matthias Nettekoven, Giorgio Ottaviani, Camille Perret, Mark Rogers-Evans, Stephan Röver, Franz Schuler, Tanja Schulz-Gasch and Christoph Ullmer	TRIAZOLOPYRIMIDINES – A NOVEL CLASS OF HIGHLY POTENT, HIGHLY SELECTIVE AND *IN VIVO* ACTIVE CB2 AGONISTS	67
12.00	**LUNCH**		
13.00 – 15.00	**POSTER SESSION 3**		P3

Oral Session 11.
Beyond THC: Cannabidiol and Other Components
Chairs: Luciano De Petrocellis and Michelle Sexton

15.00	Attila Oláh, Lídia Ambrus, Attila G. Szöllősi, Christos C. Zouboulis, Ralf Paus and Tamás Bíró	"WEED AGAINST ZIT?" – EXPLORATION OF THE MECHANISMS OF THE COMPLEX ANTI-ACNE ACTIONS OF CANNABIDIOL	68
15.15	Jeffrey C. Raber, Sytze Elzinga, Mark E. Raber, Bradley J. Douglass, Cameron Miller, Aaron Kendziorek, Justin Fischedick and Dale Gieringer	CANNABINOID AND TERPENOID PROFILING OF CANNABIS IN CALIFORNIA AND WASHINGTON	69
15.30	Richard C. Kevin, David J. Allsop, Wendy Swift and Iain S. McGregor	"SMELLS LIKE GOOD WEED": THC LEVELS IN CANNABIS MAY BE PREDICTED BY MONOTERPENE HEADSPACE CONCENTRATIONS	70
15.45	Sándor Bátkai, Partha Mukhopadhyay, Katalin Erdélyi, Jürg Gertsch and Pál Pacher	PROTECTIVE EFFECT OF THE PHYTOCANNABINOID BETA-CARYOPHYLLENE IN LIVER ISCHEMIA REPERFUSION INJURY	71
16.00	Alex Naftaly, Ester Fride (Z"L), Jürg Gertsch and Sharon Anavi-Goffer	EFFECT OF BETA-CARYOPHYLLENE ON PHENCYCLIDINE-INDUCED BEHAVIOURAL CHANGES	72
16.15	Douglas E Brenneman, Dean Petkanas and William A. Kinney	CANNABIDIOL PROVIDES PROTECTION FROM ETHANOL AND AMMONIUM TOXICITY IN A HIPPOCAMPAL MODEL OF HEPATIC ENCEPHALOPATHY	73

16.30	Andrea Chicca, Diego Caprioglio, Alberto Minassi, Vanessa Petrucci, Salome Gachet, Giovanni Appendino, Orazio Taglialatela-Scafati and Jurg Gertsch	NATURAL PRODUCT-DERIVED CANNABMIMETICS AS SOURCE OF POLYPHARMACOLOGY IN THE ENDOCANNABINOID SYSTEM	74
16.45	Alline Campos, Aline Miranda, Fatima Brant, Fabiana Machado, Francisco Guimaraes and Antonio Teixeira	CANNABIDIOL REPEATED TREATMENT INCREASES SURVIVAL AND PROMOTES RESCUE OF COGNITIVE FUNCTION IN A MURINE MODEL OF CEREBRAL MALARIA	75
17.00	Maria Ceprian, Maria Ruth Pazos, Federica Penna, Laura Jimenez-Sanchez, Martin Santos and Jose Martinez	CANNABIDIOL PROMOTES OLIGODENDROCYTE SURVIVAL AFTER HYPOXIA-ISCHEMIA IN NEWBORN RATS	76
17.15	Nadia Solowij, Erika van Hell, Samantha Broyd, Lisa-marie Greenwood, Jelena Novakovic, Camilla Beale, Dave Martelozzo, Arno Hazekamp and Rodney Croft	EFFECTS OF CBD ALONE AND IN COMBINATION WITH THC ON COGNITIVE PERFORMANCE	77
17.30	James Brodie, Vincenzo Di Marzo and Geoffrey Guy	THE THERAPEUTIC HANDSHAKE: CRAFTING MEDICINES FOR COMPLEX MALADIES	78

17.45	Manoela V. Fogaça, Alline C. Campos and Francisco S. Guimarães	THE ANXIOLYTIC-LIKE EFFECT OF CANNABIDIOL ADMINISTRATION IN CHRONICALLY STRESSED MICE IS MEDIATED BY THE ENDOCANNABINOID SYSTEM: INVOLVEMENT OF NEUROGENESIS AND AUTOPHAGY	79
18.00	Jahan P. Marcu	RESULTS FROM AUDITING MEDICAL CANNABIS FACILITIES IN THE UNITED STATES IN 2014	80
18.15	ICRS BUSINESS MEETING		
20.00	ICRS BANQUET		

POSTER SESSION 1: TOPICS A - E
DAY 1, SUNDAY, JUNE 29TH: 13:00 - 15:00

TOPIC A. CANNABINOIDS AND PATHOPHYSIOLOGY

Michiel Balvers, Pim Koelink, Aletta Kraneveld, Mieke Poland, Jocelijn Meijerink and Renger Witkamp	THE N-3 N-ACYLETHANOLAMIDE DHEA IMPROVES MANIFESTATIONS OF EXPERIMENTAL MURINE COLITIS	P1-1
Valerio Chiurchiù, Emanuela Talamonti, Alessandro Leuti and Mauro Maccarrone	THE ENDOCANNABINOID SYSTEM IS A NOVEL MODULATOR OF HUMAN MACROPHAGE PLASTICITY AND POLARIZATION	P1-2
Caroline Turcotte, Simona Zarini, Stéphanie Jean, Cyril Martin, Véronique Provost, Adam Uzieblo, Robert C Murphy and Nicolas Flamand	PROSTAGLANDIN D_2-GLYCEROL AND E_2-GLYCEROL INHIBIT HUMAN NEUTROPHIL FUNCTIONS	P1-3
Mark Jones, Marie Smith, Susan Anderson and Saoirse E. O'Sullivan	EVIDENCE THAT THE NOVEL ENDOCANNABINOID VIRODHAMINE INCREASES OSTEOBLAST PROLIFERATION: A ROLE FOR CB2 AND GPR55	P1-4
Valerie CM Shang, David A Kendall and Richard E Roberts	THE EFFECT OF CANNABINOIDS ON HUMAN BRONCHIAL EPITHELIAL CELL PERMEABILITY	P1-5

Ku-Lung Hsu, Katsunori Tsuboi, Alexander Adibekian, Holly Pugh, Kim Masuda, Sudeshna Ghosh, Manuel Sanchez-Alavez, Aron Lichtman, Bruno Conti and Benjamin F. Cravatt	INACTIVATION OF DIACYLGLYCEROL LIPASE IN INFLAMMATORY DISEASE	P1-6
Jocelijn Meijerink, Zheng Wang, Mieke Poland, Jean-Paul ten Klooster and Renger Witkamp	DOCOSAHEXAENOYL-SEROTONIN (DHA-5-HT), AN INTESTINAL CONJUGATE OF SEROTONIN AND DHA EXHIBITS ANTI-INFLAMMATORY PROPERTIES	P1-7
Michael M. McDonald, Amos Matsiko, Adolfo López Noriega, Hugo D. Kieran, Aoife Gowran, Kevin J. Mulhall, Fergal J. O'Brien and Veronica A. Campbell	THE DEVELOPMENT OF A CANNABINOID-CONTAINING COLLAGEN-GAG SCAFFOLD FOR USE IN ORTHOPAEDIC TISSUE ENGINEERING STRATEGIES	P1-8
Valerio Chiurchiù, Emanuela Talamonti, Mirko Lanuti Tiziana Bisogno and Mauro Maccarrone	ENDOCANNABINOIDS DIFFERENTIALLY MODULATE NLRP3 INFLAMMASOME ACTIVATION IN PRIMARY HUMAN MACROPHAGES	P1-9
Michel Laviolette, Marie-Chantal Larose, Caroline Turcotte, Véronique Provost, Cyril Martin, Catherine Laprise and Nicolas Flamand	METABOLISM OF EXOGENOUS ARACHIDONOYL-ETHANOLAMIDE AND 2-ARACHIDONOYL-GLYCEROL BY HUMAN EOSINOPHILS	P1-10
Amina M. Bagher, Robert B. Laprairie, Melanie E.M. Kelly and Eileen M. Denovan-Wright	INFLUENCE OF THE DOPAMINE RECEPTOR TYPE 2 (D2) ANTAGONIST ON THE CANNABINOID RECEPTOR TYPE 1 (CB1) FUNCTION	P1-11
Attila Oláh, Nóra Czakó, Levente Molnár, Stefania Petrosino, Teresa Aveta, Vincenzo Di Marzo, Béla Fülesdi and Tamás Bíró	EFFECTS OF CONTROLLED, TRANSIENT HYPEROXIA ON THE SERUM LEVELS OF DIFFERENT ENDOCANNABINOIDS – A HUMAN PILOT STUDY	P1-12

Herbert H. Seltzman, Yatendra Mulpuri and Igor Spigelman	THE PERIPHERALLY-RESTRICTED CANNABINOID RECEPTOR AGONIST PRNMI ALLEVIATES CISPLATIN INDUCED PERIPHERAL NEUROPATHY	P1-13
Mustafa Karwad, Karen L. Wright, Michael Larvin, Jonathan Lund and Saoirse E. O'Sullivan	OLEOYLETHANOLAMIDE (OEA) AND PALMITOYLETHANOLAMIDE (PEA) MODULATE INTESTINAL PERMEABILITY IN AN *IN VITRO* ISCHAEMIA/REPERFUSION MODEL	P1-14
Ya Wang, Pierluigi Plastina, Michiel Balvers, Mieke Poland, Bartolo Gabriele, Jean-Paul Vincken, Renger Witkamp and Jocelijn Meijerink	N-ACYLDOPAMINES DERIVED FROM POLYUNSATURATED OMEGA-3 FATTY ACIDS EXERT ANTI-INFLAMMATORY EFFECTS IN MOUSE MACROPHAGES	P1-15
Natalia Malek, Katarzyna Popiolek-Barczyk, Joanna Mika, Barbara Przewlocka and Katarzyna Starowicz	RATIONALE FOR TARGETING THE ENDOCANNABINOID SYSTEM TO MANAGE NEUROINFLAMMATION IN LPS-ACTIVATED PRIMARY MICROGLIAL CULTURES	P1-16
Haley A. Vecchiarelli, Catherine M. Keenan, J. Megan Gray, Mohammad Bashashati, Keith A. Sharkey and Matthew N. Hill	COLITIS ALTERS CENTRAL ENDOCANNABINOID CONTENT	P1-17
Ulrike Taschler, Martina Schweiger, Martin A. Storr, Rudolf Schicho and Robert Zimmermann	MONOGLYCERIDE LIPASE DEFICIENCY CAUSES INTESTINAL CANNABINOID RECEPTOR DESENSITIZATION	P1-18
Elizabeth A. Cairns, Michele L. Archibald, Alex J. Straiker, Pushkar M. Kulkarni, Ganesh A.Thakur, William H. Badridge and Melanie E.M. Kelly	MODIFYING CB1 RECEPTOR SIGNALLING TO REDUCE IOP IN A MOUSE MODEL OF OCULAR HYPERTENSION	P1-19

TOPIC B. Pain and TRPs

Bright N Okine, Manish K. Madasu, Fiona McGowan, Brendan Harhen, Michelle Roche and David P. Finn	DIRECT ADMINISTRATION OF N-PALMITOYLETHANOLAMIDE INTO THE RAT MEDIAL PREFRONTAL CORTEX REDUCES FORMALIN-EVOKED NOCICEPTIVE BEHAVIOUR VIA A CB1 RECEPTOR-MEDIATED MECHANISM	P1-20
Mireille Alhouayek, Baptiste Buisseret, Owein Guillemot-Legris and Giulio G. Muccioli	COMPARISON OF THE EFFECTS OF INHIBITION OF ABHD6 AND MAGL ON INFLAMMATION	P1-21
Samira Khakpour, Kevin Wilhelmsen, Alphonso Tran and Judith Hellman	INVESTIGATION OF THE ROLE OF THE TRPV1 CHANNEL IN ENDOTHELIAL INFLAMMATION	P1-22
Martin Kaczocha, Syed Azim, James Nicholson, Mario J. Rebecchi, William Galbavy, Tian Feng and Helene Benveniste	ENDOCANNABINOID LEVELS AND FUNCTIONAL DISABILITY STATUS IN PATIENTS WITH PAINFUL OSTEOARTHRITIS	P1-23
William Notcutt, Cheryl Phillips, Phillipe Lacoux, Adrian Shanks, Viji Vijayakulasingam and Laura Baldock	A RETROSPECTIVE DESCRIPTION OF THE USE OF NABILONE IN UK CLINICAL PRACTICE	P1-24
Pierluigi Plastina, Mieke Poland, Jocelijn Meijerink, Bartolo Gabriele and Renger Witkamp	HYDROXYTYROSYL OLEATE, AN ESTER ANALOGUE OF OLEOYLDOPAMINE (OLDA), SHOWS ANTI-INFLAMMATORY PROPERTIES *IN VITRO*	P1-25

Molly S. Crowe, Emma Leishman, Ramesh Gujjar, Anu Mahadevan, Matthew Banks, Heather Bradshaw and Steven G. Kinsey	ATTENUATING NEUROPATHIC PAIN THROUGH DUAL INHIBITION OF CYCLOOXYGENASE AND MONOACYLGLYCEROL LIPASE	P1-26
Iryna Khasabova, Cutler Lewandowski, Xu Yao, Justin Paz, Natalya Burlakova, Donald Simone and Virginia Seybold	ANANDAMIDE MEDIATES THE ANTIHYPERALGESIC EFFECT OF 2AG IN A MURINE MODEL OF CHEMOTHERAPY-INDUCED NEUROPATHY	P1-27
Sara R. Nass and Steven G. Kinsey	THE MONOACYGLYCEROL LIPASE INHIBITOR JZL184 ATTENUATES HYPERALGESIA INDUCED BY COLLAGEN-INDUCED ARTHRITIS	P1-28
Peggy Schneider, Laura Bindila, Beat Lutz, Rainer Spanagel and Miriam Schneider	LONG-TERM SOCIAL REJECTION DURING ADOLESCENCE ALTERS PAIN PERCEPTION AND AFFECTS THE ENDOCANNABINOID SYSTEM IN ADULT FEMALE RATS	P1-29
Luciano De Petrocellis, Stefania Petrosino, Aniello Schiano Moriello, Santiago Cerrato, Mariella Fusco, Anna Puigdemont and Vincenzo Di Marzo	“ENTOURAGE” EFFECTS OF PALMITOYLETHANOLAMIDE: ENHANCEMENT OF 2-AG ACTION AT TRPV1 CHANNELS AND OF 2-AG LEVELS *IN VITRO* AND *IN VIVO*	P1-30
Torsten Lowin, Angelika Gräber and Rainer H. Straub	ANTI-INFLAMMATORY EFFECTS OF THE CB1/CB2 AGONIST WIN55212,2 ARE DEPENDENT ON TRPV1, TRPA1 AND AMPK IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS SYNOVIAL FIBROBLASTS	P1-31
Manish K. Madasu, Bright N. Okine, Weredeselam M. Olango, Michelle Roche and David P. Finn	DIFFERENTIAL EFFECTS OF PHARMACOLOGICAL MODULATION OF TRPV1 IN THE LATERAL PERIAQUEDUCTAL GREY ON FORMALIN-EVOKED NOCICEPTIVE BEHAVIOUR IN SPRAGUE-DAWLEY AND WISTAR-KYOTO RATS	P1-32

Carmen La Porta, Simona Andreea Bura, Antoni Pastor, Rafael de la Torre, Francisco Navarrete, Jorge Manzanares and Rafael Maldonado	ROLE OF CB1 AND CB2 CANNABINOID RECEPTORS IN THE EMOTIONAL AND COGNITIVE ALTERATIONS ASSOCIATED WITH OSTEOARTHRITIS PAIN	P1-33
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Lindsay Silva, Rita Black and Diana Dow-Edwards	AGE OF EXPOSURE AFFECTS BEHAVIORAL RESPONSE TO DELTA-9-TETRAHYDROCANNABINOL DURING THE PERI-PUBERTAL PERIOD IN THE RAT	P1-34
Justine Renard, Michael Loureiro, Walter J. Rushlow and Steven R. Laviolette	LONG-TERM EFFECTS OF ADOLESCENT THC EXPOSURE ON ADULTHOOD PSYCHOPATHOLOGY	P1-35
Marcoita T. Gilbert, Scott E. Parnell, Eric W. Fish, Lorinda K. Baker and Kathleen K. Sulik	A MOUSE MODEL SHOWS SYNTHETIC CANNABINOID TERATOGENICITY AND PROMISE FOR PRENATAL DRUG CO-EXPOSURE RESEARCH	P1-36
Anja Goepfrich and Miriam Schneider	MODULATORY INFLUENCE OF THE DEVELOPING ENDOCANNABINOID SYSTEM ON COGNITIVE ABILITIES DURING ADOLESCENT BRAIN DEVELOPMENT IN RATS	P1-37
TOPIC D. CANNABINOIDS AND CANCER		
Joseph D. Manna and Lawrence J. Marnett	LYPLA2 IS A MAJOR PROSTAGLANDIN GLYCEROL ESTER HYDROLASE IN HUMAN CANCER CELLS	P1-38

Elisa Bisicchia, Filomena Fezza, Emanuela Talamonti, Mirko Lanuti, Valerio Chiurchiù, Sergio Oddi, Marco Molinari and Mauro Maccarrone	ROLE OF THE ENDOCANNABINOID SYSTEM IN AUTOPHAGY	P1-39
Tiziana Bisogno, Alberto Rainer, Luca Businaro, Emanuela Talamonti, Marcella Trombetta, Daniele Santini and Mauro Maccarrone	ANALYSIS OF ENDOCANNABINOID SYSTEM IN A 3D "CELL-ON-CHIP" MODEL OF HUMAN TUMORS	P1-40
Seok-Woo Park and Myung-Whun Sung	CYCLOOXYGENASE-2-DEPENDENT GROWTH INHIBITION OF ARACHIDONOYL ETHANOLAMIDE ON LARYNGEAL CANCER CELLS	P1-41
Stan L. Banks, Miro Golinski, Jeff Howard, Dana Hammell and Audra L. Stinchcomb	DEVELOPMENT OF A Δ^9-THC PRODRUG TRANSDERMAL DELIVERY SYSTEM FOR PREVENTION OF ACUTE AND DELAYED NAUSEA AND VOMITING ASSOCIATED WITH INITIAL AND REPEAT COURSES OF EMETOGENIC CANCER CHEMOTHERAPY	P1-42
Maria Chiara Proto, Antonio Christian Pagano Zottola, Donatella Fiore, Simona Pisanti, Paola Picardi, Elena Ciaglia, Anna Maria Malfitano, Alba D'Alessandro, Chiara Laezza, Maurizio Bifulco and Patrizia Gazzerro	ENDOCANNABINOID-INDUCED GROWTH INHIBITION OF HUMAN COLON CANCER: INVOLVEMENT OF WNT/β-CATENIN PATHWAY	P1-43
N. Rielle Capler and Lynda G. Balneaves	A REVIEW OF THE USE OF HERBAL CANNABIS AND CANNABIS EXTRACTS FOR THE TREATMENT OF CANCER AND CANCER-RELATED SYMPTOMS	P1-44

Thomas D. M. Hill, Colin G. Stott and Marnie Duncan	AN *IN VITRO* EVALUATION OF COMBINATIONS OF PHYTOCANNABINOIDS IN PANCREATIC, GASTRIC, RENAL, BLADDER AND LIVER CANCER CELL LINES	P1-45
Ghayth M. Abdulrazzaq, Sue Chan and Stephen PH Alexander	EFFECT OF N-ARACHIDONOYH GLYCIN (NAGly) AND Δ^9-TETRAHYDROCANNABINOL (Δ^9THC) ON ERK1/2 PHOSPHORYLATION IN NATIVE HEK293 CELLS	P1-46
TOPIC E. CANNABINOID RECEPTORS AND SIGNALING		
Elham Khajehali, Daniel T. Malone and Katie Leach	ALLOSTERIC MODULATION AND BIASED SIGNALLING AT CB1 CANNABINOID RECEPTORS	P1-47
Orla Haugh, Cullen McCulloch, June Penman and Andrew J. Irving	PROFILING THE ACTIVITY OF GPR55 ANTAGONISTS AGAINST RECOMBINANT AND ENDOGENOUS GPR55	P1-48
Jagjeet Mnpotra, Diane Lynch, Alan Grossfield, Nicholas Leioatts, Michael Pitman and Patricia Reggio	ROLE OF INTRACELLULAR LOOPS IN THE HYDRATION OF GDP: RESULTS FROM MOLECULAR DYNAMICS SIMULATIONS OF THE 2-AG ACTIVATED CANNABINOID RECEPTOR SUBTYPE 2 / Gi PROTEIN COMPLEX	P1-49
Khalil Eldeeb, Sandra Leone-Kabler, Lawrence C. Blume and Allyn C. Howlett	CB1 RECEPTOR INTRACELLULAR LOOP 4 MUTATION MODULATES G PROTEIN ACTIVATION AND CAMP PRODUCTION IN HUMAN NEUROBLASTOMA CELLS	P1-50

POSTER SESSION 2: TOPICS E - H
DAY 3, TUESDAY, JULY 1ST: 13:15 - 15:00

TOPIC E (CONT.) CANNABINOID RECEPTORS AND SIGNALING

Matthew W. Buczynski, Melissa A. Herman, Ku-Lung Hsu, Luis A. Natividad, Cristina Irimia, Ilham Y. Polis, Holly Pugh, Jae Won Chang, Micah J. Niphakis, Benjamin F. Cravatt, Marisa Roberto and Loren H. Parsons	CHRONIC NICOTINE EXPOSURE DIMINISHES INHIBITORY CONTROL OF VTA DA NEURONS THROUGH ENHANCED DIACYLGLYCEROL LIPASE-MEDIATED 2-ARACHIDONOYLGLYCEROL SIGNALING	P2-1
Alex Straiker, Sherry Hu, Karl Spork, Emma Leishmann and Heather Bradshaw	A GPR119-BASED SIGNALING SYSTEM IN THE MURINE EYE REGULATES INTRAOCULAR PRESSURE IN A SEX-DEPENDENT MANNER	P2-2
Richard S Priestley, Sarah A. Nickolls, Stephen P.H. Alexander and David A Kendall	CANNABINOID CB1 RECEPTOR-MEDIATED ERK1/2 RESPONSES - EVIDENCE OF $G_{I/O}$ PROTEIN-INDEPENDENT SIGNALLING AND AGONIST BIAS	P2-3
Toru Uyama, Manami Inoue, Yoko Okamoto, Naoki Shinohara, Tatsuya Tai1, Masahiro Watanabe, Iffat Ara Sonia Rahman, Kazuhito Tsuboi, Tomohito Inoue, Akira Tokumura and Natsuo Ueda	PEROXISOMAL DYSFUNCTION BY PLA/AT FAMILY PROTEINS IS NOT RELATED TO THEIR NAPE-FORMING N-ACYLTRANSFERASE ACTIVITY	P2-4
Jayendra Z. Patel, Stephen Ahenkorah, Yahaya Adams, Susanna M. Saario, Teija Parkkari, Juha R. Savinainen, Jarmo T. Laitinen and Tapio Nevalainen	LORATADINE ANALOGUES AS MAGL INHIBITORS	P2-5

Dow P. Hurst, Diane L. Lynch, Derek M. Shore, Michael C. Pitman and Patricia H. Reggio	MODELING THE ORG27569 INDUCED CB1/BETA-ARRESTIN 1 COMPLEX THAT ACTIVATES AN ARRESTIN BIASED PATHWAY	P2-6
William A. Devane and David P. Finn	PHARMACOLOGICAL CHARACTERISATION OF A BINDING SITE FOR [^{3}H]LEELAMINE	P2-7
Abhijit R. Kulkarni, Pushkar M. Kulkarni, Anisha Korde, Nicolai Zvonok, Maria Grazia Cascio, Alexandros Makriyannis, Roger G. Pertwee and Ganesh A. Thakur	DESIGN, SYNTHESIS AND BIOCHEMICAL EVALUATION OF NOVEL ELECTROPHILIC AND PHOTOAFFINITY COVALENT PROBES TO MAP THE CB1 RECEPTOR ALLOSTERIC SITE(S)	P2-8
Saoirse O'Sullivan, Sara Goodacre and Jonathan Yee	REVIEW OF EVIDENCE FOR CANNABINOID RECEPTORS AND ENDOCANNABINOID SIGNALLING IN INVERTEBRATES WITH A SPECIFIC FOCUS ON ARACHNIDS	P2-9
Carmen Rodríguez-Cueto, Mariluz Hernández-Gálvez, Cecilia J. Hillard, Patricia Maciel, Javier Fernández-Ruiz and María Gómez-Ruiz	ANALYSIS OF THE ENDOCANNABINOID SIGNALING SYSTEM IN BRAIN STRUCTURES OF SCA-3 TRANSGENIC MICE	P2-10
June Penman, Emanuel Ferreira Lopes and Andrew J. Irving	THE N-ACYL AMINO ACIDS, N-ARACHIDONOYL-L-SERINE AND N-ARACHIDONOYL GLYCINE, ACTIVATE GPR55	P2-11
Christoph Porazik, Anke Witting and Boris Ferger	SIMULTANEOUS DETERMINATION OF ENDOCANNABINOID AND PROSTAGLANDIN BIOMARKERS AND OF MONOACYLGLYCEROL LIPASE INHIBITOR EXPOSURE USING A NEW AND FAST LC-MS/MS-METHOD	P2-12
Marcus R. Goetz, Oskar Koch, Eduardo Munoz E and Bernd L. Fiebich	EFFECTS OF NOVEL SEMI-SYNTHETIC CANNABINOIDS ON CB1 AND CB2 RECEPTORS THROUGH BINDING AND SIGNALLING	P2-13

Kiri L.Wills, Kiran Vemuri, Alana Kalmar, Cheryl L. Limebeer, Alexandros Makriyannis and Linda A. Parker	DELIVERY OF THE CB1 ANTAGONIST, AM251, BILATERALLY TO THE CENTRAL NUCLEUS OF THE AMYGDALA AND ITS EFFECTS ON MORPHINE WITHDRAWAL	P2-20
Yong Liu, Ying Hu, Peng Zhong and Qing-song Liu	BDNF INTERACTS WITH ENDOCANNABINOIDS TO REGULATE COCAINE-INDUCED SYNAPTIC PLASTICITY IN MOUSE MIDBRAIN DOPAMINE NEURONS	P2-21
Regina Nelson	INTEGRAL FRAMES: INTERDISCIPLINARY TOOLS SUPPORTING CANNABIS RESEARCH	P2-22
Travis Grim, Jason Weibelhaus, Steve Negus and Aron Lichtman	C57BL6/J DEVELOP TOLERANCE TO CP55,940 IN INTRACRANIAL SELF-STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE	P2-23
Tasha Ahmad and Steven R. Laviolette	CANNABINOID CB1 RECEPTOR TRANSMISSION IN THE BASOLATERAL AMYGDALA BI-DIRECTIONALLY CONTROLS THE MOTIVATIONAL PROPERTIES OF OPIATES VIA FUNCTIONAL EXCITATORY INPUTS TO THE NUCLEUS ACCUMBENS SHELL	P2-24
Kristin M. Gabella, Margaret S. Jones, Molly S. Crowe, Sara R. Nass and Steven G. Kinsey	RIMONABANT-PRECIPITATED Δ^9-THC WITHDRAWAL ALTERS MARBLE BURYING AND STRUGGLING BEHAVIORS IN MICE	P2-25
Lena Bergström, Shima Momeni, Hanna Eriksson-Röhnisch and Erika Roman	FATTY ACID AMIDE HYDROLASE (FAAH) ACTIVITY IN DIFFERENT RAT BRAIN REGIONS AFTER TWO WEEKS TREATMENT WITH ETHANOL	P2-26

TOPIC G. METABOLISM

Tania Muller, Stephanie Troy-Fioramonti, Laurent Demizieux, Joseph Gresti, Maria Bouam, BrunoVergès and Pascal Degrace	ACTIVATION OF ADIPOSE TISSUE CANNABINOID RECEPTORS 1 (CB1R) ALTERS ANTILIPOLYTIC ACTION OF INSULIN AND INCREASES LIPOLYSIS IN MICE	P2-27
Stephanie Troy-Fioramonti, Laurent Demizieux, Joseph Gresti, Bruno Vergès and Pascal Degrace	PERIPHERAL ENDOCANNABINOID SYSTEM ACTIVATION INHIBITS INTESTINAL GLUCOSE ABSORPTION AND IMPROVES POSTPRANDIAL GLYCEMIA IN LEAN AND OBESE MICE	P2-28
Maria Scherma, Valentina Satta, Roberto Collu, Liana Fattore, Paola Fadda and Walter Fratta	PHARMACOLOGICAL MODULATION OF THE ENDOCANNABINOID SYSTEM IN A RODENT MODEL OF "ACTIVITY-BASED ANOREXIA"	P2-29
Oliver Linsell, Khaled Greish and John C. Ashton	SYNTHESIS AND CHARACTERIZATION OF A NANOMICELLE CANNABINOID FORMULATION	P2-30
Jessica Karlsson and Christopher J. Fowler	INHIBITION OF ENDOCANNABINOID METABOLISM BY THE METABOLITES OF IBUPROFEN AND FLURBIPROFEN	P2-31
Sandra Gouveia-Figueira, Jana Späth, Angela Zivkovic and Malin L. Nording	DIETARY EFFECTS ON CIRCULATING OXYLIPINS AND ENDOCANNABINOIDS	P2-32
Giovanna Morello, Roberta Imperatore, Fabiana Piscitelli, Daniela Sarnataro, Letizia Palomba, Luciano De Petrocellis, Luigia Cristino and Vincenzo Di Marzo	OREXIN-A ENHANCES 2-AG BIOSYNTHESIS VIA CB1/OX-1R HETEROMERS IN THE NEURONS OF THE MOUSE HYPOTHALAMIC ARCUATE NUCLEUS	P2-33

Julius T. Dongdem, Simon P. Dawson and Stephen P. H. Alexander	INHIBITION OF RAT LIVER FAAH ACTIVITY USING THREE DIFFERENT FATTY ACID AMIDES AS SUBSTRATES	P2-34
Antoni Pastor, Magí Farré, Montserrat Fitó, Fernando Fernandez-Aranda and Rafael de la Torre	*EX-VIVO* ENZYMATIC GENERATION OF 2-AG AND OTHER 2-MONOACYLGLYCEROLS IN PLASMA	P2-35
Caroline Turcotte, Cyril Martin, Véronique Provost and Nicolas Flamand	EXPRESSION OF ENZYMES INVOLVED IN ENDOCANNABINOID METABOLISM BY HUMAN LEUKOCYTES	P2-36
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ICRS2014 - Presidential Plenary Speaker

CANNABINOIDS REVISITED? NEW TARGETS, CHEMISTRY AND PLANT SOURCES

Giovanni Appendino, Ph.D.

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The pharmacological potential of Cannabis has long been reductively identified with the one of THC, its psychotropic constituent. While undoubtedly a major player for the medicinal exploitation of Cannabis, THC is not the only constituent of this plant having pharmacological potential, nor cannabinoids are the only class of bioactive compounds present in this plant. These issues are now well established, but less known is the fact that cannabinoids as well as compounds with cannabinoid activity are not unique to Cannabis.

Cannabinoids are the result of the biogenetic merging of a polyketide-derived alkylresorcinol and an isopropenyl residue. This leitmotiv is not uncommon in natural products, being documented in both lower (liverworts) and higher plants. In hemp cannabinoids, the isoprenyl residue is, with a single exception, of the monoterpenyl type, while the resorcinyl alkyl residue has five, or more rarely, a lower number of carbons. However, a large and diverse group of "cannabinoids" also occurs in South-African members of the genus *Helichrysum*. While cannabigerol (CBG) and its acidic precursor (pre-CBG) have both been isolated from *H. umbraculigerum* Less.,[1] most *Helichrysum* cannabinoids are derived from the phenethyl version of these compounds, where a phenyl ring replace the three terminal carbons of the classic pentyl chain of hemp cannabinoids . Surprisingly, the biological profile of these compounds is totally unknown, despite their close similarity to hemp cannabinoids and the use of some *Helichrysum* species to induce a trance state not unlike the one associated to the recreational use of hemp. The potential of *Helichrysum* cannabinoids to affect the metabotropic (CBs), ionotropic (TRPs) and trascription factors (PPARs) end-points of hemp cannabinoids will be discussed.

From a pharmacological standpoint, cannbinoids are compounds capable to interact with the metabotropic cannabinoid receptors CB_1 and CB_2. While CB_1 is rather selective in terms of natural products ligands, CB_2 is more promiscuous, and can be modulated by different structural classes of natural products. The most surprisingly ligand of CB_2 is, undoubtedly, the sesquiterpene b-caryophyllene (**3**), the only hydrocarbon capable to bind in a specific and potent way a macromolecular biological end-point, and structure-activity relationships for this interaction will be discussed, highlighting the potential of the sesquiterpene framework to modify various end-points of endogenous eicosanoids. While b-caryophyllene is a major constituent of the essential oil from hemp, this plant also contains a host of bioactive and unique phenolics capable to interact with inflammatory targets and especially the enzymatic formation of eicosanoids (PGs, LTs). This inhibitory activity on the production of inflammatory eicosanoids complements the mimicry of regulatory eicosanoids (AEA, 2AG) typical of cannabinoids, qualifying Cannabis as a source of compounds capable to selectively modulate endolipid signaling and beneficially affect its pathological imbalance in several pathological settings.

1. Bohlmann, F.; Hoffmann, E. Cannabigerol-aenlich Verbindungen aus Helichrysum umbraculigerum. *Phytochemistry* **1979**, *18*, 1371-1374.

NIDA Symposium

TUESDAY, JULY 1ST

TRP Channels: The only TR(i)P you can have on cannabinoids?

CHAIR: VINCENZO DI MARZO

Pedro Grandes
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ANATOMY AND FUNCTIONAL ROLE OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 IN THE DENTATE GYRUS OF A KAINATE-INDUCED SEIZURES MOUSE MODEL

Miren-Josune Canduela, Juan-Luis Mendizabal-Zubiaga, Amanda Sierra, Naiara Royo, Sara Peñasco, Leire Reguero, Nagore Puente and **Pedro Grandes**

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The transient receptor potential vanilloid type 1 (TRPV1) is a non-selective cation channel that acts primarily as pain sensor in the periphery but also modulates neurotransmitter release and synaptic plasticity in the brain. TRPV1 function must lay on its anatomical distribution in the peripheral and central nervous system regions where the channel has physiological roles. Nevertheless, the anatomical localization of TRPV1 is well established in the periphery, but it is a matter of debate in the brain. We have recently demonstrated a high TRPV1 localization at the dentate molecular perforant path synapses (Puente et al., 2014). However, the TRPV1 distribution at inhibitory synapses in the dentate molecular layer is not yet characterized anatomically.

To this goal, we have used TRPV1 antibodies combined with a highly sensitive pre-embedding immunogold method for high resolution electron microscopy. TRPV1 immunoparticles were in dentate granule cell dendrites receiving symmetric synapses. The labelling was mostly confined to postsynaptic membranes and was distributed at a relative short distance from the inhibitory synaptic contacts. Importantly, the TRPV1 distribution pattern at inhibitory synapses disappeared in the molecular layer of TRPV1-knockout mice.

We have also investigated the effect of an intrahippocampal injection of kainic acid (50 nl of 20 mM) on the trigger of kainate-induced seizures in WT and TRPV1-KO mice, a mouse model of medial temporal lobe epilepsy (MTLE). The behavioral score revealed that seizures were milder in TRPV1-KO than in WT during kainate-induced status epilepticus. Prior to kainate injection, a significant increase of cannabinoid 1 (CB1) receptor immunoreactivity in the inner 1/3 of the molecular layer and an increase of DAGL-α and NAPE-PLD optical densities were observed in dentate gyrus of TRPV1-KO versus WT. These findings may suggest that the absence of TRPV1 in the dentate gyrus triggers some adaptative changes that may be beneficial in the control of epileptic seizures.

Acknowledgements: Funding for P. Grandes' laboratory is provided by Ministerio de Economía y Competitividad (BFU2012-33334); The Basque Country Government grant IT764-13, and by Red de Trastornos Adictivos, RETICS, Instituto de Salud Carlos III, grant number: RD07/0001/2001. Funding for A. Sierra´s laboratory is provided by Ministerio de Economía y Competitividad (BFU2012-32089).

MODULATION OF TEMPERATURE-SENSITIVE TRP CHANNELS

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Thermosensing is initiated at sensory nerve endings, where alterations in temperature lead to swift changes in the gating of specific temperature-sensitive ion channels. This finally results in electrical signals that are conveyed to the CNS, where they are translated into a sense of temperature, ranging from ice cold to burning hot. Dysregulation of the thermal sensitivity of sensory neurons can lead to thermal allodynia, hyperalgesia and chronic pain. Temperature-sensitive ions therefore represent attractive targets for novel analgesic drugs.

In the last decades, temperature-sensitive Transient Receptor Potential (TRP) channels have been put forward as the main thermosensitive elements in sensory neurons. In this lecture, current insights into the molecular nature of TRP channels involved in cold and heat sensation will be discussed, and examples will be provided of the mechanistic basis and potential therapeutic implications of TRP channel modulation by chemical ligands.

INTERACTIONS BETWEEN THE "ENDOCANNABINOIDOME" AND THE "TRPOME": A NEW "OME" FOR PHYTOCANNABINOIDS AND LIPID MEDIATORS?

Vincenzo Di Marzo

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In 1999, it was proposed that anandamide, apart from CB1 and CB2 receptors, also activates transient receptor potential of vanilloid type-1 (TRPV1) channels. Over the last decade, this interaction has been shown to occur both in peripheral tissues and brain, during both physiological and pathological conditions. TRPV1, as well as other vanilloid-type TRP channels (namely TRPV2, TRPV3 and TRPV4), transient receptor potential of melastatin type-8 (TRPM8) channels and transient receptor potential of ankyrin type-1 (TRPA1) channels, are activated also by non-physiological high or low temperatures, and are thus grouped in that subfamily of the "TRPome" (including more than 60 members) known as "thermo-TRPs".

TRPV1 channels, can be activated also by another less abundant endocannabinoid, *N*-arachidonoyl-dopamine (NADA), and other unsaturated *N*-acyl-dopamines, and have been proposed by some authors to act as ionotropic endocannabinoid receptors. Furthermore, both anandamide and NADA antagonize TRPM8 channels. Recently, also 2-arachidonoylglycerol was found to exert activity at TRPV1, and we have confirmed this action, which, however, is exerted at concentrations higher than those required to anandamide to activate this channel. Unsaturated *N*-acyl-serotonins, which were previously known to act as inhibitors of fatty acid amide hydrolase, and hence as "indirect" CB1 and CB2 agonists, are instead *antagonists* of TRPV1 and were recently found to occur in animal tissues. Finally, several other members of the "endocannabinoidome", such as the anandamide congeners, oleoylethanolamide and palmitoylethanolamide, also activate TRPV1, whereas some *N*-acyl-taurines activate both TRPV1 and TRPV4. It is likely that also other members of the large family of endogenously occurring amides between fatty acids and amino acids or amine transmitters influence the activity of TRP channels.

Interestingly, although exhibiting in most cases very low affinity for, and functional activity at, CB1 and CB2 receptors, non-THC phytocannabinoids, such as cannabidiol, cannabigerol and cannabichromene, also interact at low micromolar or submicromolar concentrations with "thermo-TRPs", which mediate in part the pharmacological activity of these compounds. Thus, both the "endocannabinoidome" and the "phytocannabinoidome" overlap in part with the "TRPome".

I will discuss the latest discoveries on this subject from my and other laboratories and touch upon the complications of pharmacologically manipulating the endocannabinoid system in view of it being part of a wide "endocannabinoidome" with multiple signaling characteristics.

Special ICRS Speaker

OPTOGENETIC APPROACHES TO STUDYING REWARD AND SUBSTANCE ABUSE DISORDERS

Antonello Bonci, Ph.D.

National Institutes of Health

The ventral tegmental area (VTA), nucleus accumbens (NAC) and prefrontal cortex (PFC) are all part of the limbic system and play a fundamental role in motivation, reward- and drug-dependent behaviors. A few years ago, my laboratory has shown that drugs of abuse such as cocaine can produce long-term synaptic plasticity and that the duration of such plasticity is dependent upon the modality of drug or reward administration. By applying a multidisciplinary approach that includes electrophysiology, optogenetics and behavioral procedures, my laboratory has produced a series of studies aimed at defining the pathways that control and modulate reward and drug-dependent behaviors. During my presentation, I will present the latest data on the cellular mechanisms and pathways that underlie reward substance use disorders.

STUDY OF THE EXPRESSION PROFILE AND PHARMACOLOGICAL ROLE OF THE CB1 RECEPTOR IN DUCHENNE MUSCULAR DYSTROPHY (DMD) MUSCLES: A NEW OPPORTUNITY TO REINFORCE MUSCLE REPAIR AND LOCOMOTOR ACTIVITY

Fabio Arturo Iannotti[1], Enrico Mazzarella[1], Ester Pagano[2], Elisabetta Gazzero[3], Raffaele Capasso[2] and Vincenzo Di Marzo[1]

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Duchenne muscular dystrophy (DMD) is a hereditary myopathy that causes the progressive degeneration of skeletal muscle tissue. It represents one of the most common forms of muscular dystrophy, which mainly affects young males, since it is determined by alterations present in the gene encoding for the structural protein dystrophin, which is on the X chromosome. The loss of dystrophin function causes irreversible muscle damage (1). Prognosis is still poor, given that death of affected patients occurs in most cases within 10-15 years of age due to respiratory failure.

We recently showed that in both murine and human skeletal muscle cells the stimulation of CB1 receptor impairs myotube formation and inversely increases the rate of myoblast proliferation (2). The purpose of this study was to investigate the potential implication of CB1 during DMD progression. Towards this aim, we examined CB1 expression and function in skeletal muscles of Dmd^{mdx} mice, which represents the most utilized animal model of DMD (3). We found that in all the affected skeletal muscles investigated, among all the genes belonging to the endocannabinoid system, *Cnr1* and *Cnr2* showed the highest degree of up-regulation at weeks 5-6, the point of disease onset. Similar changes were observed for the *Pax7*, *Myod*, *Myf-5* genes. These genes are largely expressed in muscle satellite cells (SC) and serve to initiate and drive SC differentiation to replace injured muscle fibers (4). These changes were also detected in the muscles of children affected by DMD both at 3 and 7 years of age. Intriguingly, we found that CB1 stimulation by ACEA, anandamide and 2-AG (1-3 μM) increased the proliferation rate of primary human SC. By bio-informatic analysis we found putative consensus sites for PAX7 in both mouse and human *Cnr1* gene. By means of a luciferase reporter assay we provide preliminary evidence that functional PAX7 binding sites are located proximal to, and hence may regulate the expression of, *Cnr1*. Importantly, treatment of Dmd^{mdx} mice with the CB1 inverse agonist rimonabant (0.5mg/kg IP, 3 times a week for 3 weeks), resulted in a marked increase in locomotor activity as assessed by the rotarod assay. In conclusion, all these findings indicate a novel role for CB1 in the development of degenerative muscle disease, perhaps by affecting muscle differentiation and repair processes, thus making of this receptor a potential therapeutic target for the treatment of such disorders.

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2) Iannotti et al. (2013) *ICRS Conference – Vancouver (Canada)*
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PALMITOYLETHANOLAMIDE IN A RAT MODEL OF OSTEOARTHRITIS: ITS ANTI-INFLAMMATORY AND ANTINOCICEPTIVE EFFECTIVENESS IN COMPARISON WITH NIMESULIDE AND ACETAMINOPHEN

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Osteoarthritis (OA) is the most prevalent joint disease that reduced quality of life. Acetaminophen, and nonsteroidal anti-inflammatory drugs (NSAIDs) are employed for pain relief associated with OA. However, their prolonged use induces serious side effects. So the identification of alternative drugs is crucial for the OA pathology. Starting from previous preliminary data presented at 21st Annual Symposium on the Cannabinoids (Costa B., Russo D., Ronzulli D., and Comelli F.; Experimental osteoarthritis in rats is attenuated by oral administration of palmitoylethanolamide (2011) 21st Annual Symposium on the Cannabinoids International Cannabinoid Research Society, Research Triangle Park, NC, USA, Page 7), the aim of this present study is to confirm the anti-inflammatory, and antinociceptive efficacy of palmitoylethanolamide (PEA), an endogenous lipid analogous of the endocannabinoid anandamide, in the well known OA model induced by intrapatellar injection of MIA, and especially to compare its effect with that evoked by nimesulide and acetaminophen, in order to propose the OA treatment with PEA in clinic. PEA 50 mg/kg, nimesulide 10 mg/kg, and acetaminophen 300 mg/kg were orally administered for 21 consecutive days starting from the day after the pathology induction. As expected, rats developed a significant knee swelling, as index of inflammation. We observed that PEA was able to abolish knee swelling, as nimesulide, and acetaminophen treatment. Additionally, as further index of inflammation, the day after MIA-injection, rats developed a significant decrease in thermal withdrawal latency. Treatment of MIA rats with PEA resulted in a significant relief of thermal hyperalgesia, as observed after nimesulide, and acetaminophen administration. After MIA-injection, rats also developed mechanical allodynia, as an index of chronic pain. Treatment of MIA rats with PEA resulted in a significant, even if partial, relief of mechanical allodynia, as nimesulide, and acetaminophen treatment. However, PEA anti-allodynic effectiveness was major than that elicited by nimesulide, and acetaminophen. We also evaluated the motor functionality by a walking track analysis. In particular, according with the footprints, the SFI (Sciatic Functional Index) value was calculated: a value approximately around zero indicates a normal locomotor function, while a value close to -100 indicates a significant impairment of locomotor function. As expected, intra-articular injection of MIA resulted in a significant increase of joint discomfort. PEA treatment completely restored locomotor functionality and this effect remained stable after one week of treatment, as nimesulide and acetaminophen treatment. However, only PEA treatment preserved such an effect at the end of the treatment. In addiction, we observed a mild/moderate cartilage damage after MIA injection (large chondral erosions, and exposure of subchondral bone). Repeated PEA treatment preserved cartilage from damage, conversely to repeated nimesulide, and acetaminophen treatment. OA patients show elevated levels of pro-algogen mediators such as the nerve growth factor (NGF) in their synovial fluid. Starting from these observations, we also determined the NGF levels in the synovial fluid of MIA rats. Only PEA treatment completely restored the physiological NGF level. In conclusion, we demonstrated that the repeated administration of PEA reduced knee swelling, mechanical allodynia, thermal hyperalgesia, motor impairment, and slowed the degradation of cartilage interposition in MIA-induced osteoarthritis model. PEA efficacy was superimposable, and in some cases greater than that evoked by nimesulide and acetaminophen, two of the most drugs used for OA treatment, so suggesting a therapeutic use of PEA in clinic, without causing side effect.

Acknowledgments: authors are grateful to *Epitech group* for supporting this study.

MECHANISMS OF HUMAN EOSINOPHIL MIGRATION INDUCED BY THE ENDOCANNABINOID 2-ARACHIDONOYL-GLYCEROL

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BACKGROUND. Eosinophils are leukocytes involved in numerous inflammatory diseases such as asthma and possibly obesity. How they migrate to the tissues is not completely defined but is recognized to involve chemokines and/or bioactive lipids, noteworthy prostaglandin (PG) D_2 and 5-oxo-eicosatetraenoate (5-KETE). In mice, eosinophils might play an important role in adipose tissue maintenance. Although the chemoattractants recruiting eosinophils to the latter are unknown, it has been suggested that IL-5 might play a role. The endocannabinoid 2-arachidonoyl-glycerol (2-AG), found in the adipose tissue, activates eosinophils, is recognized to activate the cannabinoid receptors CB_1 and CB_2 and can regulate leukocyte functions through its metabolites. Herein, we investigated the mechanisms involved in the 2-AG-induced migration of human eosinophils.

RESULTS. IL-5 increased eosinophil migration induced by 2-AG from ~5% to ~40%. This was mimicked by IL-3 and GM-CSF but not by IL-13 or IFN-γ. The effect of IL-5 was rapid and blocked by the Lyn inhibitor PP2. In contrast, IL-5 did not increase the migration of eosinophils induced by prostaglandin D_2 and 5-oxo-eicosatetraenoate (5-KETE). The effect of 2-AG on migration was prevented by the CB_2 antagonists AM-630 and SR144528, but was not mimicked by anandamide or other CB_2 agonists. 2-AG, but not anandamide, also induced the biosynthesis of leukotrienes and eoxins, which was totally blocked by the 2-AG hydrolysis inhibitors MAFP and JZL-184. Using inhibitors, we determined that the 2-AG-induced migration of eosinophils involved 2-AG hydrolysis and 15-lipoxygenase, in contrast to that mediated by 5-KETE. However, lipoxin A_4, EXC_4, 15-HETE, or 15-HETE-glycerol did not recapitulate the effect of 2-AG.

CONCLUSIONS. The 2-AG-induced migration of human eosinophils requires IL-5. The effects of 2-AG likely involve MAG lipase, unidentified 15-lipoxygenase metabolites, and the CB_2 receptor.

Acknowledgements: this work was supported by grants from the Canadian Consortium for the Investigation of Cannabinoids (CT), the Canadian Institutes of Health Research (NF), the Fonds de recherche du Québec-Santé (NF), and the Natural Sciences and Engineering Research Council of Canada (FN).

PERIPHERAL TARGETING OF CB1 CANNABINOID RECEPTORS PROTECTS FROM RADIATION-INDUCED PULMONARY FIBROSIS

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Radiation-induced pulmonary fibrosis (RIF) is a severe complication of thoracic radiotherapy which limits its dose, intensity, and duration. CB1 cannabinoid receptor-mediated signaling emerges as an active promoter of liver and skin fibrogenesis; however, there is no information if CB1 cannabinoid receptors are involved in pulmonary fibrotic diseases. Here we tested a hypothesis that CB1 cannabinoid receptors take active part in the onset and progression of pulmonary fibrogenesis using a mouse model of RIF.

Our mouse model of RIF consists of 20 Gy irradiation applied to thoracic area of C57Bl/6 mice with head, abdomen, and other parts of the body shielded from irradiation. C57Bl/6 mice are sensitive to irradiation and develop RIF and die between 15 and 19 weeks post-irradiation when such a dose of irradiation is applied to thoracic area. The treatment of C57 BL/6 mice with peripherally restricted CB1 antagonist AM6545 (1 mg/kg i.p., 3x/week) from the day animals received thoracic irradiation increased animal survival and significantly delayed the onset of RIF as confirmed by decreased upregulation of markers of fibrosis and inflammation and by decreased lung tissue collagen deposition and inflammation in comparison to solvent-treated controls. The use of global CB1 knockout mice generated on the same C57BL/6 background offered similar protection from RIF. Our study provides the first evidence that radiation-induced pulmonary fibrosis can be controlled through selective targeting of CB1-mediated signaling.

Supported in part by National Institutes of Health grants HL P01 98050 to E.B, DA3801 and DA9158 to A.M., and by National Institute of Alcohol Abuse and Alcoholism Intramural Research Program to P.P.

ENDOCANNABINOID AUGMENTATION BY SUBSTRATE-SELECTIVE COX-2 INHIBITORS: MECHANISM AND *IN VIVO* PROBE DEVELOPMENT

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The development of endocannabinoid (eCB) degradation inhibitors has significantly advanced the therapeutic potential of eCB signaling for a variety of pathological conditions. We and others have previously demonstrated that COX-2 regulates brain eCB levels through oxygenation of AEA and 2-AG. While COX-2 inhibitors increase eCBs levels, they also modulate arachidonic acid (AA) and prostaglandin (PG) levels. We have developed novel probes termed "substrate-selective" COX-2 inhibitors that selectively augment eCBs without inhibiting PG production.

Substrate-selective COX-2 inhibition arises from the fact that although COX-2 is a structural homodimer, it is a functional heterodimer. Kinetic analyses have revealed that substrate-selective COX-2 inhibitors bind in the allosteric COX-2 subunit and act as non-competitive inhibitors of eCB oxygenation in the catalytic subunit. We have found that mutation of Leu-531 to Ala results in the abrogation of substrate-selective COX-2 inhibition by (*R*)-flurbiprofen, lumiracoxib, and mefenamic acid. A crystal structure of lumiracoxib bound to murine L531A reveals that this lack of inhibition is not a result of altered inhibitor binding. Taken together, these findings indicate that binding of a substrate-selective inhibitor to the allosteric COX-2 subunit causes a rotation of Leu-531 in the catalytic COX-2 subunit such that a steric clash with the glycerol head group of 2-AG occurs, leading to non-competitive inhibition of 2-AG.

Previous studies have identified LM-4131 as an *in vivo* substrate-selective COX-2 inhibitor; however, it does not have optimal *in vivo* pharmacological properties. We screened a small library of promising *in vitro* substrate-selective COX-2 inhibitors and identified lumiracoxib as a potent *in vivo* substrate-selective COX-2 inhibitor. Lumiracoxib increases brain AEA and 2-AG in acute and chronic dosing regimens up to 8 hours after treatment, but also increases the levels of AA and 2-OG. Co-treatment of lumiracoxib with the MAGL inhibitor JZL-184 abolishes the increase in AA and 2-OG, suggesting that inhibition of COX-2 by lumiracoxib leads to a transient increase in 2-AG and subsequent hydrolysis by MAGL to AA. In addition, lumiracoxib is soluble in aqueous buffers and is orally bioavailable. The duration of action of lumiracoxib and the lack of PG inhibition in chronic studies identifies lumiracoxib as an improved *in vivo* substrate-selective COX-2 inhibitor for studying eCB augmentation by COX-2.

Acknowledgements: This work is supported by the A.B. Hancock Jr. Memorial Laboratory for Cancer Research and by grants from the National Institutes of Health (CA89450, GM15431, DA031572, MH090412, and MH100096). Analytical studies were conducted in the Vanderbilt University Mass Spectrometry Research Center.

ROLE OF 5HT1A RECEPTORS ON THE NEUROPROTECTIVE AND NEUROBEHAVIORAL EFFECTS OF CANNABIDIOL IN HYPOXIC-ISCHEMIC NEWBORN PIGS

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Background: Blockade of 5HT1A receptors (5HT1AR) antagonizes the neuroprotective effects of cannabidiol (CBD) in the first 6 hours after a hypoxic-ischemic (HI) insult in newborn pigs (Pazos et al, Neuropharmacology 2013). The aim of the present work was to study whether or not 5HT1A blockade interferes with CBD neuroprotective and neurobehavioral effects in a longer period-72 h. **Methods:** 1 day-old piglets were studied for 72 h after a HI insult (carotid clamp and FiO2 10% for 20 min). Thirty min after HI piglets received vehicle (HV, n=8) or CBD 1 mg/kg single dose or in 3 repeated doses u.i.d (respectively, HC1, n=5; and HC3, n=6), alone or with the 5HT1AR antagonist WAY100630 1 mg/kg/12 h, 15 min before the corresponding CBD dose (respectively, HC1W, n=4; HC3W, n=4). Non HI piglets served as controls (SHM, n=4). Every 24 h brain activity was assessed by amplitude-integrated EEG (aEEG) and a neurobehavioral score was carried out including eating behaviour assessment. Object-related and social playfulness activity was assessed by video recording, and anxiety was quantified by the restless time during holding for aEEG recording. At the end of the experiment the brain was obtained. Proton magnetic resonance spectroscopy (H^+-MRS) was made on frozen brain samples to assess neuroprotection by neuronal and astroglial damage quantification by lactate/n-acetyl aspartate (Lac/NAA) or myoinositol/creatine (mI/Cr) ratios, respectively. **Results:** CBD treatment restored brain activity (aEEG) and neurobehavioral performance 72 h postHI (Table). CBD blunted HI-increase of Lac/NAA and decrease of mI/Cr. In addition, CBD induced an anxiolytic effect and restored playfulness. There were no differences between HI piglets receiving single dose or three doses of CBD. CBD neuroprotective effects as shown by brain activity and motor performance and H^+-MRS biomarkers were blunted by 5HT1AR antagonism in animals receiving CBD single dose but not in those receiving CBD for three days. CBD effects on anxiety and playfulness responses, however, were reversed by 5HT1AR antagonism in all CBD-treated animals no matter the dosage schedule. The 5HT1AR antagonist did not worse HI effects on vehicle-treated animals and had no effects on sham animals.

Item	SHM	HV	HC1	HC3	HC1W	HCW3
aEEG (μV)	18(2)	*14(1)*	20(3)	19(2)	***12(3)***	18(2)
Lac/NAA	0.87(0.1)	*2.17(0.4)*	0.96(0.1)	1.1(0.1)	***2.3 0.5)***	1.1(0.1)
mI/Cr	1.6 (0.1)	*1.3(0.1)*	1.42(0.1)	1.42(0.1)	***1.2(0.1)***	1.4 (0.1)
NBS (pts)	35.5(0.5	*29(2)*	35(1)	34.6(1)	***32.6(1)***	34.5(1)
Eating bhv (pts)	4.6(0.3)	*3.5(0.4)*	4.8(0.1)	4.8(0.2)	***3.1(0.3)***	4.4(0.1)
Restless (min)	3.5(1)	*6.7(1)*	*2.2(1)*	*2.4(1)*	***5.2(1)***	***6.3(1)***
Object play (%)	14(3)	*17(8)*	22(6)	24(8)	***10(2)***	***11(3)***
Social play (%)	52(6)	*23(8)*	40(6)	37(8)	***16(5)***	***15(6)***

Items measured 72 h postHI. *Italic:* p<0.05 vs. SHM. **Bold:** p<0.05 vs HC

Conclusions: 5HT1AR activation is involved in CBD neuroprotection in the first 24 hours postHI. Later on CBD is able to induce neuroprotection by 5HT1AR-independent mechanisms. 5HT1AR activation mediates anxiolytic and some behavioural effects of CBD.

Supported by FIS PI09/01900, PI1200192 and GWCRI09119

PROTECTIVE ROLE OF THE CANNABINOID RECEPTOR SYSTEM IN AN *IN-VITRO* MODEL OF AGE-RELATED MACULAR DEGENERATION (AMD)

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Accumulation in the retinal pigment epithelium (RPE) cells of A2E, a pyridinium bis-retinoid, has the potential to cause RPE cell death and may contribute to the RPE cell atrophy that is observed in AMD. The cannabinoid receptor system is presented in human RPE cells and is intimately involved in the oxidative damage process, including that associated with AMD. It has been shown that levels of endocannabinoids are significantly increased in retinal tissue from AMD donors.

This study aimed to investigate the effects on pro-inflammatory agents and specific oxidative intracellular signalling related proteins- MAPKs, by *in vitro* AMD-A2E model, in the presence and absence of cannabinoids (HU-210 and HU-308), endocannabinoid (anandamide), non-psychoactive cannabinoid (CBD) and cannabinoid antagonists (SR-141716A [CB1] and SR-144528 [CB2]). By using this unique *in vitro* model, which includes A2E-loaded RPE cells exposed to blue light irradiation, we can mimic the oxidative stress taking place in AMD and leading to inactivation of the proteasome.

Experiments were conducted to investigate the ability of cannabinoids and endocannabinoids to attenuate the effect of A2E on the pro-inflammation agent's secretion and MAPKs pathway activation. When the RPE cells were exposed to A2E and blue light, anandamide and CBD, successfully and significantly, reduced the expression and secretion of IL-8 by deactivation of the p38 MAPK. Irradiated A2E-loaded RPE cells suppressed the expression and secretion of MCP-1, while anandamide, HU-210, HU-308 and CBD lead to up-regulation of MCP-1. A significant decrease in several MAPK signals was also determined in the AMD-A2E model, in the presence of the cannabinoids. The above signals were measured in presence and absence of the selective CB1 and CB2 antagonists for pathways clarification.

The results support our hypothesis that the newly discovered cannabinoid receptor system may attenuate AMD generated by the accumulation of A2E and may contribute to delaying or arresting A2E-related toxicity to the RPE. We assume that inactivation of the proteasome by A2E is a mechanistic link between oxidative stress and altered inflammatory responses. The down-regulation of MCP-1, under extensive oxidative stress by A2E, may have physiological consequences since it was reported that MCP-1 knockout mice developed AMD-like phenotypes. Therefore up-regulation of MCP-1 by the cannabinoids is suggested for neuroprotection in AMD.

THE CANNABINOID-1 RECEPTOR IN CARDIAC FIBROSIS

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The endocannabinoid system is an emerging target in chronic cardiac diseases, owing to its involvement in fibrogenesis, inflammation and cell death. The deregulation of this system has been implicated in myocardial infarction and subsequent heart failure. We have shown previously that chronic CB_1 blockade is cardioprotective in cardiac injury models such as diabetes and doxorubicin toxicity. A recent study suggested that CB_1 antagonists improve cardiac function and reduces adverse remodeling after myocardial infarction, but the exact mechanism of these beneficial effects is still unknown. The aim of our study was to identify signaling pathways and investigate their involvement in the chronic effect of CB_1 inhibition in cardiac fibrosis and left ventricular remodeling.

In a mouse model of cardiac fibrosis, angiotensin II (AII, 3 mg/kg/day) was administered by osmotic minipumps for 14 days. CB_1 receptor antagonist (rimonabant, 5 mg/kg), or vehicle was given every second day during the AII administration period. At the end of 2 weeks, hemodynamic parameters were measured by non-invasive echocardiography. Cardiac pressure volume catheter was used to evaluate global functional parameters. Cardiac tissue was collected for histologic and biochemical evaluation. Two weeks of AII infusion significantly increased systolic pressure in all groups, irrespective of treatment. Although no difference in systolic parameters was found between the groups, cardiac dysfunction was shown by altered myocardial performance index, which was significantly prevented by CB_1 antagonist treatment ($p<0.01$). There was also a significant reduction ($p<0.05$) of the left ventricular mass in he group of mice treated with the CB_1 antagonist. Fibrosis, assessed by collagen deposition, was significantly reduced in the CB_1 antagonist group by one fold. Reduced fibrosis was confirmed by downregulation of pro-fibrotic genes e.g. CTGF and Col1a1 in the same group. In vitro studies, using activated 3T3 cells, suggest that CTGF is downregulated after rimonabant treatment. Fibrogenic activation of primary fibroblast isolated from mice hearts induced extracellular matrix genes expression e.g. Col1a1, CTGF and fibrillin, which was sensitive to CB_1 antagonist treatment.

In conclusions, we found that chronic CB_1 antagonist treatment in AII-induced mice preserved LV function and cardiac fibrosis was reduced with concomitant downregulation of fibrogenic genes. The study helps to better understand the anti-fibrotic action of chronic CB_1 treatment. Novel generation of CB_1 inhibitors, devoid of neuropsychiatric side-effects, may be therapeutically explored in chronic heart failure.

Acknowledgement: Funded by the European Commission, FP7-PEOPLE-2011-CIG (Grant Nr: 294278).

THE ENDOCANNABINOID N-ARACHIDONOYL DOPAMINE (NADA) AND WIN55,212-2 MODULATE THE INFLAMMATORY ACTIVATION OF HUMAN ENDOTHELIAL CELLS

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Recent reports have shown that cannabinoids possess immunomodulatory activity and suggest that they may be able to regulate the activation of the endothelium in response to inflammatory mediators. However, it remains unclear which receptors and what mechanisms are responsible for this modulation. Understanding the specific role of cannabinoids in endothelial cell (EC) activation is critical, since ECs are centrally involved in the pathogenesis of organ injury in acute inflammatory disorders. ECs express cytokines and chemokines, which facilitate the trafficking of leukocytes to organs, and can decrease vascular barrier function. We hypothesized that ECs express cannabinoid receptors and enzymes required for cannabinoid metabolism, and that cannabinoid signaling pathways modulate endothelial inflammatory responses. We find that primary human ECs from multiple organs express the cannabinoid receptors CB_1R, GPR18, and GPR55, as well as the ion channel TRPV1. In contrast to leukocytes, CB_2R is only minimally expressed in some EC populations. Furthermore, ECs express all of the known endocannabinoid (eCB) metabolic enzymes. Examining a diverse panel of cannabinoids, we also demonstrate that the synthetic cannabinoid WIN55,212-2 and the eCB *N*-arachidonoyl dopamine (NADA), but neither anandamide nor 2-arachidonoylglycerol, reduce EC inflammatory responses induced by bacterial lipopeptide, LPS, and TNFα. We find that endothelial CB_1 and CB_2 receptors are necessary for the effects of NADA, but not those of WIN55,212-2. Furthermore, TRPV1 appears to counter the anti-inflammatory properties of WIN55,212-2 and NADA, but conversely, in the absence of these cannabinoids, its inhibition exacerbates the inflammatory response in ECs activated with LPS. These data indicate that the eCB system can modulate the inflammatory activation of the endothelium and may have important implications for a variety of acute inflammatory disorders that are characterized by EC activation.

Acknowledgements: This work was supported by the UCSF Department of Anesthesia and Perioperative Care, the National Science Foundation Graduate Research Fellowship Program (#1144247), and the UCSF Research Evaluation and Allocation Committee (REAC) Program (Huntington Fund).

NOVEL INHIBITORS OF FATTY ACID AMIDE HYDROLASE EXERT REMARKABLE ANTI-INFLAMMATORY EFFECTS BOTH *IN VITRO* IN HUMAN KERATINOCYTES AND *IN VIVO* IN NC/TND MICE

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High prevalence of skin diseases (e.g. atopic dermatitis [AD]), characterized by pathological cutaneous inflammatory processes, highlights the necessity of invention of novel, highly targeted therapeutic approaches, preferable possessing favorable side-effect profiles. It has recently been shown that the cutaneous endocannabinoid (eCB) tone is one of the key "gate-keepers" of the cutaneous inflammatory processes (Karsak et al, Science, 2007). Since fatty acid amide hydrolase (FAAH), a key enzyme involved in the degradation of the prototypic eCB anandamide, is an important regulator of this tone, in the current study, we intended to explore the potential anti-inflammatory activity of certain novel FAAH-inhibitors (compound -440 and -479) both *in vitro* in human epidermal keratinocytes (KC) and *in vivo* in NC/Tnd mice.

First, we confirmed that FAAH is indeed expressed in KCs. Moreover, quite intriguingly, we have also found that its expression was highly increased upon Toll-like receptor (TLR)-2, -3 or -4 activation, suggesting that its up-regulation and the subsequent decrease in the eCB tone might contribute to the pro-inflammatory action of the TLR-signaling. Next, by monitoring the expression of various pro-inflammatory target genes (interleukin [IL]-1α, IL1β, IL6, IL8), whose expressional alterations specifically indicated the development of the cellular inflammatory response of KCs upon lipoteichoic acid (LTA; a TLR2-activator) treatments (24 hrs), we investigated the putative anti-inflammatory effects of the inhibition of FAAH. We found that co-administration of the novel FAAH-inhibitors was able to prevent pro-inflammatory action of LTA both at the mRNA and at the released protein levels (IL6 and -8). Since these effects were effectively abrogated by the co-antagonism of CB1 and CB2 cannabinoid receptors, it can be proposed that they were indeed mediated by the elevation of the cutaneous eCB tone. Finally, since both inhibitors showed negligible cytotoxic effects on KCs following long-term (72 hrs) administration, we tested their efficiency *in vivo* by using the well-known animal model of AD, the NC/Tnd mice. We found that both inhibitors improved clinical score and reduced ear thickness of these mice statistically significantly; moreover, compound -440 reduced scratching behavior as well.

Taken together, our *in vitro* and *in vivo* data strongly argue for that the investigated novel FAAH-inhibitors are potent anti-inflammatory agents by targeting the "very first-line" players (i.e. the epidermal keratinocytes) of the cutaneous inflammatory responses. Therefore, our findings may encourage one to systematically explore, next in appropriate clinical trials, their putative therapeutic efficiency in cutaneous inflammatory diseases such as e.g. AD.

Acknowledgements: "Lendület" LP2011-003/2011, TÁMOP 4.2.4. A/2-11-1-2012-0001 "National Excellence Program", TÁMOP-4.2.2./A-11/1/KONV-2012-0025, OTKA 101761, OTKA 105369, BMWI (ZIM KF2611301MD0).

STIMULATION OF BONE MASS BY A NOVEL METHYLATED OLEOYL SERINE DERIVATIVE

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Bone mass is determined by a continuous remodeling process, whereby the mineralized matrix is being removed by osteoclasts and subsequently replaced with newly formed bone tissue produced by osteoblasts. Several endogenous fatty acyl amides are present in bone and affect bone cells. Of these compounds, oleoyl serine (OS) was found to be the most potent anti-osteoporotic agent in both *in vitro* and *in vivo* models. OS can be rapidly hydrolyzed by amidases that limit its activity. Here we tested whether the OS activity can be enhanced by adding a methyl group adjacent to the amide group, thus hindering the enzyme action. Two such derivatives were tested, oleoyl α-methyl serine (HU-671) and 2-methyl-oleoyl serine (HU-681), in which the added methyl group is on the respective serine and fatty acid part of OS. The results show that HU-671 stimulates the number of osteoblasts in culture with a peak effect at a concentration10-fold lower than that of OS, whereas HU-681 had its peak activity at a concentration an order of magnitude higher than that of OS. OS and HU-671, inhibit *ex vivo* osteoclast formation peaking at 10^{-11} and 10^{-13} M, respectively, in a culture of bone marrow derived monocytes incubated with M-CSF and RANKL. When osteoclasts are grown on dentin slices, both compounds inhibit *ex vivo* bone resorption expressed as a reduction in the number of resorption pits. HU-681 affects neither osteoclastogenesis nor pit formation. As in the case of OS, the effects of HU-671 and HU-681 on osteoblasts are blocked by pertussis toxin and the MEK/Erk1,2 inhibitor PD98059, suggesting that all three compounds target a signaling pathway consisting of a Gi-protein coupled receptor and Erk1,2. In a mouse model for osteoporosis daily treatment for 6 weeks with OS or HU-671 completely rescues bone loss. The increase in bone density consists primarily of enhanced trabecular thickness. The most effective dose of HU-671 is 0.5 mg/kg/day, an order of magnitude lower compared to OS. The reversal of bone loss resulted from both increased bone formation and decreased bone resorption. These results, based on quantitative morphology, were confirmed by determination of the serum bone remodeling markers osteocalcin (bone formation) and type 1 collagen C-terminal crosslinks (bone resorption). Taken together, these data suggest that methylation interferes with amidase activity, thus enhancing the OS *in vitro* and *in vivo* effects by extending its availability to target cells. In addition, the present data provides a preclinical proof for further development HU-671-based anti-osteoporotic therapy. The potential advantages of such therapy are the concomitant bone anabolic and anti-resorptive activity. HU-671 has logP of 5.17, closely matching the gold standard for oral bioavailability.

DIACYLGLYCEROL LIPASE BETA: NEW EVIDENCE FOR INFLAMMATORY AND NEUROPATHIC PAIN RELIEF IN MICE

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Diacylglycerol lipase (DAGL), the enzyme responsible for generation of 2-arachidonylglycerol (2-AG), represents a particularly interesting component of the endogenous cannabinoid (endocannabinoid) system as a potential therapeutic target to treat pain. This enzyme also regulates formation of arachidonic acid in mouse peritoneal macrophages in response to inflammatory insults. As DAGL-β inhibition leads to decreases in lipopolysaccharide (LPS)-induced prostaglandins (PGs) and proinflammatory cytokines, we hypothesized that inhibiting this enzyme will reverse nociceptive behavior in laboratory animal models of inflammatory and neuropathic pain. Both the inflammatory model of intraplantar LPS (2.5 μg) and chronic constriction injury of the sciatic nerve (CCI) model of neuropathic pain produce robust increases in sensitivity to light mechanical touch, or allodynia, as assayed with the von Frey test, and increased thermal sensitivity, or thermal hyperalgesia, as assayed in the hotplate test. Both allodynia and thermal hyperalgesia are sensory-discriminative pain evoked behaviors, but do not encompass the affective-motivational aspect of pain, which is often seen clinically. Pain-depressed behavioral models may therefore be additionally beneficial in determining the therapeutic potential of experimental compounds for the treatment of pain. Here a form of pain-depressed behavior in mice, digging, was examined via the marble burying assay.

Both systemic and local intraplantar injection of the selective DAGL-β inhibitor KT109 reversed LPS-induced allodynia in a time- and dose-dependent manner. As KT109 also has off target effects at ABHD6, we tested KT195, a compound structurally related to KT109 that selectively inhibits ABHD6 but is inactive against DAGL-β. Importantly, systemic or intraplanatar injection of KT195 had no effect on LPS-induced allodynia. Moreover, DAGL-β knockout mice displayed significant reductions in LPS-induced allodynia. The anti-allodynic effects of intraplantar KT109 were verified as being local, and not systemic, as its intraplantar administration into the contralateral paw did not reduce LPS-induced hyperalgesia or allodynia. Interestingly, neither intrathecal nor intracerebroventricular injection of KT109 reversed LPS-induced allodynia, consistent with a peripheral site of action. Systemic KT109 (40 mg/kg) also reversed CCI-induced allodynia and thermal hyperalgesia. Experiments using transgenic mice indicated that these antinociceptive effects did not require CB1 or CB2 receptors. Systemically administered KT109 also restored CCI-depressed digging behavior, suggesting that inhibiting DAGL-β also reverses the negative emotional component often associated with pain. Taken together, these findings suggest that DAGL-β represents a provocative target to treat both inflammatory and neuropathic pain.

Acknowledgements: Research was supported by NIH grants: DA009789, DA017259.

POSITIVE ALLOSTERIC MODULATION OF CB_1 WITH GAT211 SUPPRESSES PACLITAXEL-INDUCED NEUROPATHIC PAIN WHILE BYPASSING UNWANTED SIDE EFFECTS OF CB_1 RECEPTOR ACTIVATION

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Activation of cannabinoid CB_1 receptors suppresses pathological pain but also produces unwanted central side effects (e.g. psychoactivity) that constrain therapeutic dosing. We hypothesized that positive allosteric modulation of the CB_1 receptor would suppress neuropathic pain produced by chemotherapy treatment without producing cardinal signs of CB_1 receptor activation (i.e. hypothermia, catalepsy, hypoactivity and tail-flick antinociception). We evaluated a positive allosteric modulator of the CB_1 receptor, GAT211, for anti-allodynic efficacy in a model of chemotherapy-induced peripheral neuropathy produced by paclitaxel treatment. Responsiveness to mechanical and cold stimulation was assessed before, during and after establishment of paclitaxel-induced neuropathic pain. Anti-allodynic efficacy of GAT211 was assessed following acute and chronic dosing initiated during the maintenance phase of paclitaxel-induced allodynia. Effects of GAT211 on cardinal signs of CB_1 rector activation were also evaluated in a modified tetrad. Acute treatment with GAT211 (20 mg/kg i.p.) failed to produce antinociception in the tail-flick test, catalepsy in the ring test, motor ataxia or hypothermia. Moreover, GAT211 did not alter basal nociceptive thresholds to mechanical or cold stimuli in the absence of paclitaxel. However, GAT211 suppressed paclitaxel-induced mechanical and cold allodynia following both acute and chronic administration. Therapeutic efficacy was preserved over a chronic dosing period of 8 days with no appreciable signs of tolerance to antinociceptive efficacy. By contrast, repeated dosing with the prototypic classical cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) produced tolerance to the antinociceptive effects of the cannabinoid over the same dosing interval. Finally, the CB_1 antagonist rimonabant (10 mg/kg i.p.) did not produce CB_1-dependent withdrawal signs in mice treated chronically with GAT211. The results of these experiments suggest that positive allosteric modulation of the CB_1 receptor with GAT211 does not produce unwanted CNS side-effects commonly associated with CB_1 receptor activation. Our results also suggest that GAT211 remains efficacious in suppressing paclitaxel-induced allodynia following both acute and chronic dosing. Thus, positive allosteric modulation of CB_1 represents an alternate route for harnessing the therapeutic potential of the endocannabinoid signaling system to suppress neuropathic pain without unwanted CNS side effects. Allosteric modulation of CB_1 thus represents an analgesic strategy that may be exploited to bypass antinociceptive tolerance and detrimental CNS side-effects observed with prototypical cannabinoid agonists such as Δ^9-THC.

Supported by DA037673 (to AGH) and DA027113 (to GAT)

ANATOMY AND FUNCTIONAL ROLE OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 IN THE DENTATE GYRUS OF A KAINATE-INDUCED SEIZURES MOUSE MODEL

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The transient receptor potential vanilloid type 1 (TRPV1) is a non-selective cation channel that acts primarily as pain sensor in the periphery but also modulates neurotransmitter release and synaptic plasticity in the brain. TRPV1 function must lay on its anatomical distribution in the peripheral and central nervous system regions where the channel has physiological roles. Nevertheless, the anatomical localization of TRPV1 is well established in the periphery, but it is a matter of debate in the brain. We have recently demonstrated a high TRPV1 localization at the dentate molecular perforant path synapses (Puente et al., 2014). However, the TRPV1 distribution at inhibitory synapses in the dentate molecular layer is not yet characterized anatomically.

To this goal, we have used TRPV1 antibodies combined with a highly sensitive pre-embedding immunogold method for high resolution electron microscopy. TRPV1 immunoparticles were in dentate granule cell dendrites receiving symmetric synapses. The labelling was mostly confined to postsynaptic membranes and was distributed at a relative short distance from the inhibitory synaptic contacts. Importantly, the TRPV1 distribution pattern at inhibitory synapses disappeared in the molecular layer of TRPV1-knockout mice.

We have also investigated the effect of an intrahippocampal injection of kainic acid (50 nl of 20 mM) on the trigger of kainate-induced seizures in WT and TRPV1-KO mice, a mouse model of medial temporal lobe epilepsy (MTLE). The behavioral score revealed that seizures were milder in TRPV1-KO than in WT during kainate-induced status epilepticus. Prior to kainate injection, a significant increase of cannabinoid 1 (CB1) receptor immunoreactivity in the inner 1/3 of the molecular layer and an increase of DAGL-α and NAPE-PLD optical densities were observed in dentate gyrus of TRPV1-KO versus WT. These findings may suggest that the absence of TRPV1 in the dentate gyrus triggers some adaptative changes that may be beneficial in the control of epileptic seizures.

Acknowledgements: Funding for P. Grandes' laboratory is provided by Ministerio de Economía y Competitividad (BFU2012-33334); The Basque Country Government grant IT764-13, and by Red de Trastornos Adictivos, RETICS, Instituto de Salud Carlos III, grant number: RD07/0001/2001. Funding for A. Sierra´s laboratory is provided by Ministerio de Economía y Competitividad (BFU2012-32089).

OVEREXPRESSION OF TRPV1 IN HEK CELLS DRIVES DRAMATIC CHANGES IN BASAL ENDOCANNABINOIDS AND RELATED LIPIDS WHICH ARE POTENTIATED WITH STIMULATION BY CAPSAICIN

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Use of expression systems such as TRPV1-transfected HEK cells are an important molecular tool to study a range of pharmacological and biochemical pathways. Our groups routinely use TPR-HEK expression systems to determine novel ligand activity as well as push our understanding of intracellular cascades with known TRP activators. One aspect of cellular signaling that has been somewhat left out of this equation is the potential bioactive lipid modulation with activation by TRPs. Here, to test the hypothesis that activation of TRPV1 will drive changes in the cellular lipidome, we treated TRPV1-transfected HEK cells (hereafter called TRPV1-HEKs) with 100nM capsaicin for 5 minutes and then quenched the reaction with 5 volumes of methanol, cells were scraped from the flask and the cell/methanol solution was spiked with 100pmols of D8NAGly and then put on ice for 2 hours. At two hours, the solution was vortexed and then centrifuged at 19,000 x g for 15 min at 24C. Methanolic extracts were partially purified on C-18 solid-phase extraction columns. Eluants were analyzed with high-pressure liquid chromatography coupled to tandem mass spectrometry using an API 3000 triple quadrupole MS. The same protocol was used to compare non-transfected HEK cells with TRPV1-HEKs.

Activation by capsaicin in TRPV1-tranfected HEK cells produced a dramatic increase in 2-AG as well as Anandamide and a variety of additional structurally related lipids, however, no significant changes were measured in arachidonic acid. Release/production of 2-AG after TRPV1 activation suggests that this may be a mechanism of action for the synergistic interactions of TRPV1 and the Endocannabinoid system. In terms of overall lipidomics findings, one of the most striking differences were observed between the basal lipid levels of the WT HEK cells and the basal levels. Screening over 80 lipids between the two cell lines reveals that 34 distinct lipids in the screen were significantly different between the WT and the TRPV1-HEKs. Most notably, the levels of AEA and related N-acyl ethanolamines were significantly increased, whereas, the level of 2-AG, arachidonic acid and PGs were significantly decreased in the TPRV1-HEKs compared to the WTs. These data highlight new findings in the signaling pathways associated with TRPV1 activation as well as demonstrating that the overexpressing of TRPV1 alone is enough to dramatically change the lipidome of the cell.

ANANDAMIDE REGULATES MATURATION AND FUNCTION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS

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Dendritic cells (DC) are the most important professional antigen-presenting cells of the immune system and regulate the balance between immunity and tolerance. They play a major role in innate immune responses to infection as well as in linking innate and adaptive immunity. They exist in two different states that reflect their functional activity: immature (immDC) and mature (matDC) dendritic cells. ImmDC have high phagocytic activity and express low levels of antigen-presenting molecules (HLA-DR) and co-stimulatory activation markers (CD80 and CD86); however matDC have a central role in T cell activation, due to low phagocytic activity and high expression levels of HLA-DR activation markers. Although DC possess a fully functional endocannabinoid system, yet no evidence for a modulatory role of anandamide (AEA) on DC maturation and activation has been reported.

ImmDC and matDC were obtained from peripheral blood monocytes and were first differentiated into immDC for 6 days with granulocyte-macrophage colony stimulator factor and interleukin-4, and then matured with LPS for 2 additional days in presence or absence of AEA. We found that AEA-treated immDCs showed a remarkable decrease in surface activation markers (HLA-DR, CD80 and CD86) and an increase in their phagocytic activity. Conversely, AEA-treated matDCs expressed significantly higher levels of such surface markers, with no significant effects on their phagocytic activity. Finally, matDC were also tested for their ability to activate T cells upon DC/T cells co-cultures and we found that T cells released reduced levels of tumor necrosis factor-α following co-culture with AEA-treated matDC. These findings demonstrate for the first time that AEA, on one hand is able to directly affect DC capacity of processing the antigens and of co-stimulating nearby T cells, on the other hand it also affects the inflammatory immune responses of those co-stimulated T cells, thus suggesting that this endocannabinoid might play a key role in the regulation of the "innate-adaptive immune axis".

LEARNING UNDER STRESS DIFFERENTIALLY AFFECTS CANNABINOID MODULATION OF SPATIAL MEMORY RETRIEVAL IN RATS

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Literature evidence shows that variation in environmental aversiveness differentially influences spatial memory processes in rats (Akirav et al., Learn Mem. 11 (2004) 188-195; Salehi et al., Learn Mem. 17 (2010) 522-530). We have previously demonstrated that cannabinoid effects on memory are dependent on the aversiveness of environmental context and on the level of stress at the time of drug administration and/or training (Campolongo et al., Front Behav Neurosci. 20 (2012) 11; Campolongo et al., Neuropsychopharmacology. 38 (2013) 1276-1286). Moreover, we also showed that glucocorticoids interact with the hippocampal endocannabinoid system in impairing contextual aversive memory retrieval (Atsak et al., Proc Natl Acad Sci U S A. 109 (2012) 3504-3509).

Here we investigated the role of the hippocampal endocannabinoid system on spatial memory retrieval in rats trained under two experimental conditions that differed with respect to their training associated stress levels. To this aim adult Sprague Dawley rats were trained in a Morris Water Maze task at two different water temperatures (19° C and 25° C) in order to elicit different levels of emotional arousal (Salehi et al., Learn Mem. 17 (2010) 522-530). To test cannabinoid effects on spatial memory retrieval, the synthetic cannabinoid agonist WIN55,212-2, the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor JZL184 were bilaterally infused into the hippocampus 1 hr before the retrieval (probe) trial in separate groups of animals. We found that WIN55,212-2 impaired memory retrieval only in rats trained under more stressful conditions (19° C). Such effect was blocked by administration of the CB1 antagonist AM251. Interestingly, URB597 did not alter spatial memory retrieval performances in any of the two experimental conditions. However, highly comparable with WIN55,212-2 effects, JZL184 impaired spatial memory retrieval only in rats trained under higher stressful conditions *via* an interaction with CB1 receptors. Consistently, rats trained under higher stress displayed an increase in hippocampal 2-AG levels, both after the training and probe trials, and alterations in CB1 affinity and in the activity of the main 2-AG degradative enzyme MAGL after the probe trial than rats trained under less stressful conditions. The present findings indicate that the hippocampal endocannabinoid system plays a key role in mediating emotional arousal effects on spatial memory retrieval, shedding light on the neurobiological mechanism involved in the differential impact of stress on memory processes**.**

THE ROLE OF ENDOCANNABINOID SIGNALING IN MEDIATING EFFECTS OF SEVERAL STRESS SYSTEMS IN MEMORY CONSOLIDATION

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Glucocorticoid hormones enhance the consolidation of long-term memory of emotionally arousing experiences. Prior studies indicated that emotional arousal induces noradrenergic activation within the basolateral complex of the amygdala (BLA) and glucocorticoids affect memory by rapidly influencing this noradrenergic activity. However, the time frame of this influence is too rapid to be compatible with the genomic mechanism of glucocorticoids and thus possibly mediated by a nongenomic mechanism. Recent evidence showed that glucocorticoids interact with endocannabinoids, a retrograde messenger system in the brain, in regulating the stress response. Here, we examined whether the endocannabinoid system is involved in regulating glucocorticoid effects on the BLA noradrenergic arousal system in enhancing memory consolidation. We found that posttraining blockade of the cannabinoid receptor type 1 (CB1) by AM251 in the BLA prevents the inhibitory avoidance memory enhancement as induced by co-infused glucocorticoid receptor agonist RU 28362 or by membrane-impermeable glucocorticoid CORT-BSA or by corticotropin-releasing factor receptor activation (CRF). These findings demonstrate that CB1 receptor activation in the BLA is essential in mediating memory enhancement induced by several neurohormonal stress systems such as glucocorticoids and CRF. Accordingly, activation of CB1 receptors by WIN55,212-2 in the BLA results in an enhancement of inhibitory avoidance memory; however, this effects is blocked by the beta-adrenoceptor antagonist propranolol co-infused into the BLA, suggesting that endocannabinoids regulate the memory-modulatory effects of glucocorticoids via fast influences on the noradrenergic system within the BLA. These findings have important implications for understanding local network functions within the BLA in regulating the effects of several neurohormonal stress responsive systems on neural plasticity and memory consolidation.

FUNCTIONAL CHARACTERIZATION OF THE ENDOCANNABINOID SYSTEM DURING ZEBRAFISH (*DANIO RERIO)* EMBRYONIC DEVELOPMENT

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Over the last two decades the endocannabinoid system (ECS) has been intensively studied, and considerable advancements have been made in the understanding of its physiological roles in different vertebrate phyla. Zebrafish (*Danio rerio*) represents a low-cost and powerful vertebrate organism for disease modeling and drug screening applications (1). Very little is known about the developmental role of the endocannabinoid system in this teleost species, even though there is evidence indicating that this lipid signaling pathway is evolutionarily conserved within zebrafish (2,3).

In order to get initial insights into the role of the ECS during zebrafish development we utilized qPCR analysis to track the expression of 14 genes including the cannabinoid receptors as well as anandamide and 2-AG metabolic enzymes at different embryonic stages. Interestingly, the gene expression analysis revealed different trends for the genes regulating these two endocannabinoids. While anandamide catabolic and metabolic enzymes showed a similar time-dependent increase, indicating no net change in anandamide levels, two 2-AG hydrolyzing enzymes (*mgll*, *ptgs2b*) displayed a time specific down-regulation during the hatching period. Mirroring the gene expression trends, LC-MS quantifications confirmed that while anandamide levels remained constant over the time periods examined, 2-AG levels had a significant time-dependent increase with a peak corresponding to the hatching period. To further understand the function of 2-AG during zebrafish development, we studied the *mgll* spatial expression profile in zebrafish larvae by whole mount *in situ* hybridization. From early somitogenesis (14 hour post fertilization, hpf) and on, *mgll* was seen in several regions of the forebrain and hindbrain including the optic tectum and retinal ganglion cells of the eye. Given the abundance of 2-AG during zebrafish development, we also performed morpholino knock-down experiments on *dagla* and *mgll* genes in order to gain insight into their respective roles in the developing brain. Analysis of the morphant phenotypes, revealed that *mgll* and *dagla* morpholinos resulted in an expansion and loss of the mildline barrier, respectively, at the level of the midbrain hindbrain boundary associated with aberrant patterns of neuronal fasciculation and axon trajectories. Intriguingly, only the *mgll*-deprived embryos displayed a retinal loss of neuronal innervation/fasciculation, but both gene morphants showed a thinner optic chiasm with respect to the control.

Our study shows for the first time that a complete and redundant ECS is able to actively produce endocannabinoids during zebrafish development. Furthermore, we provide evidence of *mgll* gene distribution inside distinct areas of the embryo CNS underling a putative role of 2-AG during neurogenesis and brain patterning. Indeed, morpholino experiments suggest a possible role of this endocannabinoid acting as paracrine mediator for axonal pathfinding and fasciculation, and possible implications in optic development.

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EFFECTS OF DELETIONS IN FAAH ON THE ENDOCANNABINOID AND WIDE-RANGING-RELATED LIPIDOME IN EIGHT REGIONS OF THE MOUSE BRAIN

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Endogenous cannabinoids 2-AG, AEA, and the AEA metabolite *N*-arachidonoyl glycine (NAGly) are all derivatives of arachidonic acid (AA), a polyunsaturated fatty acid (PUFA) that is also a substrate for the production of prostaglandins (PGs). The directionality of the relationship among these classes of AA-derived lipids is an important biochemical factor in how enzymes that regulate each of the classes of lipids affect the available pools of the others. Fatty acid amide hydrolase (FAAH) is hypothesized to be responsible for the majority of AEA hydrolysis in the brain. Even though FAAH is best known for its role in AEA hydrolysis, it can also hydrolyze other *N*-acyl amides, such as the *N*-acyl ethanolamines. Therefore, deleting FAAH may have effects on levels of other bioactive lipids that are both AA-derived as well as other classes of lipids derived from other fatty acids. This study aims to elucidate the effects of genetic deletion of FAAH on the *N*-acyl amide, 2-acyl glycerol, and PG lipidome in the mouse striatum, hippocampus, cerebellum, thalamus, cortex, hypothalamus, midbrain and brainstem. Groups of 6 FAAH knockout (KO) mice were each compared to 6 age and sex matched wild-type (WT) mice from the same C57 genetic background. Animals were sacrificed, brains were removed and targeted areas were dissected and stored at -80C. Methanolic extracts were partially purified on C-18 solid-phase extraction columns. Eluants were analyzed with high-pressure liquid chromatography coupled to tandem mass spectrometry using an API 3000 triple quadrupole MS.

Results here highlight those of AEA, 2-AG, NAGly, and PGs; however, many differences were observed in other lipids in these FAAH KO mice. Replicating previous studies, levels of AEA were higher in FAAH KO mice across all eight brain regions. *N*-arachidonyl serine was likewise increased, but only in the striatum, thalamus, and brainstem. Interestingly, 2-AG levels were *significantly reduced* with regional specificity in the cerebellum, thalamus, and cortex of FAAH KO mice. FAAH KO mice displayed lower levels of NAGly and *N*-arachidonyl GABA in all brain regions, and levels of 6 additional *N*-arachidonyl acyl amides were significantly lowered by FAAH deletion as well. However, in stark contrast to the data from MAGL deletion presented here last year, deleting FAAH had no impact on levels of PGE_2 or free AA. These data replicate and greatly extend the finding that deletion of FAAH drives changes in a range of AA metabolites and extends this to a wider range of *N*-acyl amides. This pattern of shifts in substrates and products appears largely unique to AA derivatives, as the majority of effects on the *N*-acyl amide lipidome of other fatty acid derivatives showed an increase in levels throughout. These data demonstrate that FAAH has differential effects on lipid biosynthetic and metabolic pathways that are dependent on the specific fatty acid derivatives that are interacting with the enzyme and that AA derivatives have a unique interplay with FAAH that drives a variety of different biochemical outcomes.

NOVEL CB_2 SELECTIVE CANNABINOID-ORTHOQUINONES EFFECTIVE FOR THE TREATMENT OF TRIPLE NEGATIVE BREAST CANCER AND LACKING NON-TUMOR CELL TOXICITY

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Triple-negative breast cancer is characterized by tumors that do not express estrogen receptors (ER), progesterone receptors (PR), or HER-2 receptors. This type of cancer does not respond to endocrine therapy or other available targeted agents so it represents an important clinical challenge. Consequently, there is an evident need to develop new therapeutic strategies for the management of this disease. Chemotherapy with its well-known side effects is currently used as systemic treatment for this cancer. It is why selective toxicity for cancer, but not normal cells, is crucial in the discovery of potent antitumor drugs.

In that context, new chromenopyrazolediones have been designed and synthesized as anticancer agents using the multibiological target concept that involves quinone cytotoxicity and cannabinoid antiproliferative properties. In radioligand competition binding experiments, these compounds showed to be fully selective CB_2 cannabinoid receptor ligands with affinity in the nanomolar range, with affinity for the CB_1 receptor higher than 40 μM which eliminates any side psychotropic effect derived from their activity at these receptors. Concerning their antitumor activity, they decreased cell proliferation in human a triple negative breast cancer cell line (MDA-MB-231) using MTT assays. An additional important fact is their lack of significant cytotoxicity against normal human mammary epithelial cells (HMEC). Further mechanistic studies allowed us to determine that these antitumor effects were mediated through activation of CB_2 receptors and through induction of oxidative stress. As confirmed by western blot analysis using active caspase-3 as biomarker, these compounds induced apoptosis in the aforementioned breast cancer cell line.

In summary, we have designed and synthesized a series of chromenopyrazolediones, with selectivity for the CB_2 receptor, which may serve for the development of new antitumoral therapies for the treatment of those forms of aggressive and hormone-independent breast cancer.

Acknowledgements: Supported by Spanish Grant SAF2012-40075-C02-02 and CANNAB-CM S2011/BMD-2308. P.M. is recipient of a fellowship JAE-Pre-2010-01119 from CSIC co-financed by FSE.

IDENTIFYING POTENTIAL UPSTREAM REGULATORS OF THE ENDOCANNABINOID SYSTEM IN PROSTATE CANCER

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Introduction: A high CB_1 receptor expression in prostate cancer (Pca) tumour tissue is associated with a poor disease outcome (Chung et al., Eur J Cancer 45 [2009] 174-182). There are a number of down-stream mediators of CB_1 receptor signalling in Pca (review, see Guindon & Hohmann, Br J Pharmacol 163 [2011] 1447-63), but little is known about upstream regulators of this receptor. In the present study, we have used a Bayesian network analysis of a well-characterised Pca database to identify potential candidates.

Method: The database for the study contained immunoreactive scores for tumour and non-malignant tissue for the following parameters: CB_1 receptor (CB_1R), FAAH epidermal growth factor receptor (EGFR) and its phosphorylated form (pEGFR), the related growth factor receptor ErbB2, the EGFR modulator LRIG1, platelet-derived growth factor receptor ß (PDFRß), androgen receptor (AR), von Willebrand factor (vWf), endoglin, hyaluronan and mast cell densies. Bivariate correlation analysis indicated that the CB_1IR was significantly correlated with FAAH, pEGFR, ErbB2 and LRIG1, and so these were used for the Bayesian analyses, which were conducted using the max-min hill-climbing method to create Directed Acyclic Graphs. Bootstrap analyses were conducted to assess the degree of robustness of the findings.

Results: For the non-malignant samples, two Directed Acyclic Graphs were constructed, one with three variables (CB_1R, pEGFR and LRIG1, n=263) and one with four variables (as above + FAAH, n=221). In both cases, directionality pEGFR → CB_1R was seen. For the tumour samples (n=267-274 depending upon the number of variables used), the same directionality was seen, but only when FAAH was included in the analysis. An additional pathway ErbB2 → FAAH was also found in the tumour samples.

Conclusion: The data suggest that EGFR may be an upstream regulator of CB_1 receptors. Together with data from the literature using cultured cells (e.g. Mimeault et al. Prostate 56 [2003] 1-12) the present analyses can be used to propose a model whereby EGFR, in addition to its other cellular actions (EGFR is well established as a pathogenic factor in Pca), increases CB_1 receptor densities which then respond to local endocannabinoids to feedback inhibit EGFR signalling as well as producing their own signalling responses. In tumour tissue, however, the increased expression of endocannabinoid metabolic enzymes (such as FAAH and cyclooxygenase-2) reduces the levels of the endocannabinoids, thus weakening this feedback regulatory pathway and hence allowing deleterious EGFR-mediated overactivity to proceed unchecked.

Supported by grants from the Swedish Science Research Council and the Swedish Cancer Research Foundation

BETA-ARRESTIN2 APPEARS TO MEDIATE THE ACTIVITY OF CANNABINOIDS IN FEMALE MICE IN A MANNER THAT DIFFERS FROM MALES

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Previous studies of sex differences in the responses to cannabinoids have found that generally cannabinoids produce the same effects in both sexes, but mostly minor differences in the degree of some effects exist. Many of the differences have been attributed to differences in rates of metabolism or in percent muscle mass or fat distribution between the sexes. We have found evidence that cannabinoid receptor signal transduction with respect to beta-arrestin2 differs between male and female mice and may underlie some of the observed sex differences.

We have previously reported that male beta-arrestin2 knock-out mice exhibited greater temperature depression and antinociceptive effects than wild-type mice treated with Δ^9-tetrahydrocannabinol (THC)[1]. *In vitro* studies showed that THC stimulated more [^{35}S]GTPγS binding to brain membranes from beta-arrestin2 knock-out mice than from wild-type[2]. Other agonists tested in males, including CP55940, methanandamide and O-1812, did not show any differences between those genotypes for any of these assays performed in male mice.

More recent studies in female mice have shown very different effects from what was seen in males. The antinociceptive (but not rectal temperature) effects of THC obtained in wild-type females were nearly absent in beta-arrestin2 knock-outs. Similar to male mice, the effects of CP55940 did not differ between the genotypes in female mice. Currently, *in vitro* assays to assess [3H]SR141716A and [^{35}S]GTPγS binding to brain membranes from female mice are underway.

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Acknowledgements: Beta-arrestin2 wild-type and knock-out breeder mice were developed by Dr. Robert Lefkowitz of Duke University and provided by Aron Lichtman and Dana Selley of Virginia Commonwealth University

SIGNALLING PROFILE OF CRIP1a: G-PROTEIN ACTIVATION AND SIGNAL TRANSDUCTION

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Background: Cannabinoid receptors are a family of G-protein coupled receptors that are involved in a wide range of physiological functions and diseases. Key regulators unique to cannabinoid receptors are cannabinoid receptor interacting proteins (CRIPs). Among them, CRIP1a was found to decrease the constitutive activity of the cannabinoid type-1 receptor (CB1R). The aim of this study was to gain an understanding of how CRIP1a modulates agonist-induced CB1 receptor function.

Methods: CB1R agonists, WIN55,212-2 (WIN), CP55,940 (CP), anandamide (AEA) and 2-arachydonylglycerol (2-AG) were used to investigate changes in signalling in the presence or absence of CRIP1a. Changes in K+ channel signalling were determined using a Membrane Potential Assay in AtT20-hCB1 cells inherently expressing CRIP1a. Changes in ERK1/2 phosphorylation and cAMP accumulation were determined using AlphaScreen assays in HEK293-hCB1±CRIP1a cells. Changes in G-protein activation were determined using BRET technology also in HEK293-hCB1±CRIP1a cells, transiently transfected with Rluc8- and Venus-tagged constructs.

Results: *K+ channel activation:* $CRIP_{1a}$ protein knockdown was observed in cells treated with $CRIP_{1a}$-siRNA (20nM), 48 to 72 hours post-transfection ($p<0.001$). This siRNA-induced $CRIP_{1a}$ knockdown significantly increased both AEA and 2-AG-induced K^+ channel activation ($p < 0.05$) whilst no change was observed in response to WIN and CP. *ERK1/2 phosphorylation and cAMP accumulation:* Downstream signalling studies found no significant difference in adenylyl cyclase or MAP kinase activity in response to WIN, CP, AEA and 2-AG in CRIP1a expressing HEK293-hCB1 cells compared with HEK293-hCB1 cells not expressing CRIP1a. *G-protein activation:* ERK1/2 phosphorylation assays, routinely used to evaluate ligand-induced Gi/o-mediated signalling, demonstrated that addition of Rluc8 to CB1 did not alter the potency or efficacy of G protein-coupling following activation by WIN and CP. The pEC50 values were 6.80±0.04 and 6.76±0.25 for WIN treatment and 6.97±0.12 and 6.83±0.09 for CP, for untagged and Rluc-tagged CB1R respectively. BRET kinetic studies were used to show ability of the CB1R to signal via specific Gai proteins, including Gai1, 2 and 3, in response to WIN, CP, AEA and 2-AG. Ongoing BRET studies are currently looking at changes in agonist mediated Gi/o protein activation in the presence of overexpressed CRIP1a.

Conclusions: These results suggest that $CRIP_{1a}$ modulates CB_1 receptor signalling in the ligand- and pathway-specific manner. Overall, this study provides a greater understanding of the biological link between $CRIP_{1a}$ and CB_1 and improves our knowledge of how CB_1 receptor activity can be selectively altered.

MITOCHONDRIAL CANNABINOID RECEPTORS MEDIATE SPECIFIC EFFECTS OF CANNABINOIDS *VIA* SOLUBLE ADENYLYL CYCLASE (sAC)

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INTRODUCTION: Recent evidence indicates that the cannabinoid type-1 CB1 receptor is present at brain mitochondrial membranes (mtCB1), where it can control cellular respiration, energy production and synaptic plasticity (Benard et al. *Nature Neuroscience* 2012, 15(4):558-64 and Hebert-Chatelain et al., *Molecular Metabolism - in press*). Cannabinoids exert potential therapeutic effects (e.g. analgesia), accompanied by important side effects (e.g. catalepsy and amnesia), but the specific molecular mechanisms and the brain region(s) involved are poorly understood. In this study, we asked whether mtCB1 receptors are involved in specific effects of cannabinoids.

METHODS: Biochemical, pharmacological and genetic approaches, together with stereotactic drug and virus applications and anatomical studies were used to identify the brain region(s) and the molecular mechanisms involved in specific behavioral effects of cannabinoids

RESULTS: The lipid cell-penetrant antagonist AM251 injected into the substantia nigra reticulata (SNr) blocked cannabinoid-induced analgesic and cataleptic effects. However, a peptide non-cell penetrant antagonist (hemopressin) injected in the same brain region blocked only analgesic effects, suggesting the participation of intracellular CB1 receptors in sedative effects. By biochemical studies in the brain, we found that mtCB1 receptors regulate brain bioenergetic processes via inhibition of the mitochondrial soluble adenylyl cyclase (sAC). Interestingly, intra-SNr injections of the sAC inhibitor KH7 blocked the cataleptic, but not the analgesic effect of cannabinoids. Additionally, we generated a mutant CB1 protein (DN22-CB1) lacking mitochondrial localization and function *in vitro*. Electron microscopic expression analysis of viral hippocampal re-expression of wild-type CB1 or DN22-CB1 in CB1-KO mice confirmed the lack of mitochondrial localization of DN22-CB1 *in vivo*. Importantly, viral re-expression of wild-type CB1 rescued cannabinoid-induced memory impairment in an object recognition task in CB1-KO mice, whereas re-expression of DN22-CB1 did not.

CONCLUSIONS: Cannabinoids exert analgesia independently of mtCB1 signaling. However, cannabinoid-induced catalepsy and memory impairment require mtCB1 receptor signaling. Thus, the study of smtCB1 receptors signaling represent a novel way to dissect beneficial (analgesia) from undesired side-effects (catalepsy and memory impairment) of cannabinoids.

MOLECULAR DYNAMICS STUDY OF A CB1 ENDOGENOUS ALLOSTERIC MODULATOR, LIPOXIN A4: DYNAMIC BEHAVIOR IN A LIPID BILAYER AND ENTRANCE INTO THE CB1 RECEPTOR

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The CB_1 endogenous, positive allosteric modulator, lipoxin A_4, increases the equilibrium binding and efficacy of CP55,940 and anandamide (orthosteric agonists). Interestingly, lipoxin A_4 contains a carboxyl group; our calculations strongly suggest that this carboxyl group is negatively charged at physiological pH. This negative charge makes lipoxin A_4 unique among the cannabinoids, as the majority of other CB_1 orthosteric and allosteric ligands (endogenous and synthetic) are electrostatically neutral. The purpose of this study was to use molecular dynamics (MD) simulations to investigate how this negative charge may affect lipoxin A_4's dynamic behavior in a lipid bilayer, as well as its mechanism of binding.

We have previously reported that orthosteric cannabinoids may enter the receptor from the lipid bilayer, between TMH6/7 (Hurst et al. JBC 285:17954 (2010)). However, this method of entry may depend on a ligand's ability to partition into the lipid bilayer and reside at the correct height in the bilayer. To investigate if individual cannabinoids partition differently into the lipid bilayer, we have performed MD simulations on lipoxin A_4, 2-arachidonoylglycerol (2-AG), CP55,940, and ORG27569 in a fully hydrated POPC bilayer. Simulations were run using the GPU-accelerated PME (Particle Mesh Ewald) AMBER12 package. The NPT ensemble was used to maintain temperature and pressure (T=300K, P=1.0bar). The CHARMM22 protein force field with CMAP corrections and the CHARMM 36 lipid force field were used in this study. 2-AG, CP55,940, and ORG27569 all partitioned completely into the lipid bilayer, with their headgroups/polar moieties (on average) residing in the glycerol region of the bilayer. In contrast, the charged headgroup of lipoxin A_4 resided (on average) higher up in the bilayer in the phosphocholine region. Of all the ligands studied, only lipoxin A_4 showed a tendency to partially leave the membrane by extending its headgroup into water. These results suggest that lipoxin A_4 may be able to enter CB_1 from a different portal than TMH6/7.

To investigate whether this unique behavior in the lipid bilayer impacts receptor binding, we also conducted MD simulations of lipoxin A_4 in the presence of CB_1 (embedded in a POPC lipid bilayer). In these simulations, we observed lipoxin A_4 partially leave the bilayer to reach over TMH1 and interact with the EC-3 loop, as well as the N terminus. These results suggest that lipoxin A_4 may enter the CB_1 receptor from lipid at the level of the extracellular loops, a very different approach route than seen for 2-AG, for example (Hurst et al. JBC 285:17954 (2010)). Altogether, these results may inform ligand design in the pursuit of specific binding/signaling outcomes.

[Support: RO1 DA003934 and KO5 DA021358 (PHR)]

GENETIC RESCUE OF CB_1 RECEPTORS ON MEDIUM SPINY NEURONS PREVENTS LOSS OF EXCITATORY STRIATAL SYNAPSES BUT NOT MOTOR PHENOTYPE IN R6/2 MICE

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Huntington's disease (HD) is caused by an expanded polyglutamine repeat in the huntintin (htt) protein that disrupts gene expression, protein function and neurotransmission in specific neuronal populations resulting in characteristic motor, cognitive and affective symptoms. Histopathological hallmarks observed in both HD patients and genetic mouse models include the down-regulation of striatal synaptic proteins and reduction of dendritic spines on medium spiny neurons (MSNs), both of which are thought to mediate the predominant behavioral deficiencies associated with this neurodegenerative disease.

The down-regulation of cannabinoid CB_1 receptors on MSN terminals of HD patients and mouse models represents an early event that is hypothesized to participate in disease pathogenesis, and CB_1 receptor signaling is known to play a crucial role in neuronal survival and connectivity. We developed a new genetic mouse line that allows for the cell-specific genetic rescue of CB_1 receptor expression in MSNs of R6/2 mice. Detailed histological and electrophysiological studies show that rescuing CB_1 receptor function selectively in MSNs fully rescues the loss of excitatory synaptic markers (synaptophysin, vGlut1, vGlut2, and PSD95), MSN dendritic spine density and sEPSCs in MSNs. Remarkably, despite restoration of ecitatory striatal input, motor impairment persisted in these mice. This results emphasizes the concept that behaviour is dependent on many different circuits and their interactions, and that it is not possible to predict rescue (or not) of behaviour based on function of a subset of synapses onto MSNs.

We conclude that CB_1 receptor functionality on MSNs controls the loss of striatal excitatory synapses in the R6/2 mouse model of HD, and that this loss can be uncoupled from the motor phenotype. Our results provide a deeper molecular understanding of the therapeutic potential of and limitations of cannabinoid-based therapeutics for treating patients with HD.

Acknowledgment: Funded by NIDA to NS (Grant no. DA026430)

FATTY ACID BINDING PROTEINS (FABPs) ARE INTRACELLULAR CARRIERS FOR Δ^9-TETRAHYDROCANNABINOL (THC) AND CANNABIDIOL (CBD)

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THC and CBD are hydrophobic compounds that are insoluble in aqueous media. In the blood, lipoproteins and albumin serve as carriers. To date intracellular transporters have not been described for THC and CBD. In theory they would require a carrier to transverse the aqueous milieu, for example, to enter the nucleus for signaling and the endoplasmic reticulum for catabolism. Fatty acid binding proteins (FABPs) have been identified as intracellular transporters for the endocannabinoid anandamide (AEA) and other N-acylethanolamines. We describe here how FABPs can also serve as transporters for the cannabinoids.

Computational methods were initially employed to determine the likelihood of FABP5 being a carrier for cannabinoids. MD simulation showed that both THC and CBD displayed scores consistent with appreciable binding. This analysis showed tighter binding of CBD to FABP5 than THC with $\Delta G_{binding} =$ -41.67±0.81 and $\Delta G_{binding} =$ -36.63.67±1.85, respectively. Furthermore, modeling shows that THC and CBD were accommodated in the FABP5 pocket, in a manner similar to the fatty acid palmitate.

Binding studies of THC and CBD using a fluorescent NBD-stearate displacement assay indicated that these two cannabinoids bind to FABP3, FABP5 and FABP7 with low micromolar affinities. Interestingly, THC and CBD bind the FABPs as well as a variety of other compounds including various known transport inhibitors.

We next determined if FABP5 is an intracellular transporter for THC and CBD. HeLa cells are a suitable cell-type to examine interactions of exogenous cannabinoids with FABPs and their effects upon endocannabinoid transport. Furthermore, HeLa cells, which lack FAAH were transfected with human FAAH and AEA uptake was subsequently examined. This assay measures AEA uptake that is coupled to AEA breakdown by FAAH and to intracellular transport by FABP5, the primary FABP in HeLa cells. Both THC and CBD inhibited AEA uptake, with CBD being slightly more efficacious. The greater potency of CBD as an inhibitor of AEA uptake mirrored its higher affinity (lower K_i) for FABP5. The FABP5 knockdown (shRNA), as expected, inhibited AEA uptake and neither of these two cannabinoids had any further effect upon uptake in the knockdown cells. Neither CBD nor THC inhibited human FAAH activity in cell homogenates. In conclusion, our work indicates that FABPs are intracellular carriers for Δ^9-tetrahydrocannabinol and cannabidiol. This may help explain the observations of others that CBD and THC raise endocannabinoid levels *in vivo*.

We acknowledge NIDA for making this work possible (DA016419 and DA035923).

FUNCTIONAL SELECTIVITY IN CB1R PHARMACODYNAMICS

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Introduction. Functional selectivity is a relatively recent concept in the G-protein coupled receptor field which describes the ability of a range of agonists to activate different pathways with different efficacy or potency (Kenakin et al, 2011). This has produced a major paradigm shift away from the suggestion that the receptor has a single "active" state and towards the idea that subtly different conformations of the receptor produce different downstream signalling events.

Aim. To investigate a large range of pharmacodynamic parameters of the CB1R following activation by a range of structurally diverse ligands.

Methods. The CB1R endogenous ligands: anandamide, 2-arachidonoyl glycerol; classical partial agonists: THC, BAY59-3074; and classical full agonists: CP55940, WIN55212-2 were used to investigate functional selectivity in a range of signalling assays in both HEK and AtT20 cells transfected with hCB1 receptors. Assays studied included competition binding assays, G protein activation, inhibition of cAMP accumulation, GIRK channel activation, CB1R desensitization, arrestin recruitment and internalisation.

Results and Discussion. Differences in rank order of agonist potency and efficacy suggest ligand dependent functional selectivity for some pharmacodynamic parameters. Analysis of this data using an operational model shows a strong signalling bias of all ligands towards the inhibition of cAMP compared to other Gi-linked pathways, probably reflecting amplification within this signalling pathway. Ligand specific bias was observed for GIRK activation by 2AG, with this pathway being maximally activated at very low receptor occupancy, suggesting strong intrinsic efficacy. CP55,940, often considered a "classical" cannabinoid was particularly poor at desensitizing the receptor, yet highly efficacious in internalising the receptor, suggesting these pathways are not always strongly linked together.

Kenakin T (2011). Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**(2)**:** 296-302.

CANNABIS WITHDRAWAL IN ADULTS WITH MOOD DISORDERS

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Cannabis use is prevalent among adults with mood disorders, but little is known about the experience of cannabis withdrawal in this population. We collected data about cannabis use patterns and the experience of cannabis withdrawal from 51 adults (two-thirds men) with mood disorders (26 bipolar, 24 major depression, 1 unspecified), using the Marijuana Quit Questionnaire, a 176-item, semi-structured questionnaire. The index quit attempt and subsequent withdrawal period was defined as their "most difficult" (self-defined) cannabis quit attempt without formal treatment.

42.4% of participants experienced a cannabis withdrawal syndrome (DSM-5 criteria) at some point in their life, of whom 70.4% used cannabis in response to withdrawal symptoms. At the start of their index quit attempt, participants were a mean age of 28 years (range 10-55 years) and using cannabis at least weekly. The quit attempt lasted 14.5 [41.8] months (median 2 months, range 1 day to 35 years). During the quit attempt, 95.5% of subjects reported ≥ 1 withdrawal symptom (mean [SD] 9.5 [6.1], median 9.0): the most frequently reported being irritability (76.5%), increased cannabis craving (74.5%), feeling depressed (68.6%), and feeling anxious (68.6%). The number of withdrawal symptoms was positively correlated with greater frequency and amount of cannabis use. Withdrawal symptoms often prompted actions to relieve them, including increased use of alcohol (41.5%), tobacco (48.2%), and cannabis (33.3%). These findings suggest that cannabis withdrawal syndrome is common among adults with mood disorders and is experienced similarly to other individuals with mental illness (e.g., schizophrenia) and people without serious psychiatric comorbidity. As is seen in other populations, cannabis withdrawal often serves as negative reinforcement for relapse during a quit attempt and may prompt increased use of other psychoactive substances.

Acknowledgements: Funded by the Intramural Research Program, National Institutes of Health, National Institute on Drug Abuse (NIDA) (Gorelick) and by NIDA Residential Research Support Services Contract HHSN271200599091CADB (N01DA-5-9909; Kelly, PI), with additional support from National Institute of Mental Health (NIMH) grant R03 MH076985-01 (Kelly, PI).

EFFECTS OF Δ^8-TETRAHYDROCANNABIVARIN (Δ^8-THCV) ON APPETITIVE EFFECTS OF COCAINE AND NICOTINE IN RODENTS

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Growing evidence suggests that blockade of brain cannabinoid CB_1 receptors or activation of brain cannabinoid CB_2 receptors attenuates the rewarding effects of cocaine or other addictive drugs such as nicotine. Δ^8-Tetrahydrocannabivarin (Δ^8-THCV) is a synthetic analogue of the plant cannabinoid Δ^9-tetrahydrocannabivarin (Δ^9-THCV), which exhibits CB_1 receptor antagonist and CB_2 receptor agonist profiles. Thus, dual CB_1 receptor blockade and CB_2 receptor activation might produce an additive or synergistic therapeutic anti-reward effect.

To test this hypothesis, we observed the effects of Δ^8-THCV on cocaine and on nicotine self-administration and other addiction-related behavior. We found that systemic administration of Δ^8-THCV (3, 10, 20 mg/kg, i.p.) failed to alter intravenous cocaine self-administration in wild-type or CB_2 receptor-knockout mice, but dose-dependently inhibited intravenous nicotine self-administration in alcohol-preferring rats and wild-type mice. Co-administration of Δ^8-THCV and AM630, a selective CB_2 receptor antagonist, blocked Δ^8-THCV's action in alcohol-preferring rats, and genetic deletion of CB_2 receptors (in CB_2 receptor-knockout mice) partially attenuated Δ^8-THCV's action on nicotine self-administration. Also, systemic administration of Δ^8-THCV (0.3-3 mg/kg, i.p.) inhibited nicotine-induced conditioned place preference and nicotine-seeking behavior in wild-type mice during extinction in the absence of nicotine and nicotine-associated cues. Further, CB_2 receptor-knockout mice show significantly lower levels of nicotine self-administration with longer inter-infusion intervals than their wild-type littermates, an effect similar to drug-taking behavior maintained by a higher dose of nicotine. Taken together, these findings suggest that: 1) Δ^8-THCV may have anti-nicotine, but not anti-cocaine, therapeutic effects – partially mediated by activation of CB_2 receptors; and 2) deletion of CB_2 receptors appears to enhance nicotine's rewarding effects. Further studies are required to confirm these findings.

Acknowledgement: Supported by NIDA IRP

EFFECT OF ROOM VENTILATION ON THE PHARMACODYNAMIC AND PHARMACOKINETIC EFFECTS OF SECONDHAND CANNABIS SMOKE EXPOSURE

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There has been little controlled research conducted on the effects of exposure to secondhand cannabis smoke. Most research has focused on detection of cannabinoids in biological matrices; assessment of the subjective and physiologic effects of secondhand cannabis exposure is rare. Since seminal work conducted in the 1980s, technology for toxicology testing has evolved considerably and the average potency of "street" cannabis has increased more than 3-fold.

The present study was conducted to evaluate the pharmacodynamic and pharmacokinetic effects of secondhand cannabis exposure. Across 3 experimental sessions, cannabis potency (approxmately 5% THC and 10-12% THC) and room ventilation (standard HVAC ventilation of 11 air changes per hour vs. unventilated) were studied. Six adult cannabis users and 6 drug-free adults who agreed to secondhand smoke exposure completed each session. Cannabis exposure occurred in a 910 cu.ft. plexiglass chamber. Seating position alternated between cannabis smokers and non-smokers, and smokers were allowed to smoke cannabis *ad libitum* for 60-minutes. Biological specimens and measures of subjective, cardiovascular, and cognitive performance effects [psychomotor ability (DSST), working memory (PASAT), divided attention] were obtained outside the exposure chamber at baseline and for up to 34 hours post-exposure. Here we present outcomes from non-smokers in the ventilated and unventilated test sessions during which cannabis containing 10-12% THC was smoked.

Active smokers consumed a considerable amount of cannabis in both ventilated (16.5 grams total) and unventilated (14.4 grams total) sessions. During the ventilated test session, secondhand smoke exposure did not affect subjective ratings of "drug effect", heart rate, blood pressure, or divided attention. Psychomotor ability (DSST number correct) and working memory (PASAT total correct) improved in the first hour post-exposure relative to baseline (likely indicating a learning effect). THC was detected in whole blood by highly sensitive methods (LC/MS/MS) immediately following exposure in 4 of 6 non-smokers (min/max = 0.7/0.9 ng/mL), but was not detectable 30 minutes post-exposure. The metabolite, THCCOOH, was detected immediately in 2 of 6 individuals (min/max = 0.6/0.7 ng/mL), and one individual had detectable levels in blood 30 minutes post-exposure. During the unventilated exposure session, secondhand cannabis smoke did not affect heart rate, blood pressure, or cognitive performance. Subjective ratings of "drug effect" increased immediately post-exposure [mean (±SD) score of 23 (13) on a 100-pt VAS item], and remained above zero for an average of 1.8 hours. THC was detected in whole blood immediately following exposure (min/max = 1.2/5.6 ng/mL) and for an additional hour in all participants, and remained detectable 3 hours post-exposure for one individual. THC-COOH was detected in 5 of 6 individuals, peaked 30 minutes after exposure (min/max = 2.1/5.1 ng/mL), and remained detectable for up to 22 hours post exposure in 3 individuals. Detection of cannabinoids in urine and oral fluid similarly varied as a function of room ventilation and assay sensitivity.

Study results indicate that absorption of cannabinoids may occur during extreme secondhand exposure to cannabis smoke. Room ventilation had a significant impact on the degree of exposure and resultant pharmacodynamic effects. Though participants in the unventilated condition reported low to moderate levels of subjective intoxication, impairment was not observed on the cognitive domains assessed. Whether or not cannabinoids would be detected in biological specimens obtained for drug testing from individuals passively exposed to cannabis smoke will depend on the characteristics of the matrix (blood, urine, oral fluid, hair) and the sensitivity and specificity of the methodology (screening and confirmation cutoffs used and whether target of analysis is THC or THC-COOH).

Acknowledgement. This paper was developed (in part) under contract HHSS277200800006C from the Substance Abuse and Mental Health Services Administration (SAMHSA), U.S. Department of Health and Human Services (HHS). The views, policies, and opinions are those of the authors and do not reflect those of SAMHSA or HHS. Additional support was obtained from NIH/NIDA training grant T32-DA07209.

ADVERSE PEER-EXPERIENCES THROUGHOUT ADOLESCENCE IN MALE RATS PERSISTENTLY ALTER ETHANOL INTAKE AND ENDOCANNABINOID SIGNALING IN LATER LIFE

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Peer-interactions become particularly important during adolescence and hence, human teenagers display enhanced sensitivity towards social rejection, which might contribute to the development of neuropsychiatric disorders. Neurobiological consequences of social rejection during adolescence are have not yet been well investigated,, and no appropriate animal model is available. Here, we studied the potential adverse consequences of inadequate social encounters in adolescent rats, which we propose as an operational model for adolescent peer-rejection. Male adolescent Wistar rats were either reared with other Wistar animals (control), or with age-matched rats from the less playful Fischer344 strain (inadequate social rearing; play-deprived). From day 65 on, all Wistar rats were again group-housed with same strain partners. Voluntary ethanol intake was assessed in Wistar animals throughout adolescence and in adulthood. In addition, levels of the endocannabinoids anadamide (AEA) and 2-arachidonylglycerol (2-AG), as well as protein levels of the CB1 receptor were measured in adult Wistar animals in various brain regions. Animals reared with inadequate social partners showed increased ethanol intake during adolescence as compared to controls, a feature which persisted into adulthood. The two groups exhibited differences in the expression levels of the CB1 receptor and concentration of endocannabinoids, which were most pronounced in the amygdala and the nucleus accumbens. In conclusion, adverse social experiences during adolescence result in distinct acute and persitent behavioral and neurobiological alterations which promote ethanol intake.

NALTREXONE MAINTENANCE REDUCES CANNABIS SELF-ADMINISTRATION IN CANNABIS SMOKERS

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Background: Currently, approximately 24% of patients entering treatment for substance use disorders have a diagnosis of cannabis use disorder (CUD), yet their treatment outcome is poor, with a small minority of patients achieving continued abstinence following clinical treatment. There is a clear need to improve treatment outcome for CUD and pharmacological options are one important strategy to investigate. In light of the unavailability of cannabinoid receptor antagonists for clinical study, opioid receptor antagonists offer an indirect approach to reducing cannabis' positive subjective and reinforcing effects. Preclinical studies show that opioid antagonists reduce both the discriminative stimulus and reinforcing effects of CB1 receptor agonists, suggesting that opioid receptor antagonists could reduce cannabis abuse liability. However, in daily cannabis smokers, acute pretreatment with the opioid antagonist, naltrexone (12, 25, 50, 100 mg), *increased* rather than attenuate the positive subjective effects of cannabis (Cooper and Haney, 2010). Given that chronic antagonist administration can produce different effects on drug intoxication than acute antagonist pretreatment, this placebo-controlled, human laboratory study assessed the effects of active and inactive cannabis before, during and after chronic NTX administration.

Method: Non-treatment-seeking, cannabis smokers were randomized to receive NTX (50 mg) or placebo (0 mg) for 16 consecutive days. Each participant completed 10 laboratory sessions over 3-4 weeks: before NTX administration, after a single NTX administration, after 1 and 2 weeks of daily NTX administration, and 1 week after termination of NTX administration. At each timepoint, the reinforcing, subjective, psychomotor, and cardiovascular effects of active (5.5% THC) and inactive (0.0%) cannabis were assessed. Medication compliance was ensured by: observed capsule administration $\geq$ 4 times/week, plasma naltrexone measurement, and urine riboflavin levels. Cannabis self-administration was measured by having participants choose to pay $1.00 for individual puffs of cannabis (0-3 puffs/session).

Results: Forty-nine participants, receiving either placebo (n=26M; 2F) or NTX (n=18M; 5F), completed the study. Demographic variables were comparable across the two groups, with participants reporting to smoke an average of 6 cannabis cigarettes/day, 6 days/week. The number of participants who failed to complete the study was also comparable across the 2 groups (placebo: n=8; NTX: n=9). In terms of outcome, maintenance on NTX significantly reduced cannabis self-administration compared to placebo. Those maintained on placebo had 7.6 (95% CI: 1.1, 51.8) times the odds of buying at least 1 puff of active cannabis compared to those receiving NTX. Rates of inactive cannabis self-administration were low and unaffected by NTX maintenance condition. NTX also reduced the positive subjective effects (e.g., ratings of 'Good Effect' and 'Friendly') of active cannabis relative to placebo. NTX did not significantly alter cannabis's cardiovascular effects.

Discussion: These results show that chronic naltrexone administration decreased active cannabis's reinforcing and subjective effects. These data suggest that clinical studies among patients motivated to reduce their cannabis use are warranted to determine if NTX may have use for the treatment of cannabis dependence.

This research was supported by US National Institute on Drug Abuse Grant DA19239 and DA09236

SOCIAL REWARD IS MEDIATED BY INTERACTING OPIOID AND CB1 CANNABINOID RECEPTORS IN THE NUCLEUS ACCUMBENS CORE IN ADOLESCENT RATS

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Social play is an essential form of social interaction crucial for proper development of physical, cognitive and social capacities of young mammals, it is highly rewarding and it is modulated by interactions between the endocannabinoid anandamide and endogenous opioids (Trezza et al., Trends Pharmacol. Sci. 31 (2010) 463-469). The principal endocannabinoid 2-arachidonoylglycerol (2-AG) regulates emotional behaviors in rodents (Mulvihill and Nomura, Life Sci. 92 (2013) 492-497). However, its contribution in social play and the neural substrates underlying opioid-cannabinoid interactions in the modulation of this behavior remain unknown. To address this aim, we tested the effects of systemic administration of the 2-AG hydrolysis inhibitor JZL184, which prolongs the effects of locally released 2-AG (Long et al., Nat. Chem. Biol. 5 (2009) 37-44) in social play behavior.

Systemic administration of the 2-AG hydrolysis inhibitor JZL184 (1 mg/kg; i.p.) increased social play behavior in adolescent rats. These effects were blocked by systemic pre-treatment with either CB1 cannabinoid receptor antagonist SR141617A (0.1 mg/kg; i.p.) or the opioid receptor antagonist naloxone (1 mg/kg; s.c.). Thus, increasing 2-AG levels facilitates social play through CB1 cannabinoid and opioid receptor signaling.
Our previous studies demonstrated that anandamide and endogenous opioids act within the nucleus accumbens core (NAcc) to modulate social play behavior (Trezza et al., J. Neurosci. 31 (2011) 6362-6370; Trezza et al., J. Neurosci. 32 (2012) 14899-14908). Thus, we hypothesized that this brain region also underlies the 2-AG modulation of social play in adolescent rats. In line with this hypothesis, intra-NAcc infusion of SR141716A (3 μg/0.3 μl) antagonized the enhancement of social play induced by systemic administration of JZL184 (1 mg/kg; i.p.). Interestingly, intra-NAcc infusion of naloxone (0.5 μg/0.3 μl) also counteracted the increase in social play induced by systemic treatment with JZL184 (1 mg/kg; i.p.). Thus, stimulation of CB1 cannabinoid and opioid receptors within the NAcc is necessary and sufficient for 2-AG to modulate social play. Likewise, social play enhancement by systemic treatment with the opiod agonist morphine (1 mg/kg; s.c.) was prevented by intra-NAcc infusion of SR141716 (3 μg/0.3 μl).
Altogether, these data show that opioid and CB1 cannabinoid receptors crosstalk in the NAcc underlies cannabinoid and opioid stimulation of social play behavior in adolescent rats, extending previous findings indicating complex functional interactions between these two receptors.

Acknowledgements: Funded by Veni grant 91611052 (Nederlandse Organisatie voor Wetenschappelijk Onderzoek), Marie Curie Career Reintegration Grant PCIG09-GA-2011-293589 (Seventh Framework Programme People) and Futuro in Ricerca 2010 (Italian Ministry for University and Scientific Research)

CANNABIS IN MEDICINE: A NATIONAL EDUCATIONAL NEEDS ASSESSMENT AMONG CANADIAN PHYSICIANS

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The study was conducted to determine the educational needs of Canadian physicians regarding cannabis for therapeutic purposes (CTP). A national needs assessment survey was developed based on previous survey tools. The survey was approved by the Research Ethics Board of the McGill University Health Centre Research Institute and was provided online using LimeSurvey. Several national physician organizations and medical education organizations informed their members of the survey. The target audience was Canadian physicians. We sought to identify and rank using 5-point Likert scales the most common factors involved in decision making about using CTP in the following categories: knowledge, experience, attitudes, and barriers. Preferred educational approaches and physician demographics were collected. Gap analysis was conducted to determine the magnitude and importance of differences between perceived and desired knowledge on all decision factors.

Four hundred and twenty six responses were received, and physician responses were distributed across Canada consistent with national physician distribution. The most commonly identified factors influencing CTP concerned potential risks of using cannabis for medical purposes (4.23/5) and safety, warning signs and precautions for patients using medical cannabis (4.21/5). The largest gap between perceived current and desired knowledge levels was identified for dosing and the development of treatment plans (average gap=1.78).

We have identified several key educational needs among Canadian physicians regarding CTP. These data can be used to develop resources and educational programs to support clinicians in this area, as well as to guide further research to inform these gaps.

ACTIVITY OF SYNTHETIC CANNABINOID DRUGS OF ABUSE AT G PROTEIN COUPLED CB RECEPTORS AND CANNABINOID-SENSITIVE ION CHANNEL

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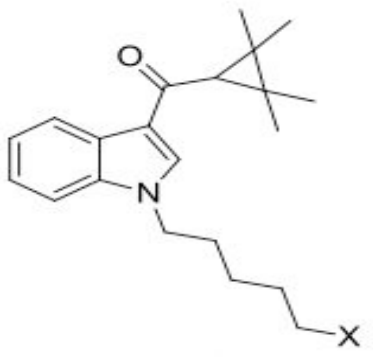
The recreational use of synthetic cannabinoids (SCs) continues to present major health and regulatory concerns. Legal restrictions on the original SCs has led to the rapid chemical evolution, with the identity of available compounds constantly changing and their pharmacological properties essentially unknown. With the instances of hospitalization and death due to the misuse of these compounds on the rise, it is important to gain knowledge on the reactivity and toxicology of these compounds. We are establishing the pharmacological profile of SCs by using high throughput membrane potential assays for hCB1 and hCB2 activation of K channels in AtT-20 cells, as well as calcium elevation assays for TRPV1 and TRPA1 activity in HEK-293 cells. Here we focus on the SCs UR-144, fluorinated XLR-11, and 5-hyroxypentyl metabolite of XLR (SB5035), and compare their activity to Δ9-THC. All three drugs were agonists at hCB1 with pEC_{50} values of 6.3 ± 0.05, 6.9 ± 0.05, and 5.5 ± 0.3 respectively. They were more potent agonists at CB2 with pEC_{50} values of 7.1 ± 0.05, 8 ± 0.15, and ~ 8.7 respectively. When compared to Δ9-THC effects (pEC_{50} value at hCB1 7.3 ± 0.05 and CB2 > 10uM). Δ9-THC is more potent and efficacious at CB1 whereas the synthetics are CB2 preferring. We tested the SCs on the related TRP channels, TRPA1 and TRPV1, both of which are targets for cannabinoids. For the TRPA1 experiments, all data was compared to the effects of maximally effective concentration of cinnamaldehyde (300μM). UR-144 (30 μM) activated TRPA1 to 38 ± 4 % of the cinnamaldehyde response. XLR-11 (pEC_{50} 5.1 ± 0.05) and SB5035 (pEC_{50} 4.95 ± 0.02) had similar efficacies to cinnamaldehyde. Both XLR-11and SB5035 were more efficacious than Δ9-THC (pEC_{50} 5.0 ± 0.3, max 75 ± 20% of cinnamaldehyde). None of the SCs were TRPV1 agonists. Surprisingly, these new SCs are significantly less potent than Δ9-THC in at least one assay of hCB1 function, they are CB2 preferring, and they are relatively potent and efficacious agonists at the noxious chemical sensing ion channel TRPA1. TRPA1 activity could conceivably mediate some of the toxicity of SCs.

UR-144; X= H

XLR-11; X= F SB5035; X = OH



EFFECTS OF CANNABIS ON THE SUBJECTIVE-EFFECT RATINGS AND PHARMACOKINETICS OF SMOKED COCAINE

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Despite the prevalence of concurrent cannabis and cocaine use, there is a paucity of controlled data addressing the interaction between these two drugs. The current study sought to establish the behavioral effects and pharmacokinetic interactions of cocaine and cannabis when smoked conjunctively. Nontreatment-seeking cocaine and cannabis smokers were recruited to participate in this within-subject, double-blind, inpatient study. Across six laboratory sessions, participants smoked inactive or active cannabis (0.0 or 5.6% THC) followed by four doses of 0, 12, or 25 mg of smoked cocaine separated by 14 minutes intervals. Throughout each session, subjective drug-effect ratings were measured and blood was drawn for pharmacokinetic analysis of plasma levels of parent drug compounds (cocaine and Δ^9-THC) and respective metabolites (benzoylecgonine [BZ] and 11-nor-9-carboxyΔ^9-THC [THC-C]).

Eight male participants who smoked cocaine (5 ± 2 days/wk) and cannabis (3 ± 1 days/wk) have completed the study. Cocaine increased subjective ratings of positive drug effects ('Liking,' Good Effect'), drug quality ('Potency,' 'High Quality'), 'High,' and engendered greater ratings of the dose's monetary value relative to placebo ($p \leq 0.05$); cocaine craving also increased under these conditions ($p \leq 0.05$). Active cannabis decreased the subjective ratings of positive drug effects, drug quality, and subjective rating of the monetary value for the high cocaine dose (25 mg; $p \leq 0.05$). However, active cannabis increased these ratings for the low cocaine dose (12 mg; $p \leq 0.05$). Cannabis decreased cocaine craving for both cocaine doses ($p \leq 0.05$). Cocaine increased plasma levels of cocaine and BZ ($p \leq 0.0001$); cannabis attenuated cocaine-induced increases of BZ but did not affect cocaine plasma levels ($p \leq 0.05$). Cannabis increased plasma THC and THCC levels ($p \leq 0.001$); cocaine did not affect these levels.

These preliminary findings suggest that cannabis may decrease the positive subjective effects of larger doses of smoked cocaine and cocaine craving, and may also alter cocaine's metabolism. By assessing the interactions between cannabis and varying doses of cocaine, this study will provide clinically relevant information regarding the rationale for why these drugs are co-abused and the health risks associated with the combination.

Acknowledgements: This research was supported by US National Institute on Drug Abuse Grant DA19239, DA09236, and DA02775.

SUBSTITUTION EFFECT IN 628 MEDICAL CANNABIS PATIENTS; RESULTS FROM THE CANNABIS ACCESS FOR MEDICAL PURPOSES SURVEY (CAMPS)

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Background
With over 628 responses so far, the Cannabis Access for Medical Purposes Survey (CAMPS) is the largest polling of Canadian medical cannabis patients to date. This paper examines self-reported cannabis substitution for alcohol, pharmaceuticals and illicit substances from the CAMPS project.

Methods
The CAMPS questionnaire is a 414 question cross-sectional survey that was made available to Canadian medical cannabis patients online and by hard copy in 2011 & 2012 in order to gather information on patient demographics, medical conditions and symptoms, patterns of medical cannabis use, cannabis substitution, and obstacles to safe access to medical cannabis. Responses were then analyzed in SPSS to identify statistically significant correlations with other patient characteristics.

Outcomes
Overall, 86.6% of patients reported substituting cannabis for at least one other substance: 80.3% (n=504) of patients stated that they used cannabis as a substitute for prescriptions drugs, 51.7% (n=325) used cannabis as a substitute for alcohol, and 32.6% (n=205) used it as a substitute for illicit substances. The main reasons cited included "better symptom management" and "less adverse side-effects". Patients who listed a greater number of symptoms were more likely to report cannabis substitution, and younger patients (below 30yrs old) were far more likely to substitute cannabis for prescription drugs, alcohol and illicit substances than older patients (50 and over).

Discussion
This study adds to a growing amount of research suggesting that a large percentage of patients who use cannabis are substituting it for pharmaceutical drugs, alcohol and illicit substances. Consequently, cannabis may be serving a harm reduction function in some patients using high rates of pharmaceuticals and/or affected by problematic substance use issues. Further research should seek to differentiate between biomedical substitution for prescription pharmaceuticals and psychoactive drug substitution for addiction, and to better elucidate the mechanisms behind both. Additionally, research into cannabis as a treatment for problematic substance use in non-patient populations should be explored.

This research was supported by a grant from the UBC Institute for Healthy Living and Chronic Disease Prevention.

LEPTIN-CONTROLLED OREXIN/ENDOCANNABINOID INTERACTIONS IN THE MOUSE PERIAQUEDUCTAL GREY: ROLE IN THE REGULATION OF THE DESCENDING ANTINOCICEPTIVE PATHWAY

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In the ventrolateral periaqueductal gray (vlPAG), activation of excitatory output neurons projecting monosynaptically to OFF cells in the rostral ventromedial medulla (RVM) causes antinociceptive responses via OFF cells stimulation and ON cell inhibition[1]. We demonstrated that this descending nociceptive pathway is under the control of cannabinoid receptor type-1 (CB1)[2]. Moreover, 2-AG deeply affects nociception via CB1 stimulation, and its concentration is higher in the PAG and RVM of wt mice during neuropathic pain[3]. Orexins are hypothalamic peptides known to modulate arousal, feeding, reward and antinociception via orexin receptors (OX-R). In obese *ob/ob* leptin knock-out mice, OX-A expression increases in the fibers projecting to vlPAG and 2-AG levels increases in the vlPAG. Recently, Ho and collaborators demonstrated that orexin-A (OX-A), by activating OX-AR (OX-A receptor) in the vlPAG of rats, stimulates the synthesis of 2-AG and retrograde inhibition of the tonically active GABAergic circuit (disinhibition) thus inducing activation of descending nociceptive pathway[4]. On this basis we hypothesized the existence of a leptin-controlled orexin/endocannabinoid interaction in the modulation of the pain network leading to nociception. In this study we have validated this hypothesis using a combination of electrophysiological (*in vivo* recording), immunohistochemical (OX-A, OX-AR and CB1 single and multiple localization), ultrastructural (CB1/OX-A immunogold labeling on symmetric or asymmetric synapses) and behavioral (nociception in the "plantar test" and in spontaneous and tail-flick-related activities of RVM neurons) approaches in wt and *ob/ob* mice.

We observed that OFF (anti-nociceptive) and ON (pro-nociceptive) cells are more and less active, respectively, in *ob/ob* compared to wt. We found a significant increase of number and intensity of OX-A fibers in the PAG of *ob/ob* mice and this was accompanied by a two-fold increase of pre-prorexin mRNA expression in the LH compared to wt. OX-AR/DAGLalpha expression colocalized in a limited subset of PAG neurons through a electron microscopy approach. Moreover, CB1 receptors were expressed at symmetric synapses to OX-AR-expressing neurons thus suggesting an heterosynaptic pathway. The pharmacological blockade of the OXR1 into the PAG produced pro-nociceptive effect in WT mice detected by both pow withrawal and ON OFF cell activity. Interestingly, in the ob/ob mice the dose of the OX-AR antagonist able to generate the pronociceptive effect were double as compared to WT mice suggesting a change of this system in the absence of leptin. On the other hand, AM251, a selective CB1 antagonist also induced pro-nociceptive effect in wt mice and needed of lower dose in the ob/ob mice suggestin a tight cross-talk between leptin-orexin and cannabinoid systems. The endocannabinoid level measurements further confirmed the data.

CONCLUSIONS: Here we provide evidence supporting that the heterosynaptic endocannabinoid spread in the vlPAG after OX-AR activation is modulated by leptin. The leptin-related increase of OX-A signalling in PAG is accompained by incresed activation of OX-AR which are GqPCRs and could initiate the GqPCR-PLC-DAGL-2AG retrograde inhibition onto tonic GABAergic transmission in the vlPAG , leading to the potentiation of antinociception. Finally, we show that, beside the feeding and arousal, the orexin system could be highly involved in the pain modulation and is activity is possibly regulated by the leptin-cannabinoid system interaction.

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IN VIVO EVALUATION OF THE PERIPHERALLY SELECTIVE CB1 RECEPTOR ANTAGONIST RTI-13329-2 IN A MOUSE MODEL OF DIET-INDUCED WEIGHT GAIN

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Cannabinoid receptor 1 (CB1R) antagonists have the potential to treat several important diseases such as obesity, drug addiction, diabetes, and liver disease. Regrettably, central nervous system (CNS) related adverse effects including depression and suicidal ideation were reported with non-tissue selective first generation CB1R antagonists. However, recent studies indicate that primarily targeting peripherally expressed CB1R with antagonists that have limited brain penetration is an attractive strategy for medications development. These compounds are expected to have limited adverse effects in patients while providing the metabolic benefits of CB1R antagonism. Over the last few years, our group has developed several novel CB1R antagonists with limited brain penetration based on the diphenyl purine scaffold of otenabant. One of these compounds, N-{1-[8-(2-Chlorophenyl)-9-(4-chlorophenyl)-9H-purin-6-yl]-4-phenylpiperidin-4-yl}methanesulfonamide (RTI-13329-2), was selected for further in vivo studies. RTI-13329-2 is extremely potent with apparent antagonist dissociation constant (Ke) ~ 2.9 nM, CB1R affinity (Ki) ~ 6.2 nM, and >150-fold selectivity for CB1R over CB2R (Fulp et al, J Med Chem, 55, 10022-32, 2012). Further, this compound has excellent metabolic stability, no noticeable interaction with hERG, and minimal effect on CYP3A4 induction. Pharmacokinetic studies indicated that this peripherally selective compound had ~10% brain penetration in both rats and mice. Consequently, RTI-13329-2 was tested in a mouse model of diet-induced weight gain. Male C57BL6 mice at ten weeks of age were fed a high-fat diet (HFD, 60% fat calories) for 4 weeks. Control mice were maintained on a normal diet (10% fat calories). Some animals on HFD were given otenabant (10 mg/kg) or RTI-13329-2 (1 or 10 mg/kg) orally once daily. Body weight and food consumption were closely monitored throughout the study. At the end of four weeks, animals were subjected to oral glucose testing prior to sacrifice. Animals on HFD gained significantly more weight than control animals. Treatment with otenabant and RTI-13329-2 abrogated weight gain in animals and improved glucose tolerance compared to animals on HFD. Livers of animals treated with otenabant and 13329-2 had significantly reduced levels of fat accumulation as well. In conclusion, RTI-13329-2 is a peripherally selective CB1R antagonist that produced beneficial effects in a rodent model of obesity and metabolic syndrome. Refinement of this class of compounds is currently underway.

Supported by 1R21AA019740-01 and RTI IR&D 0271300.073 to R. Maitra

WHERE'S MY ENTOURAGE? THE CURIOUS CASE OF 2-OG AND 2-LG

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2-Arachidonoylglycerol (2-AG) is the most abundant endogenous cannabinoid in the brain and is a high efficacy agonist at both cannabinoid receptors (CB_1 and CB_2). Over the past years the synthesis, degradation and signaling of 2-AG have been investigated in some detail. However, several other endogenous monoacylglycerols have been isolated from various tissues, but their pharmacology has not been fully explored. Two of these are 2-linoleoylglycerol (2-LG) and 2-oleoylglycerol (2-OG), also a GPR119 agonist. The current data suggest that these compounds do not bind to the cannabinoid receptors. Nor do they affect intracellular free Ca^{2+} levels or adenylyl cyclase activity in a CB_1-dependent manner. However, the presence of these compounds has been reported to potentiate the activity of 2-AG and slow its breakdown, possibly through competitive inhibition of 2-AG degradation. This phenomenon has been dubbed the 'entourage effect' and may be a means to regulate synaptic activity.

To clarify the activity of these lipids at the CB_1 receptor we employed patch-clamp and cell-based assays. For the former we used cultured autaptic hippocampal neurons, i.e. self-synapsing neurons that have the necessary cellular machinery for several forms of endocannabinoid-mediated synaptic plasticity. This includes the 2-AG-, CB_1-, and MAGL-dependent retrograde form of neuronal signaling known as depolarization-induced suppression of excitation (DSE), making it a useful model to test for a potential entourage effect. As expected, our electrophysiological data show that 2-OG and 2-LG do not inhibit neurotransmission via CB_1 when applied to autaptic neurons. However, these compounds fail to potentiate the 2-AG-dependent DSE. Instead 2-OG and 2-LG behave as antagonists at the CB_1 receptor, *attenuating* DSE. This result is inconsistent with an 'entourage effect'. Interestingly 2-OG and 2-LG do internalize CB_1 receptors in CB1-HEK cells, as shown by an on-cell western assay, indicating that these compounds do activate CB_1 receptors under some conditions. Our results suggest 1) that these compounds may serve as functional antagonists under certain conditions and, interestingly, 2) that these compounds may exhibit functional selectivity in their signaling. Our results suggest that the relationship between 2-AG and its congeners may be more nuanced than previously appreciated.

NAPE-PLD DELETION VIA AN ALTERNATIVE MECHANISM DRIVES SIGNIFICANT DECREASES IN AEA IN THE MOUSE BRAIN

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A leading hypothesis of the biosynthesis of the endogenous cannabinoid AEA is that it is dependent on the availability of *N*-acyl-phosphatidylethanolamine (NAPE), wherein the production of AEA is the result of NAPE hydrolysis by a NAPE-specific phospholipase D (NAPE-PLD). This hypothesis was challenged with data showing AEA levels were unaffected in NAPE-PLD KO mice[1]. Here, we examine this hypothesis with an alternative strain of NAPE-PLD KO mice developed by Palmiter and Luquet that demonstrate behavioral characteristics and a lower rate of conversion of exogenous NAPE to AEA that would suggest there is a signaling change in AEA upon NAPE-PLD deletion[2]. Groups of 6 NAPE-PLD KO mice age 9 months and 2 months from the Palmiter strain and 6 age and sex matched WT mice per group were sacrificed, brains were removed and 8 targeted areas were dissected and stored at -80C. Methanolic extracts were partially purified on C-18 solid-phase extraction columns. Eluants were analyzed with high-pressure liquid chromatography coupled to tandem mass spectrometry using an API 3000 triple quadrupole MS and over 70 lipids were analyzed for each sample.

Results here highlight those of AEA, 2-AG, NAGly, and prostaglandins; however, many differences were observed in other lipids in these KO mice. In both the 9 and 2 month old NAPE-PLD KO mice, levels of AEA were significantly reduced compared to WT levels in all regions assayed. Importantly, no changes in 2-AG levels in any brain region were observed in the 2 month old NAPE-PLD KO mice compared to WT (though only 6 regions have currently been examined), whereas, there were region specific increases in the 9 month old mice. Likewise, levels of the endogenous AEA metabolite, NAGly, were significantly different in a region specific manner, however, this was demonstrated in both age groups. Levels of PGE_2 increased in all areas of the 2 month old NAPE-PLD KO mice, whereas, most but not all regions showed increases relative to WT in the 9 month old mice. These data showing significant decreases in AEA are at odds with previous data from the Cravatt lab's NAPE-PLD KO mice[1]. Importantly, there are striking differences in the sequence generation between the two strains, which may account for this difference. Namely, the Cravatt strain deletes exon 4 of the NAPE-PLD gene[1], whereas the Palmiter strain deletes exon 3[2]. Investigating this phenomenon has the potential to clarify understanding of AEA biosynthesis and lead to a more directed manipulation of AEA production, which has significant therapeutic potential.

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BIDIRECTIONAL MANIPULATIONS OF 2-ARACHIDONOYLGLYCEROL CONTENT MODULATE ANXIETY-LIKE BEHAVIORS

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Stress is a major risk factor for multiple psychiatric disorders including major depression, post-traumatic stress, and anxiety disorders. Therefore, elucidation of novel approaches to mitigate the adverse effects of stress could have broad clinical applications. Disruption of the endocannabinoid (eCB) system has been implicated in various anxiety disorders and converging evidence demonstrates that stress decreases the levels of the eCB ligand, anandamide (AEA), in brain regions implicated in the pathophysiology stress-related disorders. Furthermore, augmentation of AEA content in the amygdala, a key modulator of fear and anxiety behaviors, can mitigate the deleterious effects of stress.

In contrast, the role that the most abundant eCB in the central nervous system, 2-arachidonoylglycerol (2-AG), plays in stress response physiology remains poorly understood. Here we show a transient elevation of 2-AG in the amygdala of mice exposed to foot-shock stress. This response may be an attempt to buffer against stress-induced anxiety, as further augmentation of 2-AG content following foot-shock stress is anxiolytic. Moreover, systemic inhibition of the primary 2-AG degrading enzyme, monoacylglycerol lipase, with JZL-184 reduces stress-induced corticosterone release and reverses stress-induced anxiety behaviors in two preclinical models of anxiety, the novelty induced hypophagia (NIH) and light-dark box (LD) tests. Conversely, genetic deletion of the primary 2-AG synthetic enzyme, diacylglycerol lipase α, which reduces whole brain 2-AG content without affecting AEA content, results in increased anxiety-like behaviors in the NIH and LD tests. Furthermore, using *ex vivo* slice electrophysiology, we demonstrate that JZL-184 application, which prevents 2-AG metabolism, produces a depression of excitatory field potentials in the central amygdala, which may represent a mechanism underlying the anxiolytic effect of JZL-184 in behavioral tests. These data strongly suggest that augmentation of 2-AG signaling may be an effective strategy for the treatment of anxiety disorders.

Acknowledgements: Funded by the National Institutes of Health (MH09412, MH064913, MH09412, MH064913, NS078291, AA019455, CA89450, GM15431, and DA031572), the Hobbs Foundation, and the A.B. Hancock Jr. Memorial Laboratory for Cancer Research. Behavioral testing was performed in the Vanderbilt University Murine Neurobehavioral Core. Analytical studies were conducted in the Vanderbilt University Mass Spectrometry Research Center.

CANNABIS, ANXIETY, AND PAIN: THE IMPORTANCE OF COPING STYLE

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The positive association between anxiety and pain is well-established. Cannabis-based medicines are noted for both their analgesic and anxiolytic properties, and recent surveys of users of cannabis for therapeutic purposes (CTP) indicate that the majority of patients who use cannabis primarily for pain relief also report cannabis use to alleviate anxiety. However, the extent to which the analgesic effects of cannabis vary according to levels of anxiety has not been determined. In the present study we examined the effects of anxiety on cannabis-induced analgesia among CTP users who identified chronic pain as a primary reason for CTP use. We further examined the extent to which the association between anxiety and cannabis analgesia was moderated by coping style. Among participants who reported CTP use for both pain and anxiety (n = 49), those who reported relatively greater cannabis analgesia also reported greater anxiolytic effectiveness for CTP (X^2 = 10.17, p < .01). Across all participants (n = 68), the perceived analgesic effectiveness of cannabis was not associated with anxiety (X^2 < .01, p = .98). However, moderation analyses revealed that the association between anxiety and cannabis analgesia was moderated by the dysfunctional coping styles of *behavioral disengagement* (X^2 = 5.86, p < .05) and *self blame* (X^2 = 3.90, p < .05). Follow-up analyses of these interactions indicated that anxiety was associated with greater analgesia among those who were less likely to engage in behavioral disengagement (X^2 = 4.32, OR = 1.12, p < .05) and with less analgesia among those who were higher in behavioral disengagement (X^2 = 3.45, OR = .84, p = .06). Analyses of self blame coping exhibited a similar but less pronounced pattern of association, with a positive association between anxiety and analgesia at lower self blame (X^2 = 3.83, OR = 1.13, p = .05), and no relationship among those more prone to self blame (X^2 = 2.33, OR = .95, p = .12).

Our findings help to elucidate the role of anxiety in cannabis-induced analgesia. First, we found that perceived analgesic and anxiolytic effectiveness of cannabis covaried positively among patients who use CTP to treat both pain and anxiety. Second, we found that cannabis was perceived to be a more effective analgesic among higher anxiety individuals who refrain from dysfunctional coping, and less effective among higher anxiety individuals who engage in higher levels of dysfunctional coping. These findings suggest that anxiety may play a role in the analgesic effectiveness of cannabis, and also point to meaningful heterogeneity among those who use cannabis for pain relief. These findings highlight the importance of individual differences in affective and cognitive functioning in understanding the complex relationship between cannabis and pain relief.

ENDOGENOUS CB1 ALLOSTERIC ENHANCER BALANCES AGE-RELATED COGNITIVE ALTERATIONS

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Allosteric binding sites at cannabinoid CB1 receptors were suggested 6 years ago, after experiments with synthetic molecules. Recently, we reported an endogenous molecule (lipoxin A4, LXA4) that enhances endocannabinoid signaling through allosteric modulation of these receptors in the CNS (Pamplona et al PNAS 2012). This molecule is a "first-in-class" and its discovery opens the possibility for future fine-tuning allosteric modulation of endocannabinoid signaling. Interestingly, LXA4 is also an important anti-inflammatory molecule, actually a resolvin, whose levels decrease during the aging process. Hence, we decided to investigate the potential role of LXA4 in a number of endocannabinoid-related CNS functions. Therefore, here we report that endogenous LXA4 affects mouse anxiety-like behavior and cognition in aged animals, and that absence of the LXA4-synthesizing enzyme 5-LO induces cognitive deficits in 5-LO KO mice (Leo et al PLoS One 2013). Moreover, LXA4 protects mice against the cognitive deficit induced by i.c.v. injection of beta-amyloid 1-40 peptide, a hallmark of Alzheimer's Disease. The reduction of LXA4 observed in 5-LO KO mice, as well as in aged mice contributes to the generation of cognitive deficits. As LXA4 levels decrease after 60+ years in humans, we are now pursuing the concept that LXA4 could be an important biomarker of brain vulnerability to aging-related cognitive deficit and neurodegenerative diseases in humans. This translational approach is currently being conducted in samples of over 200 patients with healthy aging, diagnosed with mild cognitive impairment or Alzheimer's Disease. LXA4 will be quantified in CSF and plasma samples, compared across these patient groups and plotted against age and cognitive performance. We expect that LXA4 may represent a novel biomarker to help identify individuals at higher risk of developing mild cognitive impairment and/or Alzheimer's disease over time, allowing pre-emptive clinical intervention.

Finnancial support: CNPQ-Brazil and FAPERJ-Brazil grants for F.A.Pamplona.

Δ^9-THC RESTORES AGE-RELATED CHANGES IN THE BRAIN

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Aging of the brain is accompanied by a progressive decline in cognitive abilities associated with reduced synaptic densities. This process is accelerated in mice lacking cannabinoid CB1 receptors. We have therefore wondered if an increase of the cannabinoid tone in older animals would protect them against the loss of cognitive functions. Two age groups of animals were therefore treated with 3 mg/kg/day Delta-^{9}THC for 28 days through osmotic minipumps. This treatment significantly improved spatial learning in the Morris water maze test as well as object location recognition in the old but not in the young mice. The improved cognitive performance was associated with increased synaptic densities and enhanced expression of synaptic marker proteins. We next investigated the gene expression profile of control and THC treated young and old mice. We found that THC treatment altered the expression of a number of age-related genes in the old but not in the young mice. The CREB and Erk signaling systems were activated in THC treated old animals shown by an enhanced phosphorylation. The beneficial effects of THC on brain ageing was mediated by CB1 receptors, because THC treated CB1 knockout mice failed to show any improvement in learning ability, increase in synaptic densities or change in gene expression.

A PERIPHERAL ENDOCANNABINOID MECHANISM FOR STRESS-INDUCED AMNESIA

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Memory consolidation is a labile process under the direct impact of emotional experiences, and little is known with regards to the underlying mechanisms. The endocannabinoid system plays an important role in the modulation of both emotions and memory, but the integration of these functions in the memory consolidation processes has not been addressed. This study investigates the involvement of type-1 cannabinoid (CB1) receptors in stress-induced amnesia for a non emotional memory. Using a model for declarative memory in mice, the novel object-recognition test, we found that stress and the arousal state of the animal determines the consolidation outcome in this memory. Such a memory trace was obliterated under physical or psychological acute stress conditions, or corticosterone administration. These amnesic-like effects of stress and corticosterone were not observed after pharmacological (both rimonabant and the peripherally acting CB1 receptor antagonist AM6545) or genetic blockade of CB1 receptors. According to this behavioral data, the c-FOS activation in different brain regions produced after the shock was also blocked by the CB1 antagonism.

Using several cell type-specific conditional CB1 receptor knockout mouse lines, we found that CB1 receptors expressed in noradrenergic dopamine β-hydroxylase-expressing neurons (DBH-CB1) are crucial for this stress-induced amnesia, whereas CB1 receptors in other brain neuronal populations were not involved in this response. Interestingly, conditional mice lacking DBH-CB1 (DBH-CB1-KO) did not increase plasma corticosterone levels induced by the shock compared to wild-type animals.

Finally, the removal of the adrenal glands prevented the amnesic-like effect of stress and the pharmacological noradrenergic modulation shows that both alpha- and beta-adrenergic receptors are involved in this stress-induced amnesia.

In summary, peripheral noradrenergic transmission determines the consolidation of non-emotional memories and this function is under the direct control of peripheral CB1 receptors. The elucidation of this mechanism opens novel therapeutic approaches for the treatment of memory- and stress-related disorders through peripherally acting drugs for CB1 cannabinoid receptors.

Aknowledgements: This study has been carried out with financial support from Ministerio de Ciencia e Innovación (#BFU2012-33500 to AO, #SAF2011-29864 to RM); Instituto de Salud Carlos III (RD06/0001/0001 to RM); PLAN E (Plan Español para el Estímulo de la Economía y el Empleo); Generalitat de Catalunya (SGR-2009-00731 to RM) and the French National Research Agency (ANR) in the frame of the "Investments for the future" Programme IdEx Bordeaux (ANR-10-IDEX-03-02).

PERSISTENT MICROGLIA ACTIVATION WITHIN THE PREFRONTAL CORTEX CONTRIBUTES TO THE DEVELOPMENT OF THE DEPRESSIVE/PSYCHOTIC-LIKE PHENOTYPE INDUCED BY ADOLESCENT THC EXPOSURE IN RATS

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Emerging evidence suggests that microglia might play a crucial role in brain development, regulating synaptic maturation and function, possibly suggesting that deficits in microglia function may contribute to synaptic abnormalities seen in some neurodevelopmental disorders.
In the present study we investigated whether the depressive-like phenotype induced by adolescent delta-9-tetrahydrocannabinol (THC) exposure in adult female rats (Rubino et al. 2008, 2009; Realini et al. 2011; Zamberletti et al. 2013) was associated with changes in microglia activation within the prefrontal cortex.
To this aim, chronic THC (or vehicle) treatment was performed between PND 35 to 45 and ionized calcium-binding adaptor molecule 1 (Iba1) expression, a marker of activated microglia, was monitored during THC treatment as well as 24 hours (PND 46), 15 days (PND 60) and 30 days (PND 75) after discontinuing it in the prefrontal cortex (PFC). The effect of chronic THC treatment on microglia was also morphologically determined by using Iba1 immunofluorescence.
Adolescent THC treatment significantly increased Iba1 expression from PND 39 to PND 46 and they were still enhanced at PND 75, suggesting the presence of persistent microglia activation following adolescent THC exposure. This hypothesis was supported by morphological studies revealing that in THC-treated rats glial cells were maintained in a chronic activated state (amoeboid morphology) till adulthood.

In order to assess whether microglia activation could play a role in determining the depressive/psychotic-like phenotype observed in adult THC-exposed animals, we inhibited microglia activation by co-administering Ibudilast, a non-selective phosphodiesterase inhibitor, concomitantly to THC treatment and animals were then submitted to behavioral testing in adulthood.
Intriguingly, co-treatment with Ibudilast and THC during adolescence prevented the development of some of the symptoms associated with adolescent THC exposure, thus suggesting that microglia activation could contribute to determine some of the behavioral alterations observed in adult THC-treated rats.
As a whole, the present findings demonstrate that the behavioral phenotype induced by adolescent THC exposure is associated with persistent microglia activation within the PFC that contribute to the development of some signs of THC-induced depressive/psychotic-like phenotype.
Ongoing studies are aimed at elucidating the neurobiological consequences of microglia activation in terms of cytokine release and CB2 receptor expression.

Acknowledgements: Funded by Dipartimento delle Politiche Antidroga (Rome, Italy). MG has a pre-doctoral fellowship from Dipartimento delle Politiche Antidroga.

A MEDICAL ETHNOGRAPHIC REPORT OF CANNABIS USE IN PEDIATRIC INTRACTABLE EPILEPSY (IE) PATIENTS

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Parents of children with severe and untreatable diagnoses are desperate for relief for their child and family systems. Because inefficacy and/or side effects of multiple drug cocktails do not provide cure or relief, some are turning to alternatives, such as CBD-rich Cannabis extractions. Due to US Federal restrictions, these patients and their families do not have access to products of traditional research processes. This report intends to describe demographics, diagnoses, current drug regimens and consequences of a population that has chosen to self-administer Cannabis as a potential therapeutic agent.

A retrospective chart review of thirteen patients was combined with a mixed-methods approach that included parent interviews and correspondence, a parent forum, and analytical data that was collated to describe general quality of life, dosing regimens and unintended consequences. Eligibility criteria included recommendation for Cannabis use in WA State under RCW 69.51 and diagnosis of intractable epilepsy by a neurologist. Additional diagnoses included transient tic disorder, autistic spectrum, and self-injury behavior. Information on demographics, quality of life, changes in seizure frequency, cognitive function, behavioral effects, co-administration with other anti-epileptic drugs (AEDs), analytical data on plant potency and constituents, preparation of Cannabis and dose (mg/kg) were collated and analyzed. Charts of thirteen pediatric patients revealed a patient age range of 2- 21. Parents sought medical help from 2-19 doctors and children had been prescribed 2-6 pharmaceutical drugs. Thirty-five adverse effects of current AEDs were self-reported with the most common of ataxia, anger/rage, behavioral problems including anger and aggression, and loss of appetite. The most common alternative therapies were herbal and nutritional supplements. About half of the parents felt they had support of their neurologist for oral Cannabis supplementation in the form of a plant concentrate diluted with oil. Qualitative changes reported by the parents included reduction of seizure frequency and severity, positive behavioral changes, improved sleep, and general improvement in quality of life for the child, parents and family units.

There is a trend amongst parents of pediatric patients with IE to seek alternative treatments. The families here independently navigated dosing a Cannabis preparation and the downward titration of AEDs, largely without the support of their medical provider. They reported positive support from behavioral interventionists and social and educational workers. This report indicates the need to enable prospective research on the potential therapeutic use of CBD-rich Cannabis preparations for pediatric patients with IE. Further, there is a need to investigate potential drug interactions and appropriate, safe supervised withdrawal from AEDs.

EXOME SEQUENCING OF FAMILIAL ATRIAL FIBRILLATION INFORMS POSITIVE TREATMENT WITH CANNABIDIOL

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Exome sequencing uncovered multiple non-synonymous variants in several calcium channel genes in a pedigree with multi-generational lone paroxysmal atrial fibrillation (AF). Calcium channel genes, which encode pore-forming subunits of ion channels, are genetically hypervariable. This variability complicates the identification and interpretation of causative variants in individuals with mid-life expression of disease. Exome sequencing was performed on a family of six to prioritize variants identified in cardiac genes that tracked with the atrial fibrillation phenotypes. Based on the affected individual's clinical presentation, we hypothesized that five non-synonymous common and novel variants in two calcium channel genes, *RYR2* and *CACNA1C*, were the candidate causative variants, hypothetically contributing to the phenotype in a polygenic manner. Published mutations in these genes have been reported to cause sudden cardiac death. Animal model studies have suggested a link between atrial arrhythmias and dysfunctional calcium handling[1]. Cannabidiol is known to regulate intracellular calcium homeostasis and in theory presents a possible theory for response in this unique case[2].

The patient failed conventional therapy with beta blockers and calcium channel blockers secondary to symptomatic bradycardia. Treatment with the antiepileptic drug cannabidiol (CBD), a non-psychotropic phytocannabinoid derivative of cannabis, was initiated by the patient. Arrhythmia occurrence was documented with ambulatory monitoring via the Alive Cor ECG monitor and patient reported symptoms. Atrial fibrillation abated with oral mucosal use of CBD at dose of 75mg/day. In the proband, atrial fibrillation, frequency and severity correlated with cannabidiol dose, with atrial fibrillation returning with doses below 25mg/3xday. This case presents a private scenario where variants in opposing calcium channels (cell membrane and sarcoplasmic reticulum) were unresponsive to non-specific calcium channel blockers but appeared to be responsive to CBD.

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EPIGENETIC REGULATION OF THE ENDOCANNABINOID SYSTEM IN HUMAN PSYCHIATRIC DISORDERS

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Alterations of the endocannabinoid system (ECS) have been associated with psychotic disorders (particularly schizophrenia), and thus they are getting more and more attention as potential diagnostic markers and/or therapeutic targets of innovative drugs.

Here, we investigated changes in DNA methylation of the major components of the ECS (*i.e.*, CB_1, CB_2, GPR55 and TRPV1 receptors, and NAPE-PLD, FAAH, DAGL and MAGL metabolic enzymes) in peripheral blood mononuclear cells (PBMCs) of patients suffering from major depressive disorder (MDD) or bipolar disease of type I (BDI) and type II (BDII). PBMCs were chosen because they are easily accessible cells, that possess the same machinery for epigenetic regulation as neuronal cells, and are known to mirror defects associated with neurological diseases.

We found a selective down-regulation of CB_1 mRNA expression in PBMCs from MDD patients only, compared to healthy controls. Such a down-regulation was not due to altered DNA methylation and correlated with histone deacetylase 1 (HDAC1) and HDAC10 expression, but not with that of HDAC5 or HDAC7. Interestingly, down-regulation of CB_1 was paralleled by a similar down-regulation of mitogen-activated protein kinase phosphatase-1 (MKP-1), that is a key control point in the neurobiology of depression.

Taken together, we suggest that CB_1 might regulate gene expression through HDAC1 and HDAC10, controlling MKP-1-dependent signal transduction.

Understanding the impact of ECS epigenetic regulation on human psychiatric disorders appears of great value for the possible design of more specific epigenetic drugs, also because those currently approved by FDA, although promising, have both a non-specific target and a genome-wide effect.

GENETIC DIFFERENCES IN THE CB1/CNR1 GENE MODULATE FEAR EXTINCTION IN HUMANS

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The endocannabinoid system plays an important role in the regulation of anxiety behavior. A large amount of animal research shows the ability of the endocannabinoid system to regulate both cue-specific and context-specific fear responses, through the modulation of cannabinoid receptor 1 (CB1) activation [1-2]. Enhancing CB1 receptor activation with CB1 receptor agonists or anandamide re-uptake inhibitors increases extinction of fear [1-2]. Decreasing CB1 receptor neurotransmission, either through pharmacological blockade or genetic deletion of the CB1 receptor, diminishes the extinction of fear [1-3]. Whether endocannabinoids play a role in acute fear relief, long-term habituation or relearning is still subject of debate. Taken together, animal literature strongly suggests that the endocannabinoid system holds great promise for development of pharmacotherapy in human anxiety disorders. Fear extinction is used as a human laboratory for exposure-based psychotherapy. However, research on the role of the CB1 receptor in the regulation of fear extinction in humans is still scarce. Recently, a study showed that administration of Δ 9–THC enhanced fear extinction [4]. Another study reported similar results using cannabidiol [5]. Additional research is needed to bridge the gap between preclinical and human research.

In a previous study from our research group we found that genetic variability in the human CB1 receptor gene may underlie individual differences in fear extinction [6]. Healthy human volunteers (N = 150) underwent a fear conditioning and extinction procedure in a virtual reality environment. Fear-potentiated startle of the eye-blink reflex was recorded to assess fear-conditioned responding. Subjects were genotyped for a polymorphism located in the promoter region (rs2180619) of the CNR1 gene. Importantly, homozygote A/A carriers displayed a complete lack of fear extinction, whereas G-allele carriers displayed robust extinction of conditioned fear. Furthermore, failure to extinguish fear resulted in a higher conditioned fear at the end of the experiment in the homozygote A/A group when compared to the G-allele carriers. In parallel to preclinical literature, no effects of rs2180619 genotype were found on the acquisition and expression of conditioned fear. These findings suggest that deficient fear extinction in A/A carriers of rs2180619, may predispose to developing anxiety disorders. Because candidate gene studies often use small samples and have relatively small effects-sizes, resulting in an increased risk for false-positives, we aim to replicate and extend on our previous findings. Therefore we have performed a replication study in a larger sample (N = 204) and included a testing session 1 week later to evaluate whether rs2180619 exerts long-lasting effects on fear extinction. Results of the ongoing analyses will be presented at ISCR2014.

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A QUESTION OF RANK: USING DNA BARCODES TO CLASSIFY CANNABIS SATIVA AND CANNABIS INDICA

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INTRODUCTION: The botanical taxa *C. sativa* (European fiber plants) and *C. indica* (Asian drug plants) should not be confused with the folk taxonomy of "sativa" and "indica" used in the medical cannabis world. Botanical taxa have rank: species > sub-species > variety. Rank allocation is vital (*e.g.,* endangered species laws concern species, not subspecies, as do drug laws), but notoriously subjective, exampled by *C. sativa* and *C. indica,* arbitrarily segregated at the rank of species, or subspecies, or variety. Animal taxonomists propose using a "DNA barcode" to allocate rank. They employ the *COI* gene sequence, and a "barcode gap" (2.7% difference between two *COI* sequences), as the threshold for separating animals at the rank of species. No barcode gap separating plant species has been proposed; botanists recommend combining two or more sequences as a potential barcode. Candidates include *rbcL, matK, trnH-psbA, trnL-trnF,* and *ITS*.

METHODS: Five sequences (*rbcL, matK, trnH-psbA, trnL-trnF, ITS*) were obtained from *C. sativa* and *C. indica.* Pairwise alignments of each sequence were made with BLAST. We quantified the difference between aligned sequences by tallying the number of nucleotide non-identities as a percentage of the total alignment. These calculations were repeated with a pair of very different species (apples and oranges), then with five pairs of related species (tomato and potato, hops and Japanese hops, buckwheat and bitter buck-wheat, trema and Jamaican trema, Asian ginseng and Himalayan ginseng). Many plant species, unlike animal species, can hybridize. So we repeated the process with four closely related species that can hybridize (radish and charlock, white poplar and black cottonwood, Asian rice and African rice, upland cotton and Pima cotton). Lastly we made five pairwise comparisons between plants classified at the rank of subspecies or variety (long-grain rice and short-grain rice, cabbage and broccoli, melon and wild melon, calamus and American calamus, Chinese tea and Assam tea). All sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank).

RESULTS: Apples and oranges (different genera) differed by 18.1±2.87% (mean of five sequences). The mean barcode gap of five related species was 3.0±0.292%. The mean barcode gap of four closely related that can hybridize was 1.0±0.134%. The mean barcode gap of five subspecies or varieties was 0.43±0.01%. *C. sativa* and *C. indica* differed by 0.41±0.26%.

DISCUSSION: We calculated a barcode gap between non-hybridizing plant species that nearly equals the animal *COI* barcode gap. The barcode gap between *C. sativa* and *C. indica* is similar to that of other pairs of plants at the rank of subspecies or variety. Contrary to recent taxonomic studies, *C. sativa* and *C. indica* should not be considered different species. We propose rank allocation as *C. sativa* subsp. *sativa* and *C. sativa* subsp. *indica*.

OREXIN/ENDOCANNABINOID/LEPTIN INTERACTION AFFECTS HYPOTHALAMIC TAU PHOSPHORILATION BY GLYCOGEN SYNTHASE KINASE-3BETA ACTIVATION

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Synaptic plasticity in the hypothalamus is coordinated by hormonal signals responding to the physiological energy status. This mechanism is strictly regulated, among other things, by the fine balance between phosphorylated/unphosphorylated proteins of axonal microtubules. Tau is a microtubule-associated protein (MAP) mainly expressed in neurons and its hyperphosphorylation decreases affinity and binding to microtubules with consequent axonal retraction, swelling and inhibition of fast transport. Glycogen synthase kinase-3 beta (GSK-3β) is an ubiquitous serine/threonine kinase that regulates numerous cellular functions and neuronal architecture, including Tau phosphorylation. Unlike other protein kinases, GSK-3β is constitutively active under resting conditions and is inactivated by extracellular signals like leptin through phosphorylation of its Ser-9 residue via Akt (Cross et al., 1995). However, GSK-3β activity is further increased by phosphorylation of Tyr-216 mediated by lysophosphatidic acid (LPA), with subsequent phosphorylation of Tau via tyrosine kinase Pyk2 (Sayas et al., 2006). LPA is a bioactive lipid mediator, which can be both biosynthetic precursor of, or produced by, the endocannabinoid 2-AG (Nakane et al., 2002).

By comparing adult leptin defective *ob/ob* mice to littermates we have previously found, in the arcuate nucleus (ARC) of the hypothalamus: (*i*) the elevation of orexin-A/hypocretin-1 (OX-A) trafficking and release; (*ii*) a ~4-fold increase of 2-AG levels as a possible consequence of OX-A receptor-1 (OX-AR) over-activation; (*iii*) a significant increase of pTau/Tau ratio, which is reversed 60 min after acute i.p. leptin injection (Cristino et al., 2013; Cristino et al., in preparation). On this basis, we hypothesized that the massive increase of hypothalamic 2-AG levels known to occur in *ob/ob* or fasted mice (Di Marzo et al., 2001) as a result of leptin deficiency, might lead to a strong increase of LPA, with subsequent hyperphosphorylation of Tau, axonal destabilization of pre-opiomelanocortin (POMC) neurons of the ARC and reduction of the food-intake inhibitory activity that these neurons exert. To test this hypothesis we examined the effects of leptin and orexin-A both on the amounts of LPA [with main focus on 2-AG-derived *sn*-1-lyso-2-arachidonoyl-PA] and on the two opposing pathways of GSK-3β phophorylation, via Akt or Pyk2, after in vivo injection of leptin (i.p., 5 mg/kg), orexin-A (i.p., 40 mg/kg) and the OX-AR antagonist, SB334867 (i.p., 30 mg/kg). Neuronal primary cultures from the ARC of wild-type P0 mice were also used to replicate in vitro the same experiments.

Our data suggest that, in *ob/ob* mice, Pyk2-mediated GSK-3β phophorylation constitutively contributes to Tau instability (i.e. Tau hyperphosphorylation) in anorexigenic POMC neurons, in manner prevented by leptin or SB-334867A treatment. Conversely, in wild-type fed mice, PI3K/Akt-mediated GSK-3β phophorylation constitutively contributes to Tau stability in these neurons, in a manner prevented by short term (12 hr) food deprivation or OX-A injection. This set of data was confirmed in vitro by time lapse study of elongation/retraction of neuronal processes from primary POMC-expressing neurons incubated with leptin or OX-A or LPA, alone or in the presence of the respective receptor antagonists. Finally, by immunofluorescence, we found significant CB1/OX-AR co-expression in the ARC due, at least in part, to the formation of CB1/OX-AR heteromers, as assessed by immunoblots, coimmunoprecipitation and FRET analysis.

We suggest that, in *ob/ob* mice, elevated 2-AG levels, due to impaired leptin and enhanced OX-A signaling, together with these two latter alterations, determine, via either LPA- or CB1-mediated mechanisms (or both) profound changes in hypothalamic connectivity through GSK-3β-mediated Tau phosphorylation in POMC neurons, contributing to feeding disinhibition.

CANNABINOID RECEPTOR 2 DEFICIENCY ALTERS NEUROINFLAMMATION IN AN ALZHEIMER'S DISEASE MOUSE MODEL

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The endocannabinoid system (eCS) encompasses two G-protein-coupled receptors, the cannabinoid receptor 1 (CB1), the cannabinoid receptor 2 (CB2), as well as their ligands and their respective synthesizing and degrading enzymes. While the CB1 is expressed primarily in the brain, the CB2 is mainly detected in immune cells. As various studies suggest a role of CB2 receptors in the modulation of microglia activity, which may be relevant to Alzheimer's disease (AD), we wanted to investigate the role of the CB2 receptor in a mouse model of AD. In AD microglia are attracted by deposits of accumulated amyloid-ß (Aβ) peptides and exhibit an activated state involving enhanced proliferation, increased expression of cell surface markers and production of chemokines and cytokines. Thus, we investigated the role of the CB2 receptor in activation of microglia cells *in vitro* as well as in the inflammatory process in an *in vivo* model in Alzheimer's disease (APP/PS1xCB2$^{-/-}$ mice). CB2$^{-/-}$ microglia showed a reduced expression of cell surface markers such as ICAM and CD40 as well as a decreased release of chemokines and cytokines, e.g. CCL2, IL-6 and TNF-α as compared to wild-type microglia. Absence of the CB2 receptor, however, did not result in a difference in Aβ phagocytosis in neonatal microglia.

APP/PS1xCB2$^{-/-}$ mice showed reduced numbers of microglia cells as well as infiltrating macrophages and lowered expression levels of pro-inflammatory chemokines and cytokines. Subsequently, the amount of soluble Aβ 40/42 was diminished in APP/PS1xCB2$^{-/-}$ in middle-aged (9 months) but not in aged (14 months) mice. Interestingly, this cutback in neuroinflammation did not affect learning and memory abilities in APP/PS1xCB2$^{-/-}$ mice.

Taken together, we show that CB2$^{-/-}$ microglia have a limited capacity to respond to pro-inflammatory stimuli, whereas the phagocytosis capacity is not influenced. The knockout of the CB2 receptor in an AD mouse model led to a significant reduction in AD-linked neuroinflammation, which did not influence Aβ plaque load or cognitive impairment. This suggests a functional impact of the CB2 in neuroinflammatory responses associated with Alzheimer's disease but independent of influencing Aβ pathology and cognitive impairment.

Funding for this conference was made possible in part by grant R13DA016280 from the National Institute on Drug Abuse. The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention by trade names, commercial practices, or organizations imply endorsement by the U.S. Government.

SELECTIVE ACTIVATION OF CB2Rs ELIMINATES VTA DOPAMINE NEURONAL BURSTING FIRING IN RODENTS

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Recently, the "peripheral cannabinoid type 2 receptors (CB2Rs)" have been detected in various brain areas, suggesting that CB2Rs are also expressed in the central nervous system and they may participate in the modulation of neuronal functions under both physiological and pathological conditions. Our previous study demonstrated that the activation of central CB2Rs significantly reduced animal cocaine seeking behavior, which indicates an important role played by CB2Rs in mesolimbic circuit for drug addiction. It is well known that drug addictive behavioral is highly correlated with VTA dopamine (DA) neuronal firing activity, especially bursting firing. However, whether central CB2Rs modulate VTA DA neuronal bursting firing is unknown. We hypothesize that the selective activation of functional CB2Rs in the VTA eliminates VTA DA neuronal bursting firing through the enhanced small-conductance Ca2+-activated K+ channels (SK). In the present study, we test our hypothesis using *in vivo* and *in vitro* electrophysiological approaches. In anesthetized mice, we performed extracellular single unit recording and found that systemic injection of CB2Rs agonist (JWH 133, i.p., 10 mg/kg) moderately reduced VTA DA neuronal firing rate but dramatically reduced bursting firing fraction in WT, but not CB2R KO, mice. In WT mice, the JWH133-induced inhibition in DA neuronal firing can be prevented or reversed by injection of CB2Rs antagonist AM630 (i.p. 10 mg/kg), suggesting that systemic JWH133 alters VTA DA neuronal bursting firing through the activation of CB2Rs. In VTA DA neurons in slice, bath-applied JWH133 (1 μm) significantly enhanced the amplitude of apamin-sensitive after-hyperpolarization, suggesting that the altered SK conductance may underlie JWH133-induced reduction of neuronal bursting firing. To elucidate this possible mechanism, we induced DA neuronal bursting firing with NMDA (30 μM) in VTA slices both in rats and mice. We found that JWH133 significantly inhibited NMDA-induced bursting firing represented as a decrease in amplitude of the inter-burst hyperpolarization potential. Importantly, this inhibition can be blocked by either AM630, or a SK selective blocker NS8593. Furthermore, JWH133 also enhanced SK current amplitude in DA neurons of VTA slices. Taken together, our results suggest that the selective activation of VTA CB2Rs significantly eliminates the bursting firing of VTA DA neurons, which is mediated through enhanced amplitude of SK currents. Therefore, our findings provide directly experimental evidence to improve our understanding, for the first time, of how CB2Rs play a critical role in drug addiction and DA associated diseases.

CHRONIC CANNABINOID CB_2 AGONIST REVERSES PACLITAXEL NEUROPATHY WITHOUT TOLERANCE, CB_1-MEDIATED WITHDRAWAL OR SIDE EFFECTS

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Mixed cannabinoid CB_1/CB_2 agonists, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), can produce tolerance, dependence, and unwanted CB_1-mediated central nervous system side effects following chronic administration. Whether the beneficial effects of repeated systemic administration of a CB_2-preferring agonist involves CB_1 receptors or produces unwanted CB_1-mediated side effects is unknown. We evaluated the anti-allodynic efficacy, possible tolerance, and cannabimimetic side effects of repeated dosing with a CB_2-preferring agonist (AM1710), in comparison to Δ^9-THC, in a model of chemotherapy-induced neuropathy produced by paclitaxel using CB_1KO, CB_2KO, and WT mice. We also investigated the site and mechanism of action of AM1710. Paclitaxel-induced mechanical and cold allodynia developed equivalently in CB_1KO, CB_2KO, and WT mice. Both AM1710 and Δ^9-THC suppressed established paclitaxel-induced allodynia in WT mice. Unlike Δ^9-THC, chronic AM1710 did not engage CB_1 activity or produce antinociception tolerance, CB_1-mediated cannabinoid withdrawal, hypothermia, or motor dysfunction. The anti-allodynic efficacy of systemic AM1710 was absent in CB_2KO mice or WT mice receiving the CB_2 antagonist AM630, administered either systemically or intrathecally. Intrathecal AM1710 also attenuated paclitaxel-induced allodynia in WT but not CB_2KO mice, suggesting a role for spinal CB_2 receptors in AM1710 anti-allodynic efficacy. Finally, both acute and chronic treatment with AM1710 decreased expression of tumor necrosis factor alpha and monocyte chemoattractant protein-1 mRNAs in the lumbar spinal cord of paclitaxel-treated WT mice. Our results highlight the therapeutic potential of CB_2 agonists for managing chemotherapy-induced allodynia with a favorable therapeutic ratio marked by sustained efficacy and absence of tolerance, CB_1-mediated withdrawal or side effects.

Acknowledgements: Funded by DA021644 (to AGH), DA011322 (to KM), DA021696 (to KM), DA035068 (to KM and AGH), and R13DA016280 (to the ICRS from NIDA grant).

THE COMBINATION OF AMITRIPTYLINE WITH FAAH OR MGL INHIBITORS IN CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY IS CB_2 MEDIATED

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Cisplatin, a platinum-derived chemotherapeutic agent, produces mechanical and cold allodynia in rodents that is reminiscent of chemotherapy-induced neuropathy observed in humans. Enzymes such as fatty-acid amide hydrolase (e.g. FAAH) and monoacylglycerol lipase (MGL) break down the body's own endocannabinoid ligands (e.g. anandamide) and 2-arachidonoyl glycerol. They represent targets for analgesic drug development. We compared antinociceptive effects of FAAH (URB597, URB937) and MGL (JZL184) inhibitors on mechanical and cold allodynia induced by cisplatin treatment. Anti-allodynic efficacy of each endocannabinoid modulator was compared with agents used clinically to treat neuropathy (i.e. the tricyclic antidepressant amitriptyline). Groups received intraperitoneal (i.p.) injections of either amitriptyline, URB597, URB937, JZL184 or vehicle. FAAH (URB597, URB937) and MGL (JZL184) inhibitors administered alone at lower dose partially reversed mechanical and cold allodynia. Pharmacological specificity was assessed by coadministering amitriptyline with FAAH or MGL modulator with either a CB_1 (AM251 3 mg/kg) or CB_2 (AM630 3 mg/kg) antagonist. Interestingly, the combination of FAAH or MGL inhibitors with amitriptyline, reversed cisplatin-evoked mechanical and cold allodynia to pre-cisplatin levels. Morevoer, this reversal of mechanical and cold allodynia by the combination of amitriptyline with FAAH or MGL inhibitors is CB_2 mediated. However, RT-PCR analysis demonstrate no changes in FAAH, MGL, CB_1 or CB_2 mRNA levels following chronic administration of amitriptyline, JZL184, URB937 or their combination. Our results suggest that both FAAH and MGL inhibitors in combination with amitriptyline attenuate mechanical and cold allodynia in a model of chemotherapy-induced peripheral neuropathy. Moreover, the combination of amitriptyline with FAAH or MGL inhibitors is mediated by cannabinoid receptors 2. Our studies suggest that the endocannabinoid system combined with amitriptyline represent a promising target for suppressing chemotherapy-induced neuropathic pain.

Supported by DA021644 and DA035068 (to AGH).

TAMOXIFEN IS AN ALLOSTERIC MODULATOR OF THE CB2 CANNABINOID RECEPTOR

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Recently, we have reported that several second- and third-generation selective estrogen receptor modulators (SERMs) are inverse agonists for the CB2 cannabinoid receptor. The purpose of the current study was to investigate the pharmacology of tamoxifen, a first-generation SERM and three of its metabolites, 4-hydroxytamoxifen, enodxifen, and N-desmethyltamoxifen, at the CB2 cannabinoid receptor.

Using human CB2 stably expressed in HEK293 cells, our experiments demonstrated that at concentrations up to 1 μM, tamoxifen and two of its metabolites (enodxifen and N-desmethyltamoxifen) failed to either modulate cAMP accumulation or compete for specific [^{3}H]CP-55,940 binding. In contrast, 4-hydroxytamoxifen enhanced forskolin-stimulated cAMP accumulation and competed for specific [^{3}H]CP-55,940 binding in a concentration-dependant manner. Furthermore, pretreatment of HEK293 cells stably expressing human CB2 with 4-hydroxytamoxifen caused a rightward, parallel shift of the concentration-response curves of the cannabinoid agonists HU-210, CP-55,940, and WIN55,212-2. However, pretreatment with endoxifen and N-desmethyltamoxifen did not alter the concentration-response curves of these cannabinoid agonists. Most importantly, we discovered for the first time that pretreatment with tamoxifen resulted in an enhancement of CP-55,940 efficacy to inhibit forskolin-stimulated cAMP accumulation without altering the efficacy of the cannabinoid agonists HU-210 and WIN-55,212-2. These data demonstrated that tamoxifen exhibits a positive allosterism on CP-55,940 efficacy. The allosteric effect of tamoxifen on CB2 is specific for CP-55,940 since the efficacy of the other two cannabinoid agonists was not altered by the pretreament of tamoxifen. In addition, the allosteric nature for the effects of tamoxifen was further validated by [^{3}H]CP-55,940 dissociation kinetic studies as there was a significant decrease in each of the slow and fast radioligand dissociation rates in the presence of tamoxifen.

In recent years, several synthetic compounds have been shown as allosteric modulators for the CB1 cannabinoid receptor. However, to our knowledge, there have been no allosteric modulators reported for the CB2 cannabinoid receptor. In this study, by identifying tamoxifen as the first allosteric modulator for CB2, we have provided the first piece of evidence indicating the existence of an allosteric site on the CB2 cannabinoid receptor. Furthermore, we have demonstrated a possible novel mechanism of action for tamoxifen, a well-known, first-generation SERM.

TARGETING CB2 RECEPTOR AS A NEW PHOTOTHERAPY APPROACH

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The success of targeted cancer therapy largely relies upon the selection of target and development of efficient therapeutic agents that specifically bind to the target. Cannabinoid CB_2 receptor (CB_2R) is considered as an attractive target for cancer treatment. Under healthy conditions, high CB_2R expression is only present in immune cells. However, CB_2R expression is up-regulated in many types of cancers, such as prostate, skin, liver, brain, colon and breast cancer.

In the current study, we chose CB_2R as a new target for phototherapy treatment and developed a new CB_2R-targeted photosensitizer, IR700DX-mbc94. We found that phototherapy treatment using IR700DX-mbc94 greatly inhibited the growth of CB_2R positive tumors, but not CB_2R negative tumors. In addition, phototherapy treatment with non-targeted IR700DX did not show significant therapeutic effect. Similarly, treatment with IR700DX-mbc94 without light irradiation or light irradiation without the photosensitizer showed no tumor-inhibitory effect. Taken together, IR700DX-mbc94 is a promising phototherapy agent with high target-specificity. Moreover, CB_2R appears to have great potential as a phototherapeutic target for cancer treatment.

Ackknowledgements: We thank Dr. Nephi Stella at the University of Washington for providing DBT cells and technical advice. This work was supported by the startup fund provided by the Department of Radiology, University of Pittsburgh. This project used the UPCI imaging facilities supported, in part, by award P30CA047904. We also thank the Grant of Shanghai Science and Technology (12DZ1940606 and 12ZR1439900) and Grant of Shanghai Municipal Health Bureau (20124195) for supporting N.J.'s work. We also thank the National Institutes of Health National Institute on Drug Abuse (NIH NIDA) for grant R01DA025612 (to X.-Q.X.).

PEPTIDE ENDOCANNABINOIDS (PEPCANS) ARE PAMs OF CB2 RECEPTORS AND INVOLVED IN THE INNATE IMMUNE RESPONSE

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Peptide endocannabinoids (Pepcans) are N-terminally extended "hemopressin peptides" endogenously present in different tissues, such as brain, with RVD-hemopressin (Pepcan12) being the major representative. We have previously shown that Pepcans are negative allosteric modulators (NAMs) at CB1 receptors (Bauer et al. J. Biol. Chem. 2012, 287, 36944-67). During receptor profiling and binding studies, we realized that unlike hemopressin, these peptides do not only bind to CB1 receptors, but also CB2 receptors. Using different *in vitro* assays, we show that Pepcans exert significant positive allosteric modulation (PAM) at CB2 receptors in the low nanomolar concentration range, both at the level of receptor binding and function (cAMP, GTPgammaS). Using our in house generated Pepcan mAb we measured levels of Pepcans in normal and inflamed peripheral tissues. To address the role of Pepcan12 during inflammation we studied its effect in macrophage polarization and differentiation, processes in which CB2 receptors play a role. We show that during constitutive CB2 receptor activation (likely produced by the 2-AG autocrine tone), Pepcan-12, which showed the most potent effect, already at low nanomolar concentrations modulates the shift from M1 to M2. Moreover, Pepcan12 inhibits osteoclastogenesis using stimulated primary human monocytes and controls endotoxin resistance in a model using human whole blood. As expected for a PAM at CB2 receptors, Pepcan12 acts synergistically with endocannabinoids in macrophage polarization in a CB2 receptor-dependent manner, though effects via CB1 receptors cannot be excluded.
Our data point to a prominent role of Pepcan-12 as endogenous modulator of CB2 receptors and in the innate immune response. To our knowledge, Pepcan-12 is the first endogenous PAM reported. Overall, our mechanistic studies with Pepcan12 point toward a key modulatory role of this peptide in the endocannabinoid system as it is a NAM for CB1 and PAM for CB2, thus an ideal physiological modulator of cannabinoid receptors in the periphery. Given the explicit aggregation behaviour of Pepcans *in vitro*, we have elaborated a protocol for the handling of these peptides. Future studies will address the site of production and receptor binding site of these peptides.

Acknowledgments: We thank the Swiss National Science Foundation for the funding of our work on peptide endocannabinoids.

REVERSING THC-INDUCED IMPAIRMENT OF VERBAL MEMORY IN HEALTHY HUMANS

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One of the most often reported cognitive deficits of acute THC administration is an impaired recall of previously learned information. The aim of the present study was to determine whether THC-induced memory impairment in humans is mediated via glutamatergic or cholinergic pathways. Fifteen occasional cannabis users participated in a double blind, placebo-controlled, 6-way cross-over study. On separate test days, subjects received combinations of pre-treatment (placebo, vardenafil 20 mg or rivastigmine 3 mg) and treatment (placebo or 150 µg THC /kg bodyweight). Cognitive tests were administered immediately after inhalation of treatment was finished and included measures of memory (Visual Verbal Learning Task; Prospective Memory Test; Sternberg Memory Test), perceptual-motor control (Critical tracking task), attention (Divided Attention Task) and motor impulsivity (Stop signal task). The results of this study demonstrate that subjects under the influence of THC were impaired in all memory tasks, critical tracking, divided attention and the stop signal task. Pre-treatment with rivastigmine attenuated the effect of THC on delayed recall and non-significantly on immediate recall. When THC was given in combination with vardenafil, there were no significant interactions in any of the tasks. The present data therefore suggest that acetylcholine plays an important role in the THC-induced memory impairment, whereas for glutamate this has not been demonstrated in this study.

Aknowledgments: This research was funded by the Netherlands Organization for Scientific Research (NWO) (Grant no: 400-05-096).

SELECTIVE STIMULATION OF CANNABIOID TYPE 2 RECEPTORS (CB2) IN MONOCYTES PREVENTS THEIR ENGAGEMENT OF BRAIN ENDOTHELIUM PROTECTING BLOOD BRAIN BARRIER

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CB_2 is highly expressed in immune cells and stimulation decreases inflammatory responses. We tested the hypothesis that selective CB_2 activation in primary human monocytes diminishes their ability to engage the brain endothelium and migrate across the blood brain barrier (BBB). In an *in vitro* BBB model, CB_2 activation in monocytes substantially decreased adhesion to and migration across monolayers of primary human brain microvascular endothelial cells (BMVEC) and attenuated BBB injury. CB_2 stimulation in monocytes downregulated active forms of integrins, LFA-1 and VLA-4. Cells treated with CB_2 agonists showed increased levels of inhibitory sites of the actin binding proteins, cofilin and VASP, upstream regulators of conformational integrin changes. Activated by relevant stimuli, small GTPases Rac1 and RhoA were suppressed by CB_2 agonists in monocytes paralleling decreased formation of lamellipodia that play a key role in monocyte migration. Intravital videomicroscopy was used to quantify adhesion of leukocytes to cortical vessels in LPS-induced neuroinflammation, following injection of *ex vivo* CB_2-activated leukocytes into mice; CB_2 agonists markedly decreased adhesion of *ex vivo* labeled cells *in vivo*. These results indicate that selective CB_2 activation in leukocytes decreases key steps in monocyte-BBB engagement suppressing inflammatory leukocyte responses and preventing neuroinflammation. Development of selective, specific, nontoxic and efficacious CB_2 agonists with improved pharmacologic properties will provide new treatment for neuroinflammatory disorders.

Funding: supported by NIH grant AA015913

CB_2 AGONISTS: HOW SELECTIVE ARE THEY?

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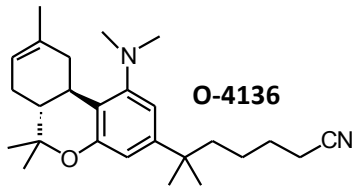
Of the two identified cannabinoid receptors, the CB_2 receptor has received far less research attention than the CB_1 receptor. Until recently, CB_2 receptors were believed to be confined to the periphery, but recent research suggests that they may also be located in the CNS, albeit their numbers and distribution may not as extensive as CB_1 receptors (Atwood & Mackie, 2010). Several structural motifs have served as templates for the development of selective CB_2 agonists that may be used to investigate these possible CNS effects. In the present study, we evaluated a series of C-1 amino and aminoalkyl substituted dibenzopyrans in a battery of in vitro and in vivo assays. Results for one of these compounds (O-4136) are presented here.

In CP55,940 displacement assays, O-4136 showed 86-fold selectivity for the CB_2 vs CB_1 receptor (CB_2 K_i=29±3.8 nM; CB_1 K_i=2491±404 nM). Further, its receptor activation profile in [^{35}S]GTPγS mirrored its binding profile, with no activation of CB_1 receptors and an E_{max}=99% (compared to E_{max}=109% for CP55,940) for the CB_2 receptor (EC_{50}=43±8 nM). In vivo, O-4136 produced characteristic cannabinoid agonist effects in the tetrad in mice and these effects were attenuated by rimonabant. In mice trained to discriminate THC from vehicle, O-4136 (30 mg/kg) partially substituted (~70%) without suppression of responding. This partial substitution was reversed by rimonabant, but not by the CB_2 antagonist SR144528.



Based upon in vitro results, O-4136 appears to be a classic CB_2-selective agonist. In contrast, in vivo results suggest that it may also have CB_1-receptor mediated effects, despite its low affinity for this receptor. Previous findings have suggested that CB_2 receptor ligands demonstrate functional selectivity in which signaling pathways they activate, with WIN55,212-2 showing the most selectivity and CP55,940 showing the least (Atwood et al., 2012). While these prior results point to the need to include diverse structural motifs in investigation of CB_2 receptor functioning, the present results emphasize the need for evaluation of putative selective ligands in both in vitro and in vivo assays. Hence, in investigation of the selectivity of CB_2 ligands, "how selective is it?" might be a less pertinent question than "*how* is it selective?"

Acknowledgements: Funded by National Institutes of Health National Institute on Drug Abuse Grant DA-003672.

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PERIPHERALLY RESTRICTED, SELECTIVE CANNABINOID CB_2 RECEPTOR AGONIST LEI-101 PREVENTS CISPLATIN-INDUCED NEPHROPATHY

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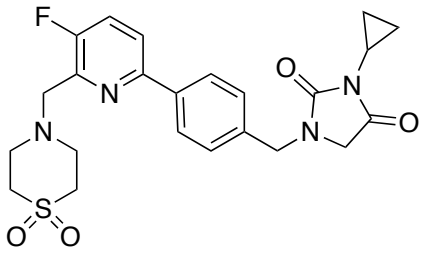
Recently, we have identified LEI-101 as a novel, peripherally restricted cannabinoid CB_2 receptor agonist. LEI-101 is a potent, selective and orally bioavailable CB_2 receptor agonist (hCB_2 pEC50 (cAMP) = 8.0; hCB_1 pEC50 < 5; F_{po} = 100%), which was active in a rat spinal nerve ligation model of neuropathic pain [1]. Here, we present the further characterization of this compound in both *in vitro* and *in vivo* models. LEI-101 is ~100-fold more selective in a CB_2 than in a CB_1 binding assay, and does not display any activity on hFAAH, MAGL, NAPE-PLD and DAGL. In a clinically relevant murine model of nephropathy (induced by the widely used antineoplastic drug cisplatin), in which the tubular injury is largely dependent on inflammation and oxidative/nitrative stress [2], we found that LEI-101 dose-dependently ameliorated kidney dysfunction and morphological damage. At 3 or 10 mg/kg (po) LEI-101 largely prevented cisplatin-induced increases in serum creatinine and blood urea nitrogen levels, improved renal histopathological injury, and attenuated oxidative/nitrative stress and inflammation in the kidney. These protective effects were absent in CB_2 KO mice, which is indicative of a CB_2-mediated effect. In addition, LEI-101 up to a dose of 60 mg/kg (po) did not exert any effects in the mouse tetrad assay of cannabimimetic activity. These results suggest that peripherally restricted CB_2 agonist LEI-101 has a good therapeutic potential in kidney and other diseases that are associated with inflammation and oxidative stress.



LEI-101

[1] Van der Stelt et al., J. Med. Chem. 2011, 54, 7450-7362
[2] Mukhopadhyay et al., Free Radic. Biol. Med. 2010, 48, 457-467.

TRIAZOLOPYRIMIDINES – A NOVEL CLASS OF HIGHLY POTENT, HIGHLY SELECTIVE AND IN VIVO ACTIVE CB2 AGONISTS

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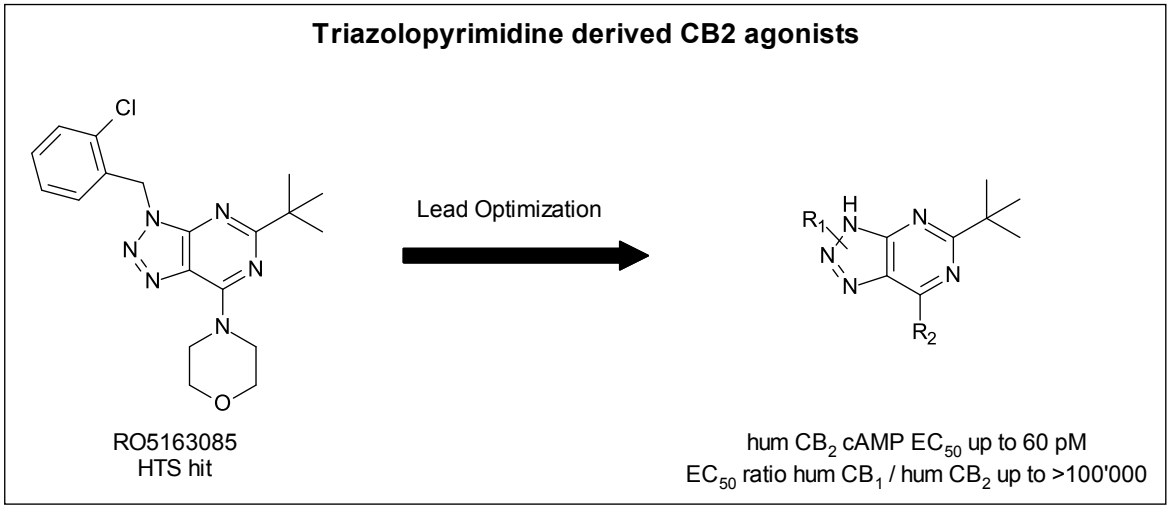
CB_2 mediated *in vivo* efficacy is often highlighted using CB_2 agonists which are not very selective against the CB_1 receptor subtype. Therefore, it is difficult to unequivocally assign the pharmacodynamics effects to CB_2 rather than CB_1 activation. We generated novel triazolopyrimidine derived CB_2 ligands highly selective against the CB_1 receptor. Starting from the potent high throughput screening hit RO5163085, which exhibited over 2'000 fold selectivity against the human CB_1 receptor in a cAMP functional assay, lead optimization provided a series of structurally novel 1- and 2-substituted triazolopyrimidines that stimulate CB2 receptors with potencies of up to 60 pM.



Details of their structure activity relationship for CB_2 and CB_1 binding and functional assays will be the subject of this communication. Additionally the physicochemical properties including solubility, membrane permeation and lipophilicity as well as metabolic stability and cytochrome P450 inhibition potential were optimized. Advanced compounds combined high *in vitro* potency with favorable early ADME properties and have been profiled in *in vivo* pharmacokinetic and efficacy studies. Selected compounds such as RO6871304 were found to protect mouse kidneys from ischemia reperfusion injury. After a period of 25 min ischemia @ 37 °C and 24 h reperfusion (n=6 per group) 10 mg/kg RO6871304 p.o. produced a statistically significant improvement of kidney function as measured by plasma creatinine levels (51% improvement; $p<0.03$). Moreover, the efficacy of RO6871304 was reflected in the reduction of relevant plasma biomarkers of kidney injury (NGAL 75%, $p<0.001$; osteopontin 85%, $p<0.001$). In addition, RO6871304 significantly reduced fibrosis in a rat unilateral ureter obstruction model (UUO) as measured by a 39% reduction in collagen I deposition 8 d after UUO (n=6, p=0.02), thereby suggesting that CB2 agonists might have beneficial effects in both acute and chronic kidney disease. These data suggest that CB_2 activation mediates protective effects in the kidney.

"WEED AGAINST ZIT?" – EXPLORATION OF THE MECHANISMS OF THE COMPLEX ANTI-ACNE ACTIONS OF CANNABIDIOL

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We have previously shown that the non-psychotropic phytocannabinoid cannabidiol (CBD) quantitatively and qualitatively normalized the "pro-acne agents" (e.g. arachidonic acid) induced lipogenesis of human SZ95 sebocytes, via the activation of transient receptor potential vanilloid-4 (TRPV4) ion channels. Moreover, we have also demonstrated that CBD exerted a unique "trinity of cellular anti-acne actions"; indeed, besides its lipostatic action, it suppressed proliferation of the sebocytes (both *in vitro* and *ex vivo* in full thickness human skin organ culture) and showed remarkable, universal anti-inflammatory effects. However, the exact mechanism-of-action of the above findings remained unclear. Therefore, in our current study, we aimed at exploring the intracellular "anti-acne" signaling pathways coupled to CBD.

First, we showed that, similar to the lipostatic effect, anti-proliferative action of CBD was mediated via the activation of TRPV4, whereas TRPV4-antagonism was unable to abolish the anti-inflammatory effect. In order to identify the signaling pathway(s) underlying the beneficial anti-acne actions, genome-wide microarray analyses were performed. These analyses (followed by confirmatory RT-qPCRs) identified TRPV4-dependent alterations in the expressions of lipid synthesis (down-regulation of nuclear receptor interacting protein-1 [NRIP1; positive regulator of the lipid synthesis in adipocytes]; and up-regulation of Rho GTPase activating protein-9 [ARHGAP9; endogenous inhibitor of the pro-lipogenic ERK-signaling]) and proliferation-related genes (down-regulation of Ki67), as well as TRPV4-independent regulation of various "immune" genes (e.g. up-regulation of tribbles homolog 3 [TRIB3; an inhibitor of the pro-inflammatory P65-NFκB pathway]). Next, we investigated the putative causative role of these newly identified target genes and the related signaling pathways in mediating the various anti-acne modalities of CBD. We found that the lipostatic activity was mediated by a TRPV4-dependent interference with the ''pro-lipogenic'' ERK1/2 mitogen activated protein kinase pathway and the down-regulation of NRIP1. Importantly, lisencing of NRIP1 mimicked the lipostatic effect of CBD. On the other hand, the anti-inflammatory action was found to be mediated by the A2a adenosine receptor-dependent up-regulation of TRIB3 and the subsequent inhibition of the pro-inflammatory P65-NFκB-signaling.

Taken together, our results demonstrate that CBD exerts its complex anti-acne effects (lipostatic, anti-proliferative and anti-inflammatory actions) on human sebocytes via two parallel signaling pathways, i.e. via the TRPV4→$[Ca^{2+}]_{IC}$↑→ARGHAP9↑ and NRIP1↓ (lipostatic/anti-proliferative effects) and the A2a→cAMP→TRIB3┤NFκB (anti-inflammatory effects) pathways. Therefore, CBD, and possibly other modulators of the identified signaling pathways, might be powerful, novel tools in the future treatment of acne vulgaris.

Acknowledgements: "Lendület" LP2011-003/2011, TÁMOP 4.2.4. A/2-11-1-2012-0001 "National Excellence Program", TÁMOP-4.2.2./A-11/1/KONV-2012-0025, OTKA 101761, OTKA 105369.

CANNABINOID AND TERPENOID PROFILING OF CANNABIS IN CALIFORNIA AND WASHINGTON

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While already well into the thousands the current number of different *Cannabis Sativa L.* strains presently available in legal markets is continuously on the rise. Consumers seeking medical relief would like to see consistency in a cultivar while some recreational users may continuously seek out novel and uniquely flavored varietals. All consumers are interested in the potential physiological impacts of each unique varietal. Numerous cultivars have received critical acclaim for their potency or general physiological impacts through international awards and press publications which popularized a specific name or national media outlets and documentaries singling out a cultivar for its' high cannabidiol content. Colorful names such as "OG Kush," "Lemon Haze," "Jack Herer," and "Charlotte's Web" have been used to represent a unique varietal. With popularity of a name comes the greater potential for its abuse by those simply seeking to capitalize on a transaction involving the cultivar.

UPLC-UV based cannabinoid and GC-FID based terpenoid chemical profiling methods coupled to principle component analysis has led us to the current understanding that there is considerable lack of standardization of cultivation methods with a concurrent lack of uniformity of chemotypic profiles across some popularized cultivar names. We have observed non-distinct chemotype profiles for "indica" and "sativa" designations, despite those terms medicinally being touted as specifically delivering a more sedative or more stimulating physiological effect respectively. Ultimately a specifically named cultivar at one dispensary is not necessarily the same product in the package at another dispensary simply because it possesses the same name. This may also be the case even week to week at the same dispensary, lending towards many different reports of the physiological impacts for a specific name and unfortunately considerable numbers of frustrated patients seeking to find relief with a specific varietal.

The latest results of our combined cannabinoid and terpenoid chemical profiling efforts across both California and Washington will be presented to provide an overview of the naming, current classifications and representative chemical profiles of the numerous cannabis cultivars available in these markets.

"SMELLS LIKE GOOD WEED": THC LEVELS IN CANNABIS MAY BE PREDICTED BY MONOTERPENE HEADSPACE CONCENTRATIONS

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The characteristic odour of cannabis is due primarily to a mixture of terpenes, with substantial variance between cannabis strains in terpene concentrations leading to markedly different odours between plants. Many consumers use smell to predict the potency of cannabis despite Δ^9-tetrahydrocannabinol (THC) and other phytocannabinoids being odourless. This link between odour and potency is plausible given common biosynthetic precursors for terpenes and phytocannabinoids within the plant, and previous research that shows a correlation between total terpene and phytocannabinoid levels (Fischedick et al., Phytochemistry 71 (2010) 2058-2073). To our knowledge, however, the association between the levels of terpenes as they occur naturally in the headspace of cannabis plant material and specific phytocannabinoids has never been directly examined. In the present study, we therefore analysed 40 cannabis samples for THC content using our previously published HPLC methods (Swift et al., PLoS One 8 (2013) e70052) and volatile terpenes using dynamic headspace sampling coupled with GC-MS.

Exploratory factor analyses revealed a three factor solution best described the data accounting for 74.88% of the variance in the terpene set. Several monoterpenes loaded strongly onto THC content, including α-pinene, β-pinene, β-myrcene, β-linalool and D-limonene (loadings between 0.5 and 0.9). Conversely sesquiterpenes such as β-caryophyllene and humulene loaded onto their own factors separate from THC. Regression analysis reveals that the monoterpene factor significantly predicts THC content ($F_{1,21}$=8.64, p=0.008), whereas the other two factors did not (p=0.4 and 0.2). Mean sample THC content (computed as THC + THC-A content) was 16.7% (SD=10.04, Range: 0.95–39.76%).

These results may reflect the fact that monoterpenes and THC have common biosynthetic precursors in the plastidial deoxyxylulose phosphate/methyl-erythritol phosphate pathway. Sesquiterpenes, on the other hand, may rely on the cytosolic mevalonate pathway. Since monoterpenes strongly influence the odour of cannabis, these results lend some support to the seemingly common consumer strategy of using odour to predict potency. This can be verified in future human psychophysical tests. Our approach might also provide an analytical method to approximate plant potency from sample headspace without further sample preparation or destruction. Interestingly, given emerging evidence of potentiation of some phytocannabinoid effects by terpenes, monoterpenes may become particularly influential when their levels rise along with increasing THC content.

PROTECTIVE EFFECT OF THE PHYTOCANNABINOID BETA-CARYOPHYLLENE IN LIVER ISCHEMIA REPERFUSION INJURY

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Liver transplantation is the ultimate therapy for end-stage liver disease. The availability of donor liver is severely limited, major approaches are underway to extend the donor criteria. Warm ischemia time is an independent risk factor for transplant dysfunction, effective novel strategies are needed to protect the liver against ischemia reperfusion injury. As a proof-of-concept, we have previously shown that activation of cannabinoid CB2 receptors in the liver protects against ischemia-reperfusion (I/R) injury. We have demonstrated the therapeutic protection for several synthetic CB2 agonists. Beta-caryophyllene (BCP) is a plant-derived natural non-toxic and potent CB2 receptor agonist that has shown an array of therapeutic effects in preclinical studies. In this study we assessed effects of systemic application of BCP in a clinically relevant rodent model of hepatic I/R injury. Hepatic I/R injury was induced by 60 min ischemia and followed by 2, 6 or 24h reperfusion in vivo.

BCP given as pretreatment before the induction of I/R, attenuated hepatic injury (measured by serum alanine aminotransferase and aspartate aminotransferase levels), decreased oxidative stress markers (tissue protein carbonyl adducts, 4-hydroxy-2-nonenal), inflammatory markers (chemokines such as CCL3 and CXCL2, TNF-α, intercellular adhesion molecule 1 (CD54) mRNA levels) and hepatocyte apoptosis (caspase 3/7 activity and DNA fragmentation). Histological evaluation revealed reduced tissue neutrophil infiltration. Protective effects of BCP against liver injury were still present when the compound was given at the onset of reperfusion.

In summary, hepatic I/R is associated with CB2 receptor activation and BCP treatment successfully decreased tissue injury and inflammation in our in vivo model. BCP treatment offers a novel therapeutic strategy against warm ischemia in liver and might be useful to preserve donor livers in the clinical setting.

EFFECT OF BETA-CARYOPHYLLENE ON PHENCYCLIDINE-INDUCED BEHAVIOURAL CHANGES

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Core evidence from laboratory and clinical research points to the involvement of the endocannabinoid system in the development of schizophrenia. Specifically, recent studies have highlighted a role for the cannabinoid CB_2 receptor in schizophrenia. In this study the effect of beta-caryophyllene, a CB_2 receptor-selective agonist, was investigated in a mouse model for schizophrenia. Phencyclidine (PCP; 5 mg/kg), an NMDA antagonist, was injected after birth to Sabra mice. The effect of beta-caryophyllene (10 mg/kg; 3 times a week for 14 days) on locomotor activity was studied in the open field test. Compared with the control group, PCP significantly decreased the exploration activity and the number of rears ($p<0.004$). Treatment with beta-caryophyllene significantly reversed the effect of PCP on rearing ($p<0.008$ vs. PCP-treated). Thus, following treatment with beta-caryophyllene the rearing activity of PCP-induced mice was not significantly different from that of the control group. The CB_2 receptor antagonist/inverse-agonist AM630 (10 mg/kg) inhibited the effect of beta-caryophyllene on rearing activity and significantly reversed the effect of beta-caryophyllene on ambulation ($p<0.04$). These results further support our previous study with HU-308 and suggest that under certain conditions, such as the inhibition of the NMDA receptors, there is a role for the CB_2 receptor in the modulation of motor activity.

Acknowledgments: This study was supported by The Institute for Psychobiology in Israel- founded by The Charles E. Smith Family, Young Investigator Award 244-09-2010 to SAG. The authors are grateful to The Daniel Turnberg UK/Middle East Travel Fellowship Scheme (to SAG), The Irving and Cherna Moskowitz Foundation (to AN) for supporting this study.

CANNABIDIOL PROVIDES PROTECTION FROM ETHANOL AND AMMONIUMTOXICITY IN A HIPPOCAMPAL MODEL OF HEPATIC ENCEPHALOPATHY

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Hepatic encephalopathy (HE) is a neuropsychiatric disorder that includes learning deficits and impairment of long-term memory. HE can be caused by chronic and excessive ethanol ingestion along with the accumulation of toxic substances that are normally removed by the liver. The pathogenesis of HE in the central nervous system includes damage to the prelimbic cortex, striatum and the hippocampus, and this pathology is believed to be mediated by the accumulation of free radicals and oxidative stress. In the present study, an in vitro model of HE has been utilized to evaluate the protective properties of cannabidiol (CBD), a substance with demonstrated protective properties against oxidative stress. For these studies, embryonic day 18 hippocampal tissue was utilized to prepare dissociated cultures consisting of a mixture of neurons and non-neuronal cells. Fluorescent assays measuring cell death (propidium iodide) and neuronal viability (CFDA) were multiplexed in the same culture well to assess the response of the toxins as well as efficacy and potency of the CBD treatment.

The amounts of ethanol and ammonium acetate required to produce a relevant and reproducible toxic response in the hippocampal cultures were determined. These toxic responses combined with the clinically determined amount of ammonia in stage 4 HE helped establish a working concentration of ammonium acetate (300 μM). An ethanol concentration of 30 mM was used to produce toxicity. Concentration-effect studies with CBD indicated EC50's of 1 μM for both toxins as estimated with the neuronal viability assay. Slightly increased EC50's for CBD were observed to prevent cell death: 4 μM. The EC90 for these protection assays ranged between 3-10 μM. The protective effects of CBD were also measured in cultures receiving combinatorial treatment with ethanol and ammonium acetate revealing EC90s of 8-14 μM. In all cases, the efficacy levels after CBD treatment brought toxin-induced changes back to control levels. Further exploration of CBD responses in hippocampal cultures indicated a significant decrease in neuronal viability (25 + 3% of control) at 100 μM. These studies suggest that CBD and CBD-like substances may provide significant protective value for the treatment of HE. Based on these results, our future CBD-related analogs will focus on safety as well as protective efficacy and enhancement of drug-like properties.

Acknowledgments: KannaLife Sciences Inc. holds an exclusive NIH license to U.S. patent #6630507 ("Cannabinoids as antioxidants and neuroprotectants") for commercialization of a target drug candidate.

NATURAL PRODUCT-DERIVED CANNABMIMETICS AS SOURCE OF POLYPHARMACOLOGY IN THE ENDOCANNABINOID SYSTEM

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To generate cannabimimetic agents that exert polypharmacology within the lipid network of the endocannabinoid system (ECS) is becoming a new concept for rational drug design. In the past decade, selective and potent pharmacological compounds have been reported for all known major targets in the ECS (CB1 and CB2 receptors, FAAH and MAGL). In recent years, several research groups have put efforts in studying polypharmacology in the ECS, mainly by modifying pre-existing molecules or by generating new chemical entities. We have approached ECS polypharmacology by starting from the unlimited source of multidiverse natural products. For example, we have investigated several chemical modifications of the widespread plant sesquiterpene β-caryophyllene, already known to be a CB2 receptor-selective agonist showing anti-inflammatory, antifibrotic, and analgesic effects *in vivo*. Structural insights into the pharmacophore of this hydrocarbon, which lacks functional groups other than double bonds, are missing. Our structure-activity study provides evidence for the existence of a well-defined sesquiterpene hydrocarbon binding site in CB2 receptors, highlighting its exquisite sensitivity to modifications of the strained endocyclic double bond of β-caryophyllene. While most changes on this element were detrimental for activity, ring-opening cross metathesis of β-caryophyllene with ethyl acrylate followed by amide functionalization generated a series of new monocyclic amides that not only retained the CB2 receptor functional agonism of β-caryophyllene with similar potency, but also reversibly inhibited FAAH (in the low micromolar range). Intriguingly, further modification of this monocyclic scaffold generated the FAAH and endocannabinoid substrate specific COX-2 dual inhibitors, which are probes with a novel pharmacological profile. This study shows that by removing the conformational constraints induced by the medium-sized ring, and by introducing functional groups in the sesquiterpene hydrocarbon of β-caryophyllene, a new scaffold with pronounced polypharmacological features in the ECS could be generated. We also investigated natural products with the aim of identifying new probes with polypharmacological features. To that aim, a library of derivatives of the lignans magnolol and 4-*O*-methylhonokiol, a selective CB2 mixed-typed biased agonist, was screened on all major ECS targets. Modification of the substituents on the biphenyl scaffold of 4-*O*-methylhonokiol and magnolol led to the identification of selective COX-2 substrate specific inhibitors with submicromolar potency showing selectivity towards endocannabinoid over arachidonic acid oxygenation. The structural and functional repertoire of cannabimimetics and their yet poorly understood intrinsic promiscuity may be exploited to generate novel probes and ultimately more effective drugs. These compounds could serve as probes to guide the *de novo* design of agents that synergistically target different components of the ECS. Moreover, novel probes differentially targeting the intricate network of lipid pathways may be useful to better understand synergy in endocannabinoid function.

CANNABIDIOL REPEATED TREATMENT INCREASES SURVIVAL AND PROMOTES RESCUE OF COGNITIVE FUNCTION IN A MURINE MODEL OF CEREBRAL MALARIA

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Cerebral malaria (CM) is a severe complication resulting from *Plasmodium falciparum* infection that causes permanent neurological and behavioral deficits after infection resolution by antimalarial drugs. Cannabidiol (CBD) is the major nonpsychotomimetic constituent of *Cannabis sativa* with neuroprotective properties. The present work aimed to determinate if CBD treatment could prevent behavioral changes found in mice infected by *Plasmodium berghei*-ANKA (PbA). Female C57Bl6 mice were infected or not with PbA (10^6 parasitized/0.2 mL PBS i.p.). On day 3 after infection (dpi) all groups received the first injection of CBD (30mg/Kg/day-7 days i.p.) or vehicle. On 5dpi, infected animals started to be treated with artesunate (32mg/kg/day-5 days i.p). Five days after completed clearance of parasitemia, all groups were submitted to the Object Recognition task (OR) and to the Elevated Plus Maze (EPM). After the EPM (18^{th} dpi) animals were sacrificed under deep anesthesia, their brains removed and prefrontal cortex (PFC) and hippocampus (HC) dissected for cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ) and neurothrophins (BDNF and NGF) determination.

Cannabidiol treatment increased the survival of PbA-infected mice. After the complete clearance of the parasitemia by artesunate, PbA vehicle treated mice displayed memory deficits in the OR (represented by decrease in the % of new object index) and exhibited an increase in anxiety-like behaviors in the EPM. Also, levels of TNF-α was found increased in HC while levels of IL-6 were found increased in PFC and HC on day 5^{th} post-infection but not after parasite clearance. Cannabidiol treatment was able to prevent long lasting anxiogenic and cognitive impairment found and in PbA mice. CBD treatment also increase BDNF expression in the HC after the complete parasite clearance. Our results indicate that CBD could be useful as an adjunctive therapy to prevent brain damage during the course of CM.

Financial support: Fapemig, CNPq.

CANNABIDIOL PROMOTES OLIGODENDROCYTE SURVIVAL AFTER HYPOXIA-ISCHEMIA IN NEWBORN RATS

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Background: Hypoxic-ischemic (HI) insults enhance the proliferation of oligodendrocyte (OL) precursors (preOL) in immature brain. Survival of those proliferating preOL, however, is very poor because those cells are particularly sensitive to oxidative stress and inflammation. This leads eventually to hypomyelinization, which plays a key role in the genesis of cerebral palsy. We described that cannabidiol (CBD) increases the number of proliferative cells in newborn rat brain after a hypoxic-ischemic (HI) insult. In the present work we aimed to determine how CBD treatment affects OL survival.

Methods: Unilateral HI brain damage was induced in newborn Wister rats (7 day-old: P7) by exposure to hypoxia (10% FiO2) for 112 min after left carotid artery electrocoagulation under anaesthesia. Ten minutes after the end of HI pups received s.c. vehicle (HV, n=18) or CBD 1 mg/kg single dose (HC, n=24). Other pups remained as controls (SHM, n= 16). One (P8), 7 (P14) or 30 (P37) days after HI rats were sacrificed, transcardially perfused with formalin 4% and their brains cut off into coronal slices for Nissl staining for a neuropathological score (NPS; 0=no damage; 5= massive tissue loss) in P8 cortex and for immunohistochemical (IHC) study on the subventricular zone (SVZ) in P8 and P14 rats: KI67 was used to detect proliferating cells, Olig-2 for glial precursors and SOX-10 for preOL. In P37 GSTπ IHC and Myelin basic protein (MBP) fluorescence in the external capsule was used to quantify the presence of mature OL.

Results: HI insult was associated with a decrease of the proliferative response in SVZ 7 days after the HI insult affecting mainly glial precursors. CBD administration prevented that decrease to occur. In particular, CBD administration led to a dramatic increase of preOL proliferation in the following hours after HI. As a result, CBD administration blunted the HI-induced decrease of long-term myelinization, presertving mature OL and myelin production.

Group	***NPS***	***KI67***		***Olig2***		***SOX-10***		***GSTπ***	***MBP***
	P14	**P8**	**P14**	**P8**	**P14**	**P8**	**P14**	**P37**	**P37**
SHM	100(10)	100(11)	100(11)	100(8)	100 (9)	100(10)	100(16)	100(28)	51.8(4)
HV	*307(22)*	94.6(11)	*65.1(10)*	81.6(14)	87.4(11)	*24.5(4)*	156(22)	*78(13)*	*38.5(7)*
HC	***217(40)***	94.1(6)	**95(20)**	92.8(5)	**106.1(10)**	**165(50)**	172(30)	**100(14)**	**52.1(7)**

Mean (SEM). Results from NPS and IHC are normalized for SHM values (%). MBP fluoresecence is normalized for the contralateral (healthy) brain hemisphere (%). *Italic: p<0.05 vs SHM.* **Bold: p<0.05 vs. HV**

Conclusions: CBD administration preserves neuroproliferation, activating preOL and preserving OL maturation after a HI insult, thus preventing HI-induced hypomyelinization.

Supported by FIS PS09/01900 and GWCRI091190-2

EFFECTS OF CBD ALONE AND IN COMBINATION WITH THC ON COGNITIVE PERFORMANCE

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The interplay between the main constituents of cannabis, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), has gained interest in recent years. Previous studies have examined effects of THC and CBD separately, generally finding opposite effects on cognition and brain function. In the current double blind crossover placebo-controlled study, the acute effects of THC and CBD alone and in combination were explored in 18 regular cannabis users and 18 non-naïve controls/irregular users (lifetime occasions of use 260 versus 20, respectively). Participants completed a baseline session, followed by 5 randomised drug sessions: 1. Placebo; 2. THC 8 mg; 3. CBD 400 mg; 4. THC 8 mg + CBD 4 mg [LoCBD+THC]; 5. THC 12 mg + CBD 400 mg [HiCBD+THC]. Drugs were solved in 100% ethanol (which served as the placebo) and vaporised using a Volcano® Vaporiser. One hour and two hours after the first administration, a top up dose was administered (1. Placebo; 2. THC 2 mg; 3. CBD 100 mg; 4. THC 2 mg + CBD 1 mg; 5. THC 2 mg + CBD 100 mg) to ensure intoxication throughout the experiment. A cognitive task battery (CogState; administered after the second top up) assessed reaction time, various memory and attention processes and cognitive flexibility. Our primary aims were to determine 1) whether high doses of CBD alone affected performance; 2) whether low and high doses of CBD combined with THC affected performance relative to THC alone; and 3) whether these effects differed according to cannabis use status.

Relative to placebo, high dose CBD alone worsened the total words recalled on a verbal learning task and tended to worsen delayed recall. LoCBD+THC impaired while HiCBD+THC improved delayed recall. CBD alone (and in combination with THC in irregular users only) also interfered with maximum learning in a paired-associates task. CBD alone worsened performance on a one card learning task, but did not alter performance when combined with THC relative to THC alone. In a set-shifting (cognitive flexibility) task, the presence of CBD with THC increased reaction times in regular users but decreased reaction times in irregular users, and LoCBD+THC decreased accuracy while HiCBD+THC increased accuracy across both groups. No other specific effects differed between groups. These findings suggest some psychoactivity of CBD and differential effects of high versus low doses of CBD when combined with THC. The results may have implications for public health campaigns to reinstate CBD into street cannabis and in considering the therapeutic efficacy of CBD.

Acknowledgements: Funded by the National Health and Medical Research Council of Australia (Project Grant 1007593) and the Australian Research Council (FT110100752). Drugs were supplied by STI Pharmaceuticals and the Volcano® Vaporiser was provided by Storz & Bickel.

THE THERAPEUTIC HANDSHAKE: CRAFTING MEDICINES FOR COMPLEX MALADIES

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There is historical recognition for the utility of the cannabis plant in human health and we now know that cannabinoids target endogenous systems in the body controlling homeostasis and auto-regulation. Modern development of pharmaceuticals involves screening of synthesised molecular libraries to identify those most potent and selective at a single receptor/disease target. However, chronic and complex diseases, particularly those with neurological/immune system involvement, are poorly treated with single focus agents, and the conventional "magic bullet" approach has been largely unsuccessful. Disease causation is multifactorial and a 'broadside' approach may be more successful in overcoming the redundancy and multi-functionality that are inherent characteristics of compensatory mechanisms in biological systems.

Despite structural similarities, individual cannabinoids have unique pharmacology. Furthermore, plants contain a hierarchy of constituents interacting synergistically; extracts are often more potent than equivalent doses of isolated compounds. Sativex, a blend of two cannabis plant extracts, is an approved prescription medicine and despite having over 300 components is still regarded a single medicinal entity by regulators. It validates a new drug development paradigm supporting the ability to pair up complementary cannabinoids to produce a polypharmacological profile able to target complex aetiopathologies. To accomplish something similar with multiple synthesised actives carries such temporal and financial burdens that their development is prohibited except within the largest disease areas.

The last two decades have witnessed a substantial increase in the amount of genetic and proteomic information available, due in part to high throughput screening, decreasing sequencing costs and the distribution of knowledge through the internet. The ability to pool this information together, coherently, will help predict the way in which a drug might act within the human body. Here we describe a model aiming to pair up the pathophysiological profile of a disease with the pharmacological profile of one or more molecules and thus predict the most appropriate combination of cannabinoids/extracts as a potential treatment. Profiles within our model are compiled using appropriately weighted information from extensive literature searches and online databases combined with proprietary in house data. A modular approach looks to include many components: receptors, enzymes, genes, organelles and more to create a system wide view rather than a selective drill down perspective. Predictions are then able to be validated in relevant in vivo models on their journey towards becoming clinical candidates.

Our model guides development of medicines with superior risk/benefit profiles compared to synthetic compounds whilst still meeting the stringent regulatory requirements expected of modern day pharmaceutical products.

THE ANXIOLYTIC-LIKE EFFECT OF CANNABIDIOL ADMINISTRATION IN CHRONICALLY STRESSED MICE IS MEDIATED BY THE ENDOCANNABINOID SYSTEM: INVOLVEMENT OF NEUROGENESIS AND AUTOPHAGY

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Chronic stress induces neuroplastic and behavioral changes that can lead to the development of anxiety disorders. Previous studies of our group indicated that repeated injection of cannabidiol (CBD), a non-psychotomimetic cannabinoid present in the *Cannabis sativa* plant, attenuates the anxiogenic-like effect induced by chronic unpredictable stress (CUS) by increasing hippocampal neurogenesis, a process that involves cell proliferation, migration, differentiation and survival. In addition, it has been shown that under cell stress conditions the recruitment of autophagy is important to promote cell differentiation, development and survival. The aim of this study was to investigate if the behavioral and pro-neurogenic effects induced by repeated administration of CBD in mice submitted to CUS are mediated by CB_1 receptors. We also evaluated if CBD effects could be related to the fatty acid amide hydrolase (FAAH) enzyme, which metabolizes endocannabinoids, and to proteins involved in autophagy, such as mTOR, Beclin-1 and LC3B. C57BL6/J mice were divided into non-stressed and stressed groups. Stressed animals were submitted to CUS during two weeks, being exposed to different randomized stressors each day. One hour after the daily stressor the animals received combined injections of vehicle, the CB_1 receptor antagonist AM251 (0.3 mg/kg), and/or CBD (30 mg/kg). On day 14, approximately 24 h after the last injection, the animals were tested in the elevated plus maze (EPM) and on day 15 were submitted to the novelty suppressed feeding test (NSF). Half of the animals of each group were decapitated and had their hippocampus extracted to perform Western Blot analysis and the other half had their brain removed, after perfusion, to perform immunohistochemistry for doublecortin staining. Results were analyzed by Student's t test, one- or two-way ANOVA followed by Duncan test. CUS decreased open arms exploration of the EPM and increased latency to feed in the NSF test, an anxiogenic-like effect. Moreover, these animals had a decrease on hippocampal cells division and migration, as well as an increase on mTOR protein expression. These responses were prevented by CBD treatment. Also, CBD diminished FAAH and increased Beclin-1 and LC3B expression, showing a pro-autophagic response. CBD effects were abolished by pre-treatment with AM251 in both animal models, and in neuronal migration and mTOR protein expression. However, the antagonist did not prevent CBD effects on cell division and FAAH expression. Altogether, these results suggest that under stress conditions CBD induces neuroprotection and prevents the behavioral effects of CUS via FAAH inhibition and CB_1 receptors activation, which recruits intracellular pathways involved in neuronal migration, differentiation and survival.

Acknowledgements: CAPES, CNPq, FAPESP, NAPNA-USP.

RESULTS FROM AUDITING MEDICAL CANNABIS FACILITIES IN THE UNITED STATES IN 2014

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Regulation is becoming mandatory in states that allow medical cannabis. The producers, manufacturers, dispensaries, and laboratories involved in this industry can operate legally in their states but function without much regulation or oversight. Due to increasing concerns over the need to standardized medicinal cannabis preparations, the American Herbal Product Association (AHPA) has created industry guidelines on manufacturing, producing, dispensing, and laboratory operation standards. Additionally, the American Herbal Pharmacopeia (AHP) completed the Cannabis monograph, a guide for the standardization of cannabis. The work of AHPA and AHP laid the foundation for a certification body called Patient Focused Certification (PFC) a project of Americans for Safe Access. AHPA and AHP guidelines are being incorporated into state level regulations as mandatory product safety standards in new state programs. PFC launched in early 2014 with facilities in several states having successfully completed the auditing process. Results from the first round of auditing of medical cannabis facilities will be discussed, including additional research on the impact of such regulations on patients, facilities, government, universities, and neighborhoods.

THE N-3 N-ACYLETHANOLAMIDE DHEA IMPROVES MANIFESTATIONS OF EXPERIMENTAL MURINE COLITIS

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Dietary n-3 fatty acids have been linked to an attenuation of inflammatory processes, but the underlying mechanisms are not completely understood. Previous work from our group has shown that dietary n-3 fatty acids increase endogenous concentrations of the n-3 fatty acid derived N-acylethanolamides (NAEs) docosahexaenoylethanolamide (DHEA) and eicosapentaenoylethanolamide (EPEA) (Balvers MGJ et al, Metabolomics 8 (2012) 1130-1147; Balvers MGJ et al, Biochim Biophys Acta 1801 (2010) 1107-1114). Using murine macrophages (Meijerink J et al, Br J Nutr 105 (2011) 1798-1807) and human peripheral blood mononuclear cell (PBMC) cultures (Balvers et al, ICRS 2013), we showed that these molecules possess anti-inflammatory properties. In addition, we have reported that DHEA is upregulated in the ileum of mice during acute inflammation (Balvers MGJ et al, Metabolomics 8 (2012) 1130-1147), suggesting that DHEA could play a role as an endogenous suppressor or modulator of inflammation. This idea brought us to investigate the effects of DHEA in a model of inflammatory diseases. The present work aimed to study whether DHEA has disease-modifying properties on the manifestations of colitis in mice, using the dextrane sulphate sodium (DSS) model. Mice received 10 or 15 mg/kg DHEA in oil *i.p.* for 7 days, whereas vehicle control mice only received oil *i.p* (n=10/group). From day 2 to day 6, DSS (2 % w/v) was added to the drinking water, which was supplied *ad libitum*. DSS is toxic specifically to the colon epithelium and causes colitis (diarrhoea, stool blood, weight loss, reduced colon length, inflammatory cell infiltration, inflamed colon) within 2-4 days. Two additional control groups received vehicle control or 15 mg/kg DHEA without DSS (-DSS; n=6/group). Animals were sacrificed at day 8 and plasma and tissues were collected. Every day, body weights were determined and stool consistency + stool blood were scored.

At day 8, the DSS treated animals receiving 10 and 15 mg/kg DHEA had significant less body weight reduction. At both day 6 and day 8, DSS groups receiving 10 and 15 mg/kg DHEA had significant better scores for stool consistency, and reduced presence of bloody stool compared to the vehicle control group. The effect was stronger at day 6 compared to day 8. Colon length was reduced in all DSS groups compared to the -DSS groups, without any effect of DHEA. Colon myeloperoxidase activity (MPO; marker of neutrophil activation) was increased in all DSS groups compared to the -DSS groups, again with no effect of DHEA. Histological evaluation of colon tissue showed signs of colon damage in all DSS groups, with the 10 mg/kg DHEA group having a significant lower colon damage score than the vehicle group. In addition, less submucosal swelling was present in the group receiving 10 mg/kg DHEA compared to the vehicle control. In conclusion, DHEA improved manifestations of DSS colitis in mice, resulting in reduced body weight loss and improved stool consistency and reduced stool blood. The effect was stronger at day 6 compared to day 8, suggesting that DHEA delayed the onset of colitis. No effects of DHEA on general colon inflammation markers were observed in the DSS model of colitis.

THE ENDOCANNABINOID SYSTEM IS A NOVEL MODULATOR OF HUMAN MACROPHAGE PLASTICITY AND POLARIZATION

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Macrophages are key players in several physiopathological processes, and their phenotype and activation status is controlled by the surrounding microenvironment. The two extremes in the spectrum of macrophage functions are represented by the classically activated (M1) and the alternatively activated (M2) phenotypes. In general, M1 macrophages have an interleukin-12 (IL-12)^{high}, IL-10^{low} phenotype, are efficient producers of proinflammatory mediators and are strong inducers of T helper-1 responses, thus mediating resistance against intracellular pathogens and tumors. Conversely, M2 macrophages show larger variations of polarization; yet the various forms of M2 macrophages (M2a, M2b, M2c) share an IL-12^{low}, IL-10^{high} phenotype and an efficient phagocytic activity, as well as they take part in T helper-2 responses, tissue repair, angiogenesis, tumor progression and immunoregulation. Since the endocannabinoid system (ECS) is largely involved in all these pathophysiological states and exerts a plethora of immunomodulatory functions, we isolated monocytes from peripheral blood of healthy donors and polarized them into M1 or into the different subsets of M2 macrophages, and the effect of anandamide (AEA) on their polarization was investigated.

Here, we show that AEA affects macrophage plasticity by skewing the proinflammatory M1 macrophages to an anti-inflammatory M2-like phenotype and, in parallel, by enhancing the anti-inflammatory phenotype of the different M2 macrophage subtypes. Furthermore we report a higher expression of NAPE-PLD and a concomitant lower expression of FAAH in M2 compared to M1 macrophages, thus reflecting their distinct responses to this endocannabinoid and suggesting that AEA metabolism may be shifted in favor of its synthesis, rather than degradation, in M2 macrophages. These findings not only suggest that the ECS could be a brand-new biomarker to characterize M1 and M2 macrophages, but also propose endocannabinoid-triggered signaling pathways as novel players either in macrophage biology or in the regulation of M1/M2 balance, advocating for the ECS as an interesting therapeutic target to treat those chronic-inflammatory diseases where such a balance is altered.

Acknowledgements: Funded by the Italian Foundation of Multiple Sclerosis (FISM) to VC (Grant 2013/R/8)

PROSTAGLANDIN D_2-GLYCEROL AND E_2-GLYCEROL INHIBIT HUMAN NEUTROPHIL FUNCTIONS

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CONTEXT. Endocannabinoids induce a profile of pro- and anti-inflammatory effects that are related, in part, to their metabolism. Our research group has demonstrated that the endocannabinoid 2-arachidonoyl-glycerol (2-AG) activates human neutrophil and eosinophil functions through its metabolites. Interestingly, endocannabinoids are substrates for the cyclooxygenase-2 and can be oxidized into prostamides (PG-EAs) and glyceryl-prostaglandins (PG-Gs). Given the structural resemblance between these metabolites and PGE_2, we postulated that PGE_2-G and PGE_2-EA, like PGE_2, would inhibit human neutrophil functions.
AIM. To determine the impact of PGE_2-G and PGE_2-EA on human neutrophils functions and the cellular mechanisms involved.
RESULTS. PGE_2-G, but not PGE_2-EA inhibited neutrophil functions such as leukotriene biosynthesis, chemotaxis, the killing of pathogens and the respiratory burst. The effects of PGE_2-G were mimicked by PGE_2 and were prevented by the EP_2 receptor antagonist AH6809. Given PGE_2-G does not bind to the EP_2 receptor, we verified if the inhibitory effect we observed was the consequence of PGE_2-G hydrolysis into PGE_2. Using deuterated PGE_2-G, we found that the latter had a half life of ~60 minutes in our neutrophil suspensions. The hydrolysis of PGE_2-G was inhibited by potassium fluoride but not MAFP or JZL-184, indicating that a serine hydrolase other than MAG lipase was involved in the hydrolysis of PGE_2-G into PGE_2. We also observed that PGD_2-G, but not $PGF_{2\alpha}$-G, also inhibits leukotriene biosynthesis by neutrophils. Importantly, PGD_2- and PGE_2-serinol amide had no effect. Finally, all the inhibitory effects of PGE_2-G on human neutrophil functions were prevented by the cAMP-dependent protein kinase inhibitor H-89, underscoring the important role of cAMP in this process.
CONCLUSIONS. PGD_2-G and PGE_2-G inhibit human neutrophil functions through their hydrolysis into PGD_2 and PGE_2, respectively. The inhibitory effects of the COX-2 metabolites PGD_2-G and PGE_2-G on human neutrophil functions partially explains the immunosuppressive role of endocannabinoids *in vivo*.

Acknowledgements: this work was supported by grants from the Canadian Consortium for the Investigation of Cannabinoids (CT), the Canadian Institutes of Health Research (NF), the Fonds de recherche du Québec-Santé (NF), and the *Natural Sciences and Engineering Research Council* of Canada (NF).

EVIDENCE THAT THE NOVEL ENDOCANNABINOID VIRODHAMINE INCREASES OSTEOBLAST PROLIFERATION: A ROLE FOR CB_2 AND GPR55

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Research has linked the endocannabinoid system to having a role in bone maintenance and growth. CB_2 receptor activation increases osteoblast proliferation, TRPV1 is thought to activate both osteoclasts and osteoblasts, and GPR55 receptor is suggested to be important in osteoclast proliferation and function. Many endocannabinoids have been identified locally in bone tissue, such as anandamide, 2-arachidonoylglycerol, oleoylethanolamide and palmitoylethanolamide. Previous research in our department indicated that the endocannabinoid virodhamine increased osteoblast proliferation and differentiation at 10 μM; the present study continues this work to determine whether this response is concentration-dependent, and to identify the mechanisms involved.

Human osteoblasts were grown to confluence *in vitro* and treated with a range of concentrations of virodhamine for 24 h. Analyses were carried out to determine its impact on proliferation (DNA assay) and differentiation (alkaline phosphatase (ALP) assay). A second set of experiments compared virodhamine treatment alongside a range of cannabinoid receptor antagonists to identify its mechanism of action.

Lower concentrations of virodhamine (10nM and 100nM) caused significant increases in DNA content ($P<0.05$-0.01) and ALP production ($P<0.05$-0.0001) in human osteoblasts, as analysed by one-way ANOVA with Dunnett *post hoc* test comparing all concentrations against the vehicle control. This significant increase was not maintained when ALP production was normalised to DNA content, indicating that increased ALP production was due to increased cell numbers. Antagonists to the CB_2 (AM630) and GPR55 (CID16020046) receptors attenuated the effects of virodhamine at a concentration of 10nM ($P<0.05$). Antagonists to CB_1 (AM251), TRPV1 (capsazepine), PPARα (GW6471) and PPARγ (GW9662) receptors had no effect on the effects of virodhamine.

This study provides the first evidence that endocannabinoid virodhamine at least in part through the CB_2 and GPR55 receptors, plays a role in osteoblast proliferation.

THE EFFECT OF CANNABINOIDS ON HUMAN BRONCHIAL EPITHELIAL CELL PERMEABILITY

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Studies in humans revealed up-regulated release of an endogenous cannabinoid, anandamide, in response to inhalation of an allergen (Zoerner et al., 2011). In contrast, previous clinical trial findings suggest anti-inflammatory and broncho-relaxant properties of the phytocannabinoid, Δ^9-tetrahydrocannabinol (THC). Bronchial epithelial injury and remodelling in respiratory diseases, such as asthma and COPD result in the loss of barrier function, thus heighten the sensitivity to environmental insults. The aim of this study was to identify the effects of cannabinoids on bronchial epithelial cell permeability using Calu-3 cell line as an *in vitro* model. Calu-3 human bronchial epithelial cells were cultured for 21 to 28 days in 12-transwell plates at air-liquid interface to allow development of tight junctions. Changes in epithelial permeability were measured using transepithelial electrical resistance (TEER) at various time points. Cells were treated with anandamide (30 μM) either alone, or in the presence of TNF_α (10 ng/mL). A selective inhibitor of fatty acid amide hydrolase (FAAH), URB597 (1 μM) and methanandamide (100 nM) were used to distinguish whether the effect on TEER was caused by metabolites or anandamide itself. In other experiments, cells were pre-incubated with THC for one hour, prior to exposure to TNF_α. The role of cannabinoid receptors in the response to THC was determined by pre-incubation with CB_1 antagonist AM251 (100 nM) or CB_2 antagonist SR144528 (1 μM). Western blotting was used to determine expression of CB receptors from TEER. Data were presented as %TEER over time by calculating the relative change in resistance from basal reading. Anandamide alone produced a reduction in TEER, significant after 4 hours (85±5%, n=5) and maintained for 48 hours. A similar effect was seen with TNF_α alone (73±8% after 2 hours, n=5, $p<0.01$ compared to vehicle control, two-way ANOVA). Pre-treatment with URB597 (1 μM) prevented the anandamide-induced reduction in TEER (%TEER with anandamide alone = 81±9%, t = 2.5 hours, compared to 91±7% in the presence of URB597 1 μM n=5, $p<0.01$ two-way ANOVA). Methanandamide did not result in any significant TEER changes. THC (30 μM) prevented TNF_α-induced reduction in TEER (%TEER with TNF_α alone = 62.4±13.0%, t = 4 hours, compared to 86.2±3.8% in the presence of THC n=5, $p<0.01$ two-way ANOVA). The effect of THC was attenuated by AM251 (100 nM; $p<0.05$) and SR144528 (1 μM; $p<0.001$). Expression of both CB_1 and CB_2 receptors in Calu-3 cells was confirmed by Western blotting. These data suggest that the reduction in transepithelial resistance by anandamide, indicative of increased epithelial permeability, is caused by its metabolites rather than anandamide itself. Conversely, THC reverses the reduction in transepithelial resistance caused by TNF_α, through an effect at CB_1 and CB_2 receptors. Hence, THC may have potential therapeutic role in inflammation-induced changes in airway epithelial cell permeability.

Reference

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Acknowledgement: This project is funded by the School of Life Sciences, University of Nottingham

INACTIVATION OF DIACYLGLYCEROL LIPASE IN INFLAMMATORY DISEASE

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Selective chemical probes of 2-arachidonoylglycerol (2-AG)- and anandamide- degradative enzymes have been instrumental in elucidating the pathophysiological functions of endocannabinoids. In sharp contrast, the biosynthetic mechanisms and enzymes responsible for production of these lipid transmitters remains poorly understood. Although diacylglycerol lipases DAGL-alpha and DAGL-beta (DAGLA and DAGLB, respectively) have been identified as candidate 2-AG biosynthetic enzymes, functional studies aimed at examining DAGL-regulated pathways in native biological systems has been hindered by the lack of appropriate chemical tools to perturb their activities *in vivo*. To address this problem, I will describe the application of activity-based protein profiling (ABPP) for the discovery and development of the first selective and *in vivo*-active small-molecule inhibitors for DAGLB, lead DAGLA inhibitors, and paired negative-control and tailored activity-based probes for the functional analysis of DAGLs in living systems. We utilize our newly developed chemical probes in conjunction with functional proteomic/metabolomics methods to show that DAGLB inactivation lowers 2-AG production in mouse peritoneal macrophages, as well as arachidonic acid and eicosanoids in a manner that is distinct and complementary to cytosolic phospholipase-A2. A corresponding reduction in lipopolysaccharide (LPS)-induced TNF-alpha release was observed, indicating that DAGLB serves as a key metabolic hub within a lipid signaling network that regulates proinflammatory responses in macrophages. Consistent with our biological findings in peritoneal macrophages, I will also describe more recent and unpublished data in mouse models showing that pharmacological/genetic inactivation of DAGLB produces anti-allodynic and anti-anapyrexia effects in LPS-induced inflammation. Our results support a novel role for DAGLB in coordinating lipid-signaling networks involved in systemic inflammation and may represent a novel therapeutic target for long-term treatment of pain.

Acknowledgements: This work was supported by the US National Institutes of Health DA017259 (B.F.C.), DA033760 (B.F.C.), MH084512 (B.F.C.), the Skaggs Institute for Chemical Biology, Dainippon Sumitomo Pharma (K.T.), and a Hewitt Foundation Postdoctoral Fellowship (K.-L.H.).

DOCOSAHEXAENOYL-SEROTONIN (DHA-5-HT), AN INTESTINAL CONJUGATE OF SEROTONIN AND DHA EXHIBITS ANTI-INFLAMMATORY PROPERTIES

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Due to their presumed beneficial effects in health and disease, *n*–3 LC-PUFAs are receiving much interest. Recently, we provided evidence for an alternative mechanism underlying the anti-inflammatory effects of *n*–3 LC-PUFA, namely via the DHA-derived *N*-acylethanolamine DHEA (Meijerink et al., Br. J. Pharm **169** (2013) 772-783). Now, we present a second endogenous DHA-derived conjugate, with serotonin, which displays potent immune-modulating properties. DHA-5-HT, is found in gut tissue where most of the body's serotonin resides. Levels of intestinal DHA-5-HT are paralleled by the DHA content in the diet as was shown in a mice study (Verhoeckx et al., BBA **1811** (2011) 578-586). To investigate the immune modulatory activities of DHA-5-HT, its effects on several cytokines, chemokines and key inflammatory mediators were studied in RAW264.7 macrophages stimulated with LPS. Immune-modulation of inflammatory mediators and enzymes (Nitric oxide, IL6, MCP-1, CCL20, COX-2 and PGE_2) was assessed using different methods, like Griess assay, ELISA, Q-PCR and EIA.

Among a series of structurally related fatty-acid serotonin conjugates, DHA-5-HT turned out to be the most effective component in reducing nitric oxide (NO) release from stimulated macrophages. An NO reduction of approximately 80% was elicited by 2.5 μM DHA-5-HT. Both EPA-5-HT and OA-5-HT were also found to be effective in decreasing NO in this assay whereas the precursor DHA did not evoke any response. DHA-5-HT dose-dependently reduced levels of a number of cytokines and chemokines, including IL6, MCP-1 and CCL20. At 100 nM DHA-5-HT significantly reduced levels of the cyclooxygenase 2 (COX-2) metabolite PGE_2, an effect found to be regulated at the gene-expression level, as mRNA levels of the key inflammatory enzyme COX-2 were strongly down regulated by DHA-5-HT as well. However, none of the following receptors, CB_1, CB_2, PPARγ, nor the β2-adrenergic receptor were found to be involved in mediating the immune-modulatory effects of DHA-5-HT. Further studies are undertaken to elucidate the mechanism(s) of action. It can be concluded that the endogenously formed intestinal DHA conjugate of serotonin, DHA-5-HT, has strong immune modulating properties, which raises interesting opportunities in relation to intestinal diseases with an inflammatory component, both from a nutritional and a pharmacological perspective.

Acknowledgement: Authors work is supported by VLAG graduate school.

THE DEVELOPMENT OF A CANNABINOID-CONTAINING COLLAGEN-GAG SCAFFOLD FOR USE IN ORTHOPAEDIC TISSUE ENGINEERING STRATEGIES

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Cartilage tissue is frequently damaged yet lacks an intrinsic capacity for self-repair. Therefore, tissue engineering strategies are of interest to promote cartilage repair in diseases such as osteoarthritis. We have previously reported that a collagen-glycosaminoglycan (CG) scaffold supports the differentiation of MSCs along the chondrogenic line[1]. We are currently investigating whether the CG scaffold can be augmented with drug eluting properties to overcome current limitations such as poor penetration of cells into the core of the scaffold, death of a subpopulation of the seeded cells, and the requirement to use high concentrations of chondrogenic growth factors. The endocannabinoid system comprises two G-protein coupled receptors, CB_1 and CB_2, their endogenous ligands, and the enzymes involved in the synthesis and degradation thereof. There is evidence to suggest that activation of CB_1 and CB_2 may regulate MSC motility[2] as well as improving MSC viability and promoting differentiation of MSCs along the chondrogenic line[3]. The endocannabinoid system also possesses anti-inflammatory[4] and anti-oxidative[5] properties in addition to their well-documented analgesic capabilities[6], which may be of benefit in osteoarthritis.

We have developed a novel CG scaffold containing the phytocannabinoid, cannabidiol (CBD). The release profile of CBD from the scaffolds was analysed using high performance liquid chromatography (HPLC) and demonstrated the successful loading of CBD (1 – 50 μM) onto the CG scaffolds, with the drug strongly adhering to the scaffolds when incubated in an aqueous solution. MC3T3 cells, an osteoblast-like cell line, are able to adhere to the scaffold and appeared healthy in the scaffold. In parallel 2-dimensional studies, MC3T3s were treated with pro-inflammatory (IL-1β; 2.5 $ng.ml^{-1}$) and oxidative (H_2O_2; 0.05 μM) insults, which, in combination, act to mimic the microenvironment of the osteoarthritic joint, either in the presence or absence of CBD (5 μM) for 24 h. Following treatment, cell viability was assessed using TdT dUTP Nick-End Labelling (TUNEL). There was a significant increase in the rate of apoptosis in cells treated with IL-1β and H_2O_2 ($p<0.01$, ANOVA Newman-Keuls *post-hoc*, n=4 cultures) and this increase was abrogated in the presence of CBD. These data suggest that CBD is capable of protecting against pro-inflammatory and oxidative stresses similar to those observed in the osteoarthritic joint. This cytoprotective property of CBD may help to improve the viability of patient-derived cells loaded onto the CBD-eluding CG scaffolds thereby facilitating the generation of a superior cartilage construct for use in orthopaedic tissue engineering strategies and these translational studies have recently commenced.

Acknowledgments: Funded by Science Foundation Ireland

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ENDOCANNABINOIDS DIFFERENTIALLY MODULATE NLRP3 INFLAMMASOME ACTIVATION IN PRIMARY HUMAN MACROPHAGES

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Innate immunity is characterized by its ability to recognize a wide range of pathogens through a limited number of receptors, mainly TLRs, and the recent NLRs, which consist of soluble proteins that survey the cytoplasm for "danger signals" that advertise the presence of intracellular invaders, forming the so-called inflammasomes. Inflammasomes are molecular complexes that activate inflammatory caspases, which are involved in the maturation of cytokines of the IL-1 family. On the basis of the recent evidences demonstrating the important role of the endocannabinoid system in the modulation of several immune responses, we investigated whether N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2AG) may interfere with inflammasomes activation.

Our findings show that AEA and 2AG have opposite effects on NLRP3-inflammasome activation. In particular, AEA inhibited NLRP3-inflammasome dependent IL-1β production in a CB_2-dependent manner and 2AG exerted an activatory effect through both CB_1 and CB_2 receptors. Furthermore, AEA reduced NLPR3 expression at both mRNA and protein level whereas 2AG enhanced such effect. Overall, our findings account for a new homeostatic role of the endocannabinoid system in the fine-tuning of those feedback loops that are crucial for either initiation or resolution phases of inflammation, thus hinting at novel therapeutic opportunities for the treatment of several inflammatory diseases.

METABOLISM OF EXOGENOUS ARACHIDONOYL-ETHANOLAMIDE AND 2-ARACHIDONOYL-GLYCEROL BY HUMAN EOSINOPHILS

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BACKGROUND. Eosinophils are leukocytes involved in numerous inflammatory diseases such as asthma and possibly obesity. How they migrate to the tissues is not completely defined but it involves chemokines and/or bioactive lipids, noteworthy prostaglandin (PG) D_2 and 5-oxo-eicosatetraenoate (5-KETE), and possibly 2-arachidonoyl-glycerol (2-AG). In this respect, we documented that the combination of IL-5 and 2-AG induces an eosinophil migration comparable to that of CCL11, a chemokine inducing the selective recruitment of eosinophils. Noteworthy, the 2-AG-induced eosinophil migration was prevented by 15-lipoxygenase (LO) inhibitors, suggesting an involvement of the 15-LO pathway in the regulation of eosinophil functions by endocannabinoids. We thus postulated that 2-AG and arachidonyl-ethanolamide (AEA) were metabolized by the 15-LO.

RESULTS. We developed and analytical HPLC method that separates numerous AEA-, 2-AG-, and arachidonic acid (AA)-derived 15-LO metabolites as well as cysteinyl-leukotrienes (LT). AEA was mainly metabolized by eosinophils into 15-HETE-EA. This metabolism of AEA was concentration- and time-dependent. Moreover, it not modulated by platelet-activating factor (PAF), which activates the 5-LO pathway. In absence of PAF, human eosinophils mainly metabolized 2-AG into 15-HETE. In presence of PAF, human eosinophils mainly metabolized 2-AG into 15-HETE, and LTC_4. Another set of experiments was also performed in which eosinophils were incubated in presence of the 2-AG hydrolysis inhibitors MAFP or JZL-184. Under these conditions, 2-AG was mainly metabolized into a mixture of 15-HETE-*sn2*-glycerol and 15-HETE-*sn1*-glycerol. PAF did not modulate the levels of the glyceryl metabolites of 15-HETE we observed.

CONCLUSIONS. AEA and 2-AG are metabolized by human eosinophils. AEA is mainly metabolized by the 15-LO pathway. 2-AG can be hydrolyzed into AA and eicosanoids from the 5- and the 15-LO pathways. When 2-AG hydrolysis is prevented, 2-AG is mainly metabolized by the 15-LO pathway. This study underscores the importance to characterize the biological functions of endocannabinoid-derived 15-LO metabolites in the regulation of inflammation in order to define the impact of systemic MAG lipase inhibition in the regulation of inflammation.

Acknowledgements: This work was supported by grants from the Canadian Consortium for the Investigation of Cannabinoids (CT), the Canadian Institutes of Health Research (NF), the Fonds de recherche du Québec-Santé (NF) and its Respiratory Health Network (MCL, NF, ML, CL), and the Natural Sciences and Engineering Research Council of Canada (NF).

INFLUENCE OF THE DOPAMINE RECEPTOR TYPE 2 (D_2) ANTAGONIST ON THE CANNABINOID RECEPTOR TYPE 1 (CB_1) FUNCTION

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The cannabinoid receptor type 1 (CB_1) is able to activate several signaling pathways through the activation of different G proteins as well as arrestin-2. The CB_1 and dopamine receptor type 2 (D_2) oligomerize, providing unique pharmacology *in vitro* and *in vivo*. Co-expression of CB_1 and D_2 receptors in HEK cells and treating them with both CB_1 or D_2 agonists lead to an accumulation of cAMP, while stimulation with either receptor agonist lead to an inhibition of cAMP. In addition, receptor agonists may modulate subcellular localization, receptor expression and homo- and heterodimer ratios of CB_1 and D_2 receptors. Patients treated with typical antipsychotics such as haloperidol (D_2 antagonist) might be exposed to cannabinoids for medical or recreational purposes. The present study aimed to investigate the effect of the D_2 antagonist haloperidol on the $G\alpha_i$- protein coupling and arresting-2 recruitment to CB_1 in the presence of CB_1 agonists in a cell model of striatal neurons.

Using bioluminescence resonance energy transfer ($BRET^2$), we confirmed that CB_1 and D_2 receptors form heterodimers in striatal neurons. Pre-assembled CB_1-$G\alpha_i$ complexes were detected by $BRET^2$ in cells expressing CB_1-GFP^2 and $G\alpha_i$-Rluc fusion proteins. The cannabinoid agonist arachidonyl-2'-chloroethylamide (ACEA) caused a rapid and transient activation of the $G\alpha_i$ G protein. Haloperidol had no effect on ACEA-induced $G\alpha_i$ activation in the absence of D_2 receptors. When the D_2 receptors were co-expressed with CB_1-GFP^2 and $G\alpha_i$-Rluc, haloperidol reduced ACEA-induced $G\alpha_i$ activation. Haloperidol treatment significantly decreased $G\alpha_i$- dependent ERK activation in response to ACEA as detected using In-cell-Western analysis. In addition, ACEA treatment resulted in a slow and sustained arrestin-2 recruitment to CB_1, which was reduced in the present of haloperidol. These results demonstrated another level of complexity and regulation of the signaling of CB_1 and D_2 receptor complexes.

Acknowledgments: Funded by a partnership grant from CIHR, Nova Scotia Health Research Foundation (NSHRF), and the Huntington Society of Canada (HSC) (ROP-97185). Scholarship support to AB is provided by King Abdul Aziz University of Saudi Arabia.

EFFECTS OF CONTROLLED, TRANSIENT HYPEROXIA ON THE SERUM LEVELS OF DIFFERENT ENDOCANNABINOIDS – A HUMAN PILOT STUDY

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It has been described in multiple human and animal studies that several drugs, commonly used in anesthesiology clinical practice, can significantly modify the plasma levels of certain endocannabinoids (eCBs; e.g. anandamide [AEA] or 2-arachidonoylglycerol [2-AG]). However, to date, there are no data about either the effects of assisted respiration or of alterations of partial oxygen pressure, on the plasma eCB levels. Therefore, in our current pilot study, we aimed at investigating the putative alterations of the plasma levels of different eCBs and related mediators (anandamide, 2-AG, palmitoylethanolamine [PEA] and oleoylethanolamine [OEA]) in patients requiring mechanical ventilation.

Three patients, hospitalized at the intensive care unit, requiring assisted ventilation but lacking known respiratory diseases, were involved and a total number of four experiments were performed (in order to investigate the intra-individual reproducibility of the results, the experimental procedure was repeated two hours later in the case of one patient). The examination period was 1 hr long, during which both arterial and venous (from the radial artery and from the superior vena cava, respectively) blood samples were obtained every 10 minutes (from min 0 to min 60). Samples were immediately centrifuged and the plasma was stored at -80°C until analytical eCB-determination. Following 5 min of normoxia (achieved by applying 100 mmHg partial O_2 tension [FiO_2: 0.2]), hyperoxia was induced for 30 min (by increasing partial O_2 tension to 200 mmHg [FiO_2: 0.4]), which was followed by normoxia (100 mmHg partial O_2 tension [FiO_2: 0.2]) until the end of the surveillance period. We found that arterial and venous plasma levels of anandamide, 2-AG and OEA showed no significant differences during the observation. Moreover, due to the high inter-individual differences, no characteristic changes in the levels of the above eCBs could be concluded (arterial anandamide tended to increase whereas arterial OEA showed a slight decline). Moreover, neither arterial nor venous plasma levels of 2-AG appeared to be influenced by hyperoxia. However, of great importance, we identified a robust difference between venous and arterial PEA plasma levels (885. 08± 202.4 vs. 16.7± 3.9 pmol/mg lipid, respectively). Moreover, during the first 15 minutes of hyperoxia, venous PEA levels dramatically decreased (to 306.4±20.8 pmol/mg lipid).

The marked veno-arterial differences in PEA concentrations suggest that PEA is constitutively "used-up" by the lungs. Knowing that palmitic acid is a key component of the pulmonar surfactant dipalmitoylphosphatidylcholine, the results of our pilot study implicate that PEA may act as a substrate for pulmonary surfactant production, arguing for a novel and unusual mechanism for the cross-talk between peripheral tissues and the respiratory tract.

Acknowledgements: "Lendület" LP2011-003/2013.

THE PERIPHERALLY-RESTRICTED CANNABINOID RECEPTOR AGONIST PRNMI ALLEVIATES CISPLATIN INDUCED PERIPHERAL NEUROPATHY

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Painful peripheral neuropathy as a consequence of cancer chemotherapy is a severe and dose limiting side effect associated with the use of antineoplastic agents such as cisplatin, paclitaxel and vinca alkaloids. Current medications for pain alleviation often lack efficacy and exhibit undesirable side effects. Preclinical studies have demonstrated the analgesic effectiveness of brain-penetrant cannabinoids in the treatment of chemotherapy induced peripheral neuropathy (CIPN). A major impediment to the widespread use of these analgesics is their central nervous system (CNS) mediated psychotropic side effects, which can potentially be circumvented by the development of cannabinoid receptor (CBR) agonists that do not appreciably cross the blood-brain barrier. We recently succeed in the development of several such compounds, which exhibited potent effects in alleviating chronic inflammatory and neuropathic pain symptoms without CNS side effects (Seltzman et al, ICRS, 2013). Here we examined one of these compounds (4-{2-[-(1E)-1[(4-propylnaphthalen-1-yl)methylidene]-1H-inden-3-yl]ethyl}morpholine, PrNMI) for effectiveness in alleviating the painful symptoms of mechanical and cold allodynia in a rat model of CIPN.

CIPN was induced in rats by 1/week administration of cisplatin (3 mg/kg, i.p.) for 4 weeks. Oral administration of PrNMI dose-dependently suppressed mechanical and cold allodynia symptoms with complete symptom suppression at 3 mg/kg. Daily oral administration at 1 mg/kg consecutively for two weeks resulted in similar daily suppression of mechanical allodynia implicating little, if any, tolerance development. Intraplantar injection (0.25 mg/kg) completely suppressed CIPN symptoms, suggesting peripheral sensory nerve terminals as the main sites of PrNMI's anti-allodynic action. PrNMI co-administration with selective CB1R or CB2R blockers revealed mainly CB1R contribution to its analgesic effects. CNS side effects assays compared the brain-permeant CB1R agonist HU-210 at doses that alleviate neuropathy symptoms to PrNMI, its analogs, and vehicle. While HU-210 exhibited strong CNS side effects at systemic doses that relieve neuropathy symptoms, PrNMI and its analogs showed complete lack of side effects in the assays that test for catalepsy, hypothermia and motor incoordination.

These results suggest that the potency, peripheral selectivity, in vivo efficacy, and absence of CNS side effects of this novel class of CBR agonists identify them as a potentially viable treatment for CIPN pain symptoms.

Acknowledgments: Support contributed by the Academic Senate Faculty Grant (I.S.) and UCLA School of Graduate Studies Stipend (Y.M.)

OLEOYLETHANOLAMIDE (OEA) AND PALMITOYLETHANOLAMIDE (PEA) MODULATE INTESTINAL PERMEABILITY IN AN *IN VITRO* ISCHAEMIA/REPERFUSION MODEL

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Adaptive cardiovascular responses accompanying shock reduce gastrointestinal (GI) tract perfusion and thus tissue oxygenation. Impaired tight junctional function increases GI permeability with loss of integrity of the vital protective GI luminal-vascular barrier. We previously reported that endocannabinoids, including oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), modulated GI permeability under normal and inflammatory conditions in an *in vitro* model. We hypothesised that OEA and PEA might also modulate GI permeability in an ischemia/reperfusion model.

Caco-2 cell monolayers were grown on transwell inserts. Transepithelial electrical resistance (TEER) was recorded to measure permeability. Hypoxic conditions were mimicked using the GasPak™ EZ Anaerobe Pouch System (Beckton-Dickinson, Oxon, UK), for 4h (recoverable intention) or 6 h (non-recoverable) after which media was refreshed and inserts returned to the incubator. OEA and PEA were applied to monolayers either apically or basolaterally. Potential targets were probed using antagonists to transient receptor potential vanilloid subtype-1 (TRPV1) receptors and peroxisome proliferator-activated receptor-α (PPARα). Appropriate vehicles were applied to control inserts.

Hypoxia was associated with falls in TEER of approximately 35% after 4h and 50% after 6h, indicating abnormally increased permeability. When applied pre-hypoxia, apical OEA inhibited TEER falls, but basolaterally further increased permeability over hypoxic effects alone. Basolateral PEA inhibited TEER falls (P<0.001, ANOVA with Dunnett's post-hoc test), restoring permeability towards normal, but had no effect apically. After 4h hypoxia apical OEA still inhibited TEER falls attenuating hypoxic increased permeability, an effect inhibited by a TRPV1 antagonist. After 4h hypoxia, basolateral PEA still inhibited TEER falls (P<0.001) restoring permeability towards normal, inhibited by a PPARα antagonist (P<0.001). After 6h hypoxia, basolateral PEA accelerated restoration of normal permeability whereas OEA has no effect.

This study demonstrates that in a recoverable model of intestinal ischemia and reperfusion, apical OEA (via TRPV1) and basolateral PEA (via PPARα) attenuated increased permeability *in vitro*. In the non-recoverable model, only basolateral PEA was associated with significant restoration of normal permeability. The effects observed may, at least partly, explain protective effects of OEA and PEA observed during GI ischaemia *in vivo (Di Paola et al., 2012)*.

Reference: Di Paola, R.,Impellizzeri, D.,Torre, A.,Mazzon, E.,Cappellani, A.,Faggio, C.,Esposito, E.,Trischitta, F.,Cuzzocrea, S. 2012. Effects of palmitoylethanolamide on intestinal injury and inflammation caused by ischemia-reperfusion in mice. *J Leukoc Biol,* 91**,** 911-920.

N-ACYLDOPAMINES DERIVED FROM POLYUNSATURATED OMEGA-3 FATTY ACIDS EXERT ANTI-INFLAMMATORY EFFECTS IN MOUSE MACROPHAGES

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N-acyldopamines are a class of endogenous compounds that have been shown to possess bioactivity (Navarrete *et al*., Biochem. Pharmacol. ***79*** (2010) 1805–1814; Jin *et al*., Eur. J. Lipid Sci. Technol ***115*** (2013) 1284–1293). In particular, *N*-arachidonoyldopamine (NADA) was discovered as an endocannabinoid by binding to cannabinoid receptor 1 (CB_1) and transient receptor potential V1 (TRPV1); *N*-oleoyldopamine (OLDA) was reported as a capsaicin-like lipid with full TPRV1 agonist activity. However, *N*-acyldopamines derived from the polyunsaturated omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have received less attention so far (Meijerink *et al*., BJP ***169*** (2013) 772–783). Here, we report the development of an effective enzyme-based approach to synthesize *N*-eicosapentaenoyldopamine (EPDA) and *N*-docosahexaenoyldopamine (DHDA) (Fig. 1). Subsequently, the potential anti-inflammatory properties of EPDA and DHDA were evaluated by measuring each compound's effects on nitric oxide (NO), MCP-1, CCL20 and IL6 production from lipopolysaccharide (LPS)-stimulated RAW264.7 cells, using a Griess assay and ELISA.

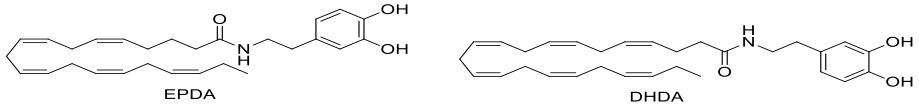

Fig. 1. Structure of *N*-eicosapentaenoyldopamine (EPDA) and *N*-docosahexaenoyldopamine (DHDA)

Synthesis of EPDA and DHDA was achieved with a one-step enzyme-catalysed *N*-acylation of dopamine with the fatty acids. In our method, *Candida antarctica* Lipase B was selected as the optimal catalyst and 2-methyl-2-butanol as the solvent of choice for this reaction. The resulting *N*-acyldopamines DHDA and EPDA significantly reduced the release of nitric oxide (NO) from lipopolysaccharide (LPS)-stimulated macrophages in a concentration-dependent way, whereas their parent compounds dopamine, DHA and EPA were ineffective. Moreover, the production of the inflammatory chemokines monocyte chemotactic protein-1 (MCP-1), macrophage-inflammatory protein-3α (CCL20) and the cytokine interleukin-6 (IL6) was dose-dependently (0.1 μM-2.5 μM) inhibited by both compounds. Taken together, our data suggest that both *N*-acyldopamines derived from polyunsaturated fatty acids may be part of another class of immune-modulating n-3 fatty acid-derived lipid mediators. It will be interesting to determine their endogenous presence and mechanisms of action in order to elucidate their physiological role.

Acknowledgements: Authors work is supported by VLAG graduate school and European Commission, Fondo Sociale Europeo (FSE), Calabria Region.

RATIONALE FOR TARGETING THE ENDOCANNABINOID SYSTEM TO MANAGE NEUROINFLAMMATION IN LPS-ACTIVATED PRIMARY MICROGLIAL CULTURES

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Neuroinflammation occurs when microglia-altered response leads to pathological changes in the central nervous system, thus it plays a key role in conditions of chronic pain. Therapy of this disorder is unsatisfactory and often limited to partial symptom relief. Currently one of the main approaches, to develop new treatments, focus on the unique long-term consequences of nerve injury involving microglial activation. There is considerable evidence supporting a role for endocannabinoids (EC), particularly anandamide (AEA), in the modulation of chronic pain. Moreover there is a functional EC system present in microglial cells and activation of cannabinoid receptors (CB1 and CB2) has been implicated in the control of its immune-related functions. Although AEA activates primarily CB1 and CB2, it might also acts on other targets (i.e. PPARα, PPARγ, "orphan" GPR55/18). Therefore in order to increase our understanding of the role of EC system modulation in LPS-induced microglial activation we explored their possible valuable therapeutic role to manage neuroinflammation as well as its possible mechanisms of action.

Primary cultures of microglial cells were prepared from 2-days-old Wistar rat pups. Adherent cells were incubated for 48 h in culture medium before compounds treatment. Phenotype was confirmed with *c1q* gene expression as well as IBA-1 and OX-42 immunostaining. Cultures contamination of astrocytes was excluded by measurements of *gfap* expression. We examined the influence of compounds on microglial cells activation by measuring NO production 24h post treatment. Cell viability assays (MTT and LDH) were performed to asses toxicity of tested compounds. mRNA and protein analysis were conducted for pro- and anti-inflammatory cytokines and EC-related molecules possibly affected by the treatment. Immunohistochemistry was used to determine the presence of examined receptors. Results were normalized to cells treated with vehicle.

LPS-induced NO release was significantly attenuated after pretreatment with AEA. This effect was not blocked by co-treatment with AM-251, a CB1 antagonist. Administration of AM-630, CB2 antagonist, partially abolished effects of AEA on microglial cells, also it strongly upregulated expression of pro-inflammatory cytokines. Neither treatment with PPARα/γ antagonists (GW 6471 and GW 9662 respectively), nor with GPR18/55 agonist, O-1602, did not altered NO production in activated primary microglial cultures. Interestingly *c1q*, a marker of microglial activation, was downregulated only after treatment with O-1602. GPR18/55 agonist also upregulated the expression of anti-inflammatory cytokine *il-10*. On the other hand GPR18/55 antagonist (CID 16020046) attenuated NO production in tested cells, but did not interfere with C1q expression. Obtained results correlated with *inos* and *cox2* mRNA expression in microglial samples.

Our results suggest that AEA counteracts inflammation through CB2 receptor, possibly by inhibiting both *inos* and *cox2* expression levels. Antagonism of GRPs might contribute to AEA actions. Dissimilar we showed that activation of GPR55 on primary microglial cells blocks its activation, but failed to affect LPS-induced inflammation. Our results leads to conclusion that EC system plays a crucial role both in management of neuroinflammation and microglial activation, but different receptors are involved in control of these processes. Further investigation might provide a new mechanism of action of AEA to limit neuroinflammation.

Acknowledgments: Supported by LIDER/29/60/L-2/10/NCBiR/2011, SONATA BIS NCN/2012/07/E/NZ7/01269, OPUS NCN/2011/03/B/NZ4/00042 and statutory funds.

COLITIS ALTERS CENTRAL ENDOCANNABINOID CONTENT

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The relationship between stress and inflammation bears significant clinical relevance, as there is a large degree of comorbidity between stress-related mental illnesses (i.e. depression or anxiety disorders) and chronic inflammatory diseases (i.e. inflammatory bowel diseases). Currently, the mechanisms linking stress and inflammation are not well understood. However, given the relationship between stress exposure and the exacerbation of an array of inflammatory conditions, determining the mechanisms linking stress and inflammation are of utmost therapeutic importance. The endocannabinoid system is involved in activation and termination of the stress response, as well as emotional behavior. Downregulation of the endocannabinoid system may be related to the development of mood and anxiety disorders. To date, however, there is no knowledge regarding whether central endocannabinoid content is altered by sustained peripheral inflammation, and whether this system could be involved in changes in stress and emotionality associated with peripheral inflammation. To examine this question, we employed an animal model of colitis, which produces a state of systemic inflammation and is known to modulate emotional behavior, and assessed its effects on central endocannabinoid content.

We used a well-established model of rodent colitis, in which adult male Sprague Dawley rats were administered a single intracolonic enema of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 0.5 ml, 50 mg/ml in 50% ethanol) or vehicle (0.5 ml 50% ethanol). 7 days after administration, rats were rapidly decapitated and corticolimbic brain regions (amygdala, hippocampus, hypothalamus and prefrontal cortex) were extracted and analyzed for anandamide and 2-arachidonoylglycerol (2-AG) levels by mass spectrometry. TNBS treated rats exhibited significant elevations in colonic inflammation as indicated by elevated levels of myeloperoxidase activity (MPO) and increased macroscopic tissue damage scores compared to vehicle treated. Similar to what we have seen following chronic stress, anandamide levels were decreased in the amygdala, hypothalamus and prefrontal cortex, but not in the hippocampus. In the amygdala, this decrease in anandamide levels was associated with an increase in the maximal hydrolytic activity of fatty acid amide hydrolase, a phenomenon that we have established contributes to the generation of anxiety. Interestingly, 2-AG levels were elevated within the hypothalamus, but no other brain region. Furthermore, there were no differences in locomotor activity at 7 days post-administration between vehicle and TNBS animals, indicating the amenability of this model to subsequently investigate the role of endocannabinoid signaling in inflammation-induced alterations in emotional behaviour.

MONOGLYCERIDE LIPASE DEFICIENCY CAUSES INTESTINAL CANNABINOID RECEPTOR DESENSITIZATION

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The endocannabinoid system (ECS) is known to affect gastrointestinal motility. Monoglyceride lipase (MGL) is expressed throughout the gastrointestinal tract where it strongly determines the level of the endocannabinoid 2-arachidonoyl glycerol (2-AG). Blockade of MGL causes an increase of 2-AG, and reduces the availability of arachidonic acid. Thus, MGL inhibition is associated with complex changes in lipid signaling and can affect inflammatory and regulatory processes by different mechanisms.
The aim of this study was to investigate whether mice globally lacking MGL (MGL-ko) show alterations in intestinal function. We found that MGL-ko mice displayed normal gut motility as compared to their wild-type littermates under normal conditions. However, cannabinoid receptor (CBR) agonists reduced whole gut transit in wild-type but not in MGL-ko mice suggesting desensitization of CBR. To elucidate whether the lack of sensitivity to CBR agonist treatment was caused by central action or direct desensitization of the gut, we performed *ex vivo* electrical field stimulation studies. Similar as observed *in vivo*, ileum segments of MGL-ko mice were insensitive to WIN 55,212 stimulation. To apply pathophysiological conditions, we induced a mild intestinal inflammation in mice using LPS. Under these conditions, wild-type mice showed increased stool weight while MGL-ko mice did not display any signs of intestinal hypermotility.
In conclusion, our data suggest that MGL strongly influences 2-AG levels in the intestine. 2-AG accumulation does not cause hyper-activation of the ECS, but is associated with severe desensitization of intestinal CBR.

Acknowlegdements: This research was supported by grants of the Austrian Science Funds, FWF, projects P21296, P 22771, P 25633, and by the DK Molecular Enzymology (W901-B05 DK).

MODIFYING CB1 RECEPTOR SIGNALLING TO REDUCE IOP IN A MOUSE MODEL OF OCULAR HYPERTENSION

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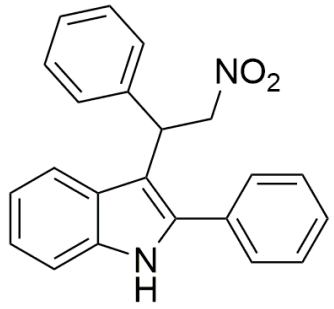
Glaucoma is a multifactorial disease involving cupping of the optic nerve and selective loss of retinal ganglion cells (RGCs). The most important modifiable risk factor in glaucoma is intraocular pressure (IOP), and reducing IOP is the main therapeutic goal of glaucoma therapies. However, despite IOP management many patients continue to show progressive vision loss suggesting that additional therapies directed at RGC neuroprotection may be beneficial. Activation of cannabinoid receptor signalling pathways, including CB1, reduces IOP in rabbits, rodents, and primates, and is neuroprotective in models of optic nerve injury. However, long-term administration of cannabinoids may be undesirable due to potential psychotropic effects and tachyphylaxis. Positive allosteric modulators (PAMs) of the CB1 receptor may provide another mechanism to develop novel glaucoma therapeutics that can both lower IOP and decrease RGC loss. CB1 PAMS have reduced potential for side effects and receptor desensitization. Our experiments examined the IOP lowering effects of a novel positive allosteric modulator, GAT211, using an ocular hypertensive (OH) model.

OH was induced into the left eye of C57Bl/6 mice by injection of ~5 μm magnetic epoxy beads into the anterior chamber of the eye. Right eye served as control. Using the average of 10 measurements, baseline IOP was measured using rebound tonometry (TonoLab) one day prior to OH induction, and every second day until IOP reached >30% of baseline (3-7 days). Once this criterion was met, baseline IOPs were taken, 5 μL of drug was topically applied, and a follow-up recording was measured 30 minutes later. Subsequently, animals were euthanized and the eyes enucleated for post-mortem analysis.



GAT211

3-(2-nitro-1-phenylethyl)-2-phenyl-1*H*-indole

Our results demonstrate that where mice have a >30% increase in IOP, 5 mM GAT211 application results in an average reduction of IOP of 13.5 mmHg in OH mice eyes, and 2.0 mmHg in contralateral control eyes. Control eyes receiving no treatment had an average IOP reduction of 1.0 mmHg 30 minutes after the initial measurement, while control eyes receiving vehicle had an average IOP reduction of 0.7 mmHg.

Conclusions from this preliminary study indicate the CB1 PAM, GAT211, can lower IOP in OH, although GAT211 had more limited hypotensive actions in mice with normal pressures. Further experiments will be examining chronic dosing with GAT211 in both control and OH mice as well as well as any potential neuroprotective effects of CB1 PAM treatment, as measured by increased RGC survival in OH eyes.

Acknowledgments: Funded by the Canadian Institutes of Health Research FRN 97768 (MK)

DIRECT ADMINISTRATION OF *N*-PALMITOYLETHANOLAMIDE INTO THE RAT MEDIAL PREFRONTAL CORTEX REDUCES FORMALIN-EVOKED NOCICEPTIVE BEHAVIOUR VIA A CB_1 RECEPTOR-MEDIATED MECHANISM

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N-Palmitoylethanolamide (PEA) is an endogenous agonist at the nuclear hormone transcription factors peroxisome proliferator activated receptor (PPAR)-α and PPAR-γ. Previous studies have reported rapid antinociceptive effects of PEA in animal models of inflammatory and neuropathic pain, which are inconsistent with transcription-dependent mechanisms. Given that both PEA and the endocannabinoid anandamide (AEA), are endogenous substrates of the enzyme fatty acid amide hydrolase (FAAH), we hypothesised that exogenous administration of PEA could provide a sparing effect on FAAH-mediated hydrolysis of AEA, and that the rapid antinociceptive effects of PEA may reflect enhanced local CB_1 receptor-mediated signalling. Here, we investigated the pharmacological effects of direct administration of PEA into the medial prefrontal cortex (mPFC), an area associated with the modulation of affective and cognitive components of pain, on formalin-evoked nociceptive behaviour and hindpaw oedema in rats and the role of cannabinoid signalling. Adult male SD rats (n=7-10 per group) received intra-mPFC injections of PEA (6nmoles/0.5μL) or vehicle and/or the CB_1 receptor antagonist AM251 (1.25nmoles/0.5 μL), via bilaterally implanted stainless steel guide cannulae, 10 minutes prior to intraplantar formalin (2.5%) injection. Nociceptive behaviour was assessed for 60 minutes and analysed using Ethovision XT software. Post-mortem brain tissues were harvested for histological verification of injection sites and measurement of levels of AEA, 2-arachidonoyl glycerol 2-AG, *N*-oleoylethanolamide (OEA) and PEA in the mPFC by liquid chromatography-tandem mass spectrometry.

Intra-mPFC administration of PEA significantly and rapidly attenuated the first and early second phases (0-25 and at 35 minutes post-formalin injection) of formalin-evoked nociceptive behaviour compared with vehicle-treated controls. The effects of PEA alone were significantly reversed by co-administration with AM251 at 10-20 minutes, post-formalin injection. Co-administration of PEA with AM251 reduced late second phase (45-50 minutes) formalin-evoked nociceptive behaviour compared with vehicle-treated controls. Administration of AM251 alone transiently attenuated formalin-evoked nociceptive behaviour at 10-20 and at 35 minutes post-formalin injection compared with vehicle-treated controls. Mass spectrometric analysis revealed a significant increase in PEA levels, a strong trend for increased AEA levels, but no change in OEA or 2-AG levels, in the mPFC of rats that received PEA microinjections compared with vehicle-treated controls. Neither PEA nor AM251 treatment nor a combination of both had any effect on hindpaw oedema.

Taken together, these data suggest that one mechanism mediating the rapid antinociceptive effects of PEA injected into the mPFC may be the elevation of local AEA levels and signalling via CB_1 receptors. However, the potential contribution of other receptor targets for both PEA and AEA warrants further investigation.

Acknowledgements: This work was funded by Science Foundation Ireland (10/IN.1/B2976) and NUI Galway's College of Science.

COMPARISON OF THE EFFECTS OF INHIBITION OF ABHD6 AND MAGL ON INFLAMMATION

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2-arachidonoylglycerol (2-AG) exerts anti-inflammatory effects in many settings. Most of the effects of 2-AG have been investigated through increasing its endogenous levels by inhibiting monoacylglycerol lipase (MAGL). A few studies have also implicated the more recently annotated enzyme α/β-hydrolase domain 6 (ABHD6) in the control of 2-AG levels. We have shown that in macrophage cell lines in culture, ABHD6 is more expressed than MAGL and is able to control 2-AG levels and macrophage activation. Here we thought to compare the effects of inhibiting MAGL and ABHD6, in the same setting, on 2-AG levels and inflammation in vivo.

Inflammation was induced in mice by administration of the endotoxin lipopolysaccharide (LPS – 300µg/kg, i.p.). Mice received the enzyme inhibitors (JZL184 for MAGL and WWL70 for ABHD6) one hour before LPS and were sacrificed 4 hours after LPS. The effects of inhibiting 2-AG hydrolyzing enzymes were assessed in several tissues: brain, spinal cord, lung, spleen, liver, colon and adipose tissue. Expression of pro-inflammatory cytokines and chemokines (IL-1β, IL-6 and MCP-1) as well as 2-AG biosynthesis and hydrolysis enzymes was measured by qRT-PCR. 2-AG levels were also measured by HPLC-MS.

Inflammation reduced MAGL mRNA expression in the liver, lung and spleen, while ABHD6 expression was decreased in the liver and lung. DAGL mRNA expression was decreased in the same tissues as MAGL. Inhibition of ABHD6 and MAGL did not have the same effects on 2-AG levels in the tissues assessed. MAGL inhibition generally increased 2-AG levels, whereas ABHD6 inhibition led to a more modest increase in 2-AG levels in some tissues such as the liver and lung, and no increase in other tissues such as the colon and brain. Inhibition of ABHD6 exerted anti-inflammatory effects in all the tissues assessed, except in the colon. MAGL inhibition on the other hand only reduced the expression of pro-inflammatory mediators in some tissues such as the liver, spleen and adipose tissue.

In conclusion, ABHD6 inhibition leads to an increase in 2-AG levels in vivo in some tissues, however all the effects of ABHD6 inhibition on inflammation are not mediated by this increase in 2-AG levels.

As ABHD6 exerts anti-inflammatory (or pro-inflammatory) effects in tissues where 2-AG levels are not increased, this opens up the way to study other pathways responsible for the effects of ABHD6 inhibition on inflammation. The fact that MAGL inhibition, despite increasing 2-AG levels in all the tissues, does not exert the same effects as ABHD6 inhibition, reinforces the notion that 2-AG is not responsible for all the effects of ABHD6 inhibition. Mechanistic studies in this context are underway.

Acknowledgment:FRS-FNRS, Belgium (CC 1.5034.10F and FRSM 3.4569.12 to GGM)

INVESTIGATION OF THE ROLE OF THE TRPV1 CHANNEL IN ENDOTHELIAL INFLAMMATION

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The transient receptor potential cation channel V1 (TRPV1) is activated by noxious stimuli (e.g. high temperature and low pH), plant compounds (e.g capsaicin), and endocannabinoids (e.g. anandamide (AEA) and *N*-arachidonoyl dopamine (NADA)). The identification of AEA and NADA as endogenous TRPV1 agonists led to hypothesis that TRPV1 has a role in physiological homeostasis, including inflammation. Paradoxically, however, reports over the past 10 years have indicated both protective (i.e. anti-inflammatory) and pro-inflammatory roles for TRPV1. While TRPV1 is predominantly expressed on sensory neurons, it is also found on non-neuronal cell types. Therefore, our lab aimed to test the hypothesis that TRPV1 has a role in endothelial cell (EC) inflammatory biology.

ECs are centrally involved in the pathogenesis of organ injury in acute inflammatory disorders, such as sepsis. ECs express cytokines and chemokines, which facilitate the trafficking of leukocytes to organs, and decrease vascular barrier function. Treatment of ECs with microbial and endogenous inflammatory agonists, including bacterial lipoproteins, lipopolysaccharide (LPS), or TNF, induces EC expression of inflammatory mediators. Our initial results show that TRPV1 is highly expressed in ECs from several different vascular beds. Additionally, we find that the endocannabinoid NADA induces Ca^{2+} flux into human microvascular ECs, consistent with a physiological function for TRPV1. Interestingly, when endothelial TRPV1 is inhibited by capsazepine or AMG 9810, or depleted by RNAi-mediated knockdown in human ECs, or removed by genetic deletion of *Trpv1* in mouse ECs, we find that both baseline and LPS-induced endothelial inflammation are exacerbated. Our results indicate that TRPV1 antagonizes the endothelial inflammatory response. We are currently investigating these observations more thoroughly, and examining the roles of NADA and AEA in endothelial TRPV1 regulation. Furthermore, we are currently expanding our studies to determine the role of other TRP channels EC inflammatory activation. We hope that elucidating the role of TRPV1 in EC inflammation will allow us to better understand the therapeutic potential of cannabinoids and other TRP channel modulators in acute inflammatory diseases, such as sepsis.

Acknowledgements: Funded by the Department of Anesthesia and the Research Evaluation and Allocation Committee (REAC) of the School of Medicine, University of California San Francisco

ENDOCANNABINOID LEVELS AND FUNCTIONAL DISABILITY STATUS IN PATIENTS WITH PAINFUL OSTEOARTHRITIS

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The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) serve as agonists at cannabinoid receptors and regulate pain and inflammation. Previous studies have demonstrated that activation of cannabinoid receptors reduces pain in preclinical models of osteoarthritis (OA), suggesting that the endocannabinoid system may serve as a therapeutic target for the treatment of OA pain. There is still very limited knowledge of endocannabinoid levels in OA patients, with only one study reporting changes in the endocannabinoid tone in the synovial fluid of OA patients. Clearly, a systematic analysis of endocannabinoid levels in key tissue compartments of humans is warranted for further understanding of how this system may relate to OA pain.

The goal of this study was to examine whether serum, cerebrospinal fluid (CSF), and synovial fluid endocannabinoids correlate with functional pain disability status associated with OA. A secondary goal was to examine associations between endocannabinoid levels and pro-inflammatory cytokine profiles. Patients (N = 36) with painful end-stage OA undergoing total knee arthroplasty were recruited for the study. Functional disability status was scored using the pain disability questionnaire (PDQ) while endocannabinoid and cytokine (leptin, TNFα and IL-6) analyses was performed by liquid chromatography-mass spectrometry and enzyme-linked immunosorbent assay, respectively.

Our results indicate that serum endocannabinoid levels do not correlate with serum cytokines. However, serum AEA positively correlates with CSF TNFα (spearman $r = 0.58$, $p < 0.05$) while serum palmitoylethanolamide (PEA) negatively correlates with leptin in the synovial fluid (spearman $r = -0.68$, $p < 0.01$). In the CSF, 2-AG levels are negatively correlated with leptin (spearman $r = -0.48$, $p < 0.01$) while in the synovial fluid 2-AG levels positively correlate with IL-6 (spearman $r = 0.68$, $p < 0.01$). Lastly, we examined correlations between PDQ scores and serum, CSF, and synovial fluid levels of PEA, AEA, 2-AG, and oleoylethanolamide. Our results indicate that PDQ scores do not correlate with endocannabinoid levels in any of the compartments examined. These results indicate that while endocannabinoid levels correlate with a subset of inflammatory cytokines involved in acute and chronic pain, the endocannabinoid tone itself does not appear to directly impact functional pain disability status (as measured by the PDQ) in patients with OA scheduled for total knee arthroplasty surgery.

Acknowledgements: This work was sponsored by internal funds from the Department of Anesthesiology.

A RETROSPECTIVE DESCRIPTION OF THE USE OF NABILONE IN UK CLINICAL PRACTICE

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Nabilone, a synthetic cannabinoid analogue, is licensed in the UK for chemotherapy induced nausea and vomiting (CINV), but is thought to be frequently used off-label for intractable chronic pain and spasticity in conditions such as multiple sclerosis (MS) and neuropathic pain.

This multicentre observational study aimed to describe the use of nabilone in the UK for licensed and non-licensed indications. The study was undertaken in two phases; a 'pilot study' (in 3 NHS hospitals) and an 'extension study' (5 sites). Sites were selected following identification by the manufacturer as high-prescribing centres. Data on patient characteristics, previous therapies, dosing regimens and benefits/side-effects were collected retrospectively from the medical records of patients prescribed nabilone on/after 1st January 2005.

Results are presented for 250 patients, initiated on nabilone 13th January 2005-13th March 2013, mean 31.1 months (standard deviation, (SD) 22.8) from nabilone initiation to data collection. Where n<250, data were missing from the medical records.

51% (127/250) patients were male. At nabilone initiation the mean (SD) age was 48.0 (11.3) years; prior symptom duration 8.8 (8.4) years; 86% (201/233) had been prescribed ≥3 other classes of medication for the same symptoms and 22% (26/116) had evidence of previous/concomitant cannabis use (this was only collected in the extension study centres). MS was the most common distinct condition for which nabilone was prescribed (19%, 48/247). Nabilone was most commonly prescribed for pain (91%, 212/232) and spasticity (19%, 45/232) and only 3 patients for CINV. The most common starting dose was 1mg daily (61%, 147/241); this was also the most common established dose (54%, 104/194). Overall 64% (159/250) patients were recorded as having benefitted from nabilone; most common benefits were pain relief (n=130), improved sleep (n=76) and spasticity relief (n=24). Benefits were recorded for 87% (53/61) patients starting on <1mg, 60% (88/147) starting on 1mg and 57% (16/28) starting on ≥2mg of nabilone daily. 33% (82/250) patients were recorded as having experienced adverse effects (most commonly drowsiness, fatigue, dysphoria). The estimated mean (SD) cost of nabilone was £3,160 (£2,365)/patient/year.

In conclusion, almost all nabilone use was for unlicensed indications, primarily pain of various aetiology and MS symptom relief. Although most patients had experienced symptoms for many years and had been prescribed a number of other previous medications, over 60% derived benefit and adverse effects were not materially different from those of other CNS-active medications. There was no evidence to suggest increased benefit from higher starting doses. Incomplete recording was highlighted as a problem for a number of data fields collected in the study. Careful and complete documentation is important, particularly when a drug is being used off-license.

Acknowledgements: This study was funded by Meda Pharmaceuticals and supported by pH Associates.

HYDROXYTYROSYL OLEATE, AN ESTER ANALOGUE OF OLEOYLDOPAMINE (OLDA), SHOWS ANTI-INFLAMMATORY PROPERTIES IN VITRO

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Hydroxytyrosol is one of the major biophenolics found in olives and extra-virgin olive oil with well known antioxidant properties. In addition, it exhibits several other biological activities, reducing the risk of coronary heart disease and atherosclerosis and showing antimicrobial, antitumor, and anti-inflammatory activities (Granados-Principal et al., Nutr. Rev. 68 (2010) 191-206). Lipophilic hydroxytyrosol derivatives have been suggested to be more active than hydroxytyrosol itself because of their increased metabolic stability and ability to pass membranes (Burattini et al., Food Chem. Toxicol. 55 (2013) 248–256). In this work, we determined the potential anti-inflammatory properties of hydroxytyrosyl oleate (Figure 1). Further to being a lipophilic derivative of hydroxytyrosol, hydroxytyrosyl oleate is an ester analogue of *N*-oleoyldopamine, an endogenous fatty acid amide, that has been reported to show anti-inflammatory and immunomodulatory activities (Jin et al., Eur. J. Lipid Sci. Technol. 115 (2013) 1284–1293).

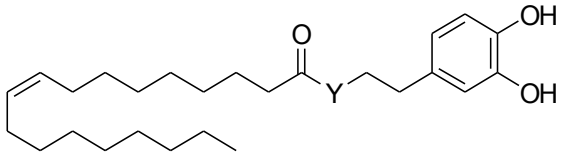

Figure 1: Structure of Hydroxytyrosyl Oleate (Y = O) and *N*-Oleoyldopamine (Y = NH).

Hydroxytyrosol was subjected to lipase-catalyzed acylation with oleic acid methyl ester. The resulting hydroxytyrosyl oleate was evaluated for its ability to reduce the release of nitric oxide (NO) by lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The inhibitory effect was further assessed at the gene expression level using quantitative RT-PCR to determine inducible NO synthase (iNOS) and the inflammatory cytokine interleukin-1β (IL1β) mRNA expression.

At the tested concentrations (0.5-5 μM), hydroxytyrosyl oleate reduced the production of NO in a concentration-dependent way, whereas its parent compounds hydroxytyrosol and oleic acid were ineffective. Inhibition was also found to take place at a transcriptional level, as gene expression of iNOS and IL1β was inhibited by hydroxytyrosyl oleate.

We conclude that lipophylization of hydroxytyrosol with an oleic acid moiety led to the formation of a compound that may be more effective than hydroxytyrosol in the modulation of inflammation.

We are currently investigating the mechanisms of action of hydroxytyrosyl oleate and whether it can be formed *in vivo*.

Acknowledgements: *Research presented in this publication was financially supported by European Commission, Fondo Sociale Europeo (FSE), Calabria Region and Graduate School VLAG.*

ATTENUATING NEUROPATHIC PAIN THROUGH DUAL INHIBITION OF CYCLOOXYGENASE AND MONOACYLGLYCEROL LIPASE

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Neuropathic pain is a life altering condition characterized by altered nerve function that often presents as allodynia, the painful perception of typically non-noxious stimuli. Neuropathic pain is commonly treated with GABA analogues, steroids, or non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are commonly used analgesics that inhibit one or more cyclooxygenase (COX) enzymes. However, chronic COX inhibition also causes negative side effects including gastrointestinal inflammation and increased risk of cardiac events. Like NSAIDs, monoacylglycerol lipase (MAGL) inhibition has analgesic and anti-inflammatory properties. The present study investigated the analgesic effects of inhibiting both MAGL and COX. Mice were subjected to the chronic constriction injury (CCI) model of neuropathic pain and then administered the MAGL inhibitor, JZL184 (1-40 mg/kg, i.p.), the nonselective COX inhibitor, diclofenac sodium (1-100 mg/kg, i.p.), or vehicle and tested for mechanical and acetone-induced cold allodynia. Then, both drugs were coadministered at various doses in ratios of 1:3, 1:1, and 3:1 parts of either compound's ED_{50}. Isobolographic analyses revealed that JZL184 and diclofenac synergistically attenuated mechanical allodynia and had an additive interaction in reducing cold allodynia. The CB_1 antagonist, rimonabant (3 mg/kg, ip), but not the CB_2 antagonist, SR144528 (3 mg/kg, ip), blocked the analgesic effects of the JZL184/diclofenac combination in mechanical allodynia. Neither antagonist blocked analgesia in cold allodynia. Brainstem and lumbar spinal cord levels of prostaglandins $F_{2\alpha}$ and E_2 were significantly reduced by diclofenac, although anandamide and 2-AG were not affected by any treatment. Thus, the observed behavioral changes were not caused by gross alterations of whole tissue levels of prostaglandins, endocannabinoids, or arachidonic acid. These data support the idea of dual MAGL/COX inhibition as a therapeutic approach for reducing neuropathic pain.

Acknowledgements: Supported by the National Institutes of Health (DA018224).

ANANDAMIDE MEDIATES THE ANTIHYPERALGESIC EFFECT OF 2AG IN A MURINE MODEL OF CHEMOTHERAPY-INDUCED NEUROPATHY

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Cisplatin treatment of various cancers is limited by development of painful peripheral neuropathy. This neuropathy is modeled in mice by daily treatment with cisplatin (1 mg/kg, i.p., 7 days). Previously we determined that increased hydrolysis of anandamide (AEA) contributes to mechanical hyperalgesia in this model, and facilitation of AEA signaling reduces the hyperalgesia (Khasabova et al., *J. Neurosci*., 2012). We extended these studies to address 1) whether a decrease in 2-arachidonoyl glycerol (2AG) signaling also occurs in this model, 2) whether inhibition of 2AG hydrolysis reduces the hyperalgesia, and 3) the cellular mechanism underlying the effect of 2AG.

Reduced levels of 2AG in the skin and dorsal root ganglia (DRGs) of mice treated with cisplatin indicated a reduction in 2AG signaling in cisplatin-treated mice with mechanical hyperalgesia. A 2-fold increase in mRNA for monoacylglycerol lipase (MGL) in the skin was associated with the reduction in 2AG. Acute intraplantar (i.pl.) injection of JZL184 (10 μg), an inhibitor of MGL, attenuated the cisplatin-induced hyperalgesia. This effect was paralleled by acute local treatment with 2AG (18 μg, i.pl.), supporting the conclusion that the effect of JZL184 was most likely due to an increase in the level of 2AG. The anti-hyperalgesic effect of each drug was blocked by co-administration of a CB1 (AM281), but not a CB2 (AM630), receptor antagonist. We reasoned that the CB1 receptor-mediated effect of 2AG occurred at the level of the sensory neuron, but 2AG had no direct effect on the depolarization of dissociated murine DRG neurons *in vitro*. Therefore we tested the effect of the drugs on ^{3}H-AEA uptake. Co-incubation with 2AG reduced ^{3}H-AEA uptake by dissociated DRGs in a concentration-dependent manner, and this effect was paralleled by co-incubation with JZL184 (1 μM). Together these data indicate that increasing peripheral levels of 2AG attenuated mechanical hyperalgesia in cisplatin-treated mice indirectly, by reducing the transport of AEA. Peripherally restricted inhibitors of 2AG hydrolysis may be useful in combination with inhibitors of AEA hydrolysis in the treatment of painful peripheral neuropathies produced by chemotherapy.

Acknowledgments: Funded by CA138684 (V.S.S.) and DA011471 (D.A.S.)

THE MONOACYGLYCEROL LIPASE INHIBITOR JZL184 ATTENUATES HYPERALGESIA INDUCED BY COLLAGEN-INDUCED ARTHRITIS

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Rheumatoid arthritis (RA) is the most prevalent chronic inflammatory joint disease, affecting approximately 1% of the world population. This autoimmune disease is characterized by pain, stiffness, swelling, and breakdown of cartilage in synovial joints. Current RA analgesic treatments are ineffective or induce negative side effects. Cannabinoids have antihyperalgesic and anti-inflammatory properties; however, the challenge remains to harness the medical potential of cannabinoids without inducing negative psychoactive effects. An alternative approach focuses on the endogenously produced cannabinoids (endocannabinoids). 2-arachidonoyl glycerol (2-AG) is the most prevalent endocannabinoid and is catabolized by the enzyme monoacylglycerol lipase (MAGL). Pharmacological inhibition of MAGL increases brain levels of 2-AG and significantly decreases acute inflammatory pain. The present study tested the hypothesis that MAGL inhibition decreases hyperalgesia, and pain suppressed behavior caused by collagen-induced arthritis (CIA), a well-established animal model of inflammatory arthritis. We investigated the antihyperalgesic effects of the selective MAGL inhibitor JZL184 on CIA-induced thermal hyperalgesia in the hotplate and tail immersion tests, as well as on spontaneous locomotor activity. JZL184 (8 or 40 mg/kg, ip) significantly attenuated hyperalgesia in both the hotplate and tail immersion assays. CIA significantly decreased spontaneous locomotor activity, and this pain suppressed behavior was reversed by JZL184 (8 mg/kg, ip). These results suggest that MAGL inhibition may be a promising strategy for the treatment of pain caused by inflammatory arthritis.

LONG-TERM SOCIAL REJECTION DURING ADOLESCENCE ALTERS PAIN PERCEPTION AND AFFECTS THE ENDOCANNABINOID SYSTEM IN ADULT FEMALE RATS

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During adolescence the focus of social interaction shifts, and peers become increasingly important. In humans, a heightened sensitivity toward rejection by peers has been reported especially in female teenagers. Such experiences of social rejection have been shown to alter pain sensitivity, but the neurobiological and behavioral consequences of social rejection are not completely understood, as no valid animal model is available yet. We have previously proposed a novel animal model for peer-rejection in adolescent rats by rearing the animals with inadequate play partners. Rearing of adolescent female Wistar rats with an age-matched partner from the less playful Fischer344 strain (play-deprived), from weaning until the end of adolescence, was found to decrease social play during adolescence and various social skills in adulthood. Additionally, we found that pain sensitivity was significantly decreased in the adult females of the play-deprived condition. Furthermore, we analyzed brain regions connected to pain processing as well as social behaviors for alterations in levels of endocannabinoids (anandamide, 2-arachidonoylglycerol) and protein levels of the cannabinoid type 1 receptor (CB1R) and of the anandamide degrading enzyme fatty acid amide hydrolase (FAAH). We detected various alterations in the endocannabinoid system, which were most pronounced in the amygdala. Here, we detected a significant increase in anandamide levels and a decrease in FAAH protein levels in female rats which had been reared in the play-deprived condition throughout adolescence.

The present results indicate that the experience of social rejection (i.e. play deprivation) due to inadequate social rearing conditions during adolescence affects pain perception and social behavior on the long-term. Activation of the endocannabinoid system may occur during inadequate peer-interaction as a countermeasure to deal with the negative experience of adverse and insufficient social play, which then persists into adulthood.

Acknowledgments: Funded by the CRU 256 of the DFG

“Funding for this conference was made possible in part by grant R13DA016280 from the National Institute on Drug Abuse. The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention by trade names, commercial practices, or organizations imply endorsement by the U.S. Government.”

"ENTOURAGE" EFFECTS OF PALMITOYLETHANOLAMIDE: ENHANCEMENT OF 2-AG ACTION AT TRPV1 CHANNELS AND OF 2-AG LEVELS *IN VITRO* AND *IN VIVO*

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Palmitoylethanolamide (PEA) is an endogenous anandamide (AEA) congener, which can be biosynthesized together with AEA by similar anabolic pathways, and degraded by fatty acid amide hydrolase as well as *N*-acylethanolamine acid amidohydrolase (NAAA). We and others have previously shown that PEA can enhance the levels and/or actions at both cannabinoid and transient receptor potential vanilloid type-1 (TRPV1) channels of AEA ("entourage" effect). However, PEA can also activate directly TRPV1, peroxisome proliferator activate receptor-α and orphan G-protein coupled receptors such as GPR119 and GPR55. Very recently, 2-arachidonoylglycerol (2-AG), previously thought to be only weakly active at TRPV1 channels, was suggested by electrophysiological and pharmacological experiments to act as a physiologically relevant activator of this channel, and, thereby, to take part in the phospholipase C-mediated activation of TRPV1. We have investigated here if PEA, as well as its synthetic analogue adelmidrol (azelaoyl-diethanolamide), can also enhance 2-AG actions at TRPV1 channels *in vitro* and/or the levels of this endocannabinoid/endovanilloid *in vitro* or *in vivo*.

We performed measurements of intracellular Ca^{2+} in human embryonic kidney cells (HEK-293) stably over-expressing human TRPV1. In agreement with previous data using patch-clamp electrophysiology and mesenteric artery vasodilation, 2-AG dose-dependently elevated intracellular Ca^{2+} in these cells (efficacy 57.4±2.1% of 4 μM ionomycin, EC_{50} = 0.9±0.1 μM), whereas it failed to evoke Ca^{2+} response in untransfected HEK-293 cells. The selective TRPV1 receptor antagonist 5-iodoresiniferatoxin (0.1 μM) blocked the Ca^{2+} response induced by 2-AG. Importantly, 2-AG, like all TRPV1 agonists including AEA, desensitized TRPV1 to the effect of capsaicin (0.1 μM) on intracellular Ca^{2+} (IC_{50} = 0.75±0.04 μM). Importantly, 2-AG was ~3.5-fold less potent than AEA at both activating and desensitizing TRPV1. PEA (1-5 μM) only slightly enhanced 2-AG activation of TRPV1-mediated intracellular Ca^{2+} increase, but dose-dependently and significantly increased 2-AG-induced TRPV1 desensitization to capsaicin 0.1 μM (IC_{50} from 0.75±0.04 to 0.45±0.02 μM, with PEA 2 μM). Adelmidrol (1-50 μM) had no effect.

We next measured the effect of PEA and adelmidrol (10- 20 μM, 40 min, 6h and 24 h incubation at 37°C) on 2-AG levels in human HaCaT keratinocytes, where PEA was previously shown to produce TRPV1-mediated anti-inflammatory actions. At all time points PEA, at the optimal concentration of 10 μM, elevated by nearly 3-fold the amounts of 2-AG as compared to vehicle-treated keratinocytes. Adelmidrol (10 μM) was inactive in HaCaT cells, but instead increased 2-AG levels (by 2-fold) in cultured dog keratinocytes. Adelmidrol also strongly increased the levels of PEA in both human and dog keratinocytes.

Finally, we measured plasma endocannabinoid levels after a single oral administration of ultramicronized PEA to either human volunteers (300 mg, after 2, 4 and 6 h from administration) or spontaneously *Ascaris suum* hypersensitive Beagle dogs (10 mg/kg, 1, 2, 4 and 8 h from administration), in which PEA was previously shown to produce skin anti-inflammatory actions. We observed that, in correspondence with the expected peak of plasma PEA levels (1 h and 1- 2 h in humans and dogs, respectively), and/or immediately thereafter, the plasma levels of 2-AG were also significantly elevated (by up to 2-fold and ~20-fold in humans and dogs, respectively) to an extent commensurate to the elevation of PEA levels (2 and ~12-fold in humans and dogs, respectively). No effect on AEA levels was observed.

These findings indicate that, in addition to the previously observed similar effect on AEA *in vitro*, PEA exerts significant "entourage" effects also on 2-AG, by both enhancing it levels *in vitro* and *in vivo*, and its action at TRPV1 channels *in vitro*. These observations may help explaining why several anti-inflammatory or analgesic effects of PEA can be attenuated by cannabinoid receptor or TRPV1 channel antagonists, and provide yet another mechanism of action for the multi-faceted pharmacological properties of this pleiotropic lipid mediator.

ANTI-INFLAMMATORY EFFECTS OF THE CB1/CB2 AGONIST WIN55212,2 ARE DEPENDENT ON TRPV1, TRPA1 AND AMPK IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS SYNOVIAL FIBROBLASTS

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In rheumatoid arthritis (RA), synovial fibroblasts (SF) secrete large amounts of IL-6, IL-8 and matrix metalloproteinases (MMPs) which are crucial for cartilage destruction. RASFs are sensitive to the action of cannabinoids and they express cannabinoid receptors type I and II (CB1 and CB2) and the transient receptor potential channels type vanilloid (TRPV1) and ankyrin (TRPA1). The synthetic cannabinoid WIN55212,2 demonstrated strong anti-inflammatory effects in monocytes and synovial fibroblasts only in high concentrations in a non-cannabinoid receptor dependent manner. In this study we assessed the ability of WIN55212,2 to modulate cytokine and MMP-3 production but also cell viability and proliferation over a wide concentration range (10-12M to 10-5M) under hypoxic conditions in synovial fibroblasts from RA and OA donors.

MMP-3, IL-6 and IL-8 were determined by ELISA. Cell proliferation was assessed by CTB, cell viability was determined by LDH assay.

WIN55212,2 robustly reduced TNF-induced IL-6, IL-8 and MMP-3 production in a concentration-dependent manner. From 10-12M to 10-6M WIN55212,2 showed a bell-shaped dose-response curve with a maximal inhibition at 10-9M (~40% decrease in cytokine production), while higher concentrations almost completely inhibited cytokine production. Cell viability was unaltered, but cell morphology changed in response to WIN55212,2 in concentrations above 4µM. Effects of WIN55212,2 were not dependent on activation of either CB1 or CB2, since antagonists (CB1: CP945598, 1µM; CB2: JTE907, 1µM) were not effective. The effects of WIN55212,2 were attenuated by the TRPV1 antagonist capsazepine (1µM), the TRPA1 antagonist A907079 (1µM) and the AMPK activator metformin (10µM).

The synthetic cannabinoid WIN55212,2 exhibits anti-inflammatory effects in synovial fibroblasts independent of CB1 and CB2. Our results indicate a TRPV1/TRPA1/Calcium (AMPK) dependent mechanism that might be coupled to cellular energy status since decreasing serum content and hypoxia augmented the effects of WIN55212,2 on production of IL-6, IL-8 and MMP-3.

DIFFERENTIAL EFFECTS OF PHARMACOLOGICAL MODULATION OF TRPV1 IN THE LATERAL PERIAQUEDUCTAL GREY ON FORMALIN-EVOKED NOCICEPTIVE BEHAVIOUR IN SPRAGUE-DAWLEY AND WISTAR-KYOTO RATS

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The Wistar-Kyoto (WKY) rat is a stress-hyperresponsive strain that exhibits a hyperalgesic phenotype, compared with the Sprague-Dawley (SD) strain (Burke et al., Neuroscience, 171 (2010) 1300-13). We have recently shown that hyperalgesia in WKY rats is mediated, at least in part, by impaired endocannabinoid signalling in the rostral ventromedial medulla (Rea et al., Pain, 155 (2014) 69-79). TRPV1 within the midbrain periaqueductal grey (PAG) plays a key role in regulating nociceptive behaviour via modulation of neuronal activity in the rostral ventromedial medulla (Palazzo et al., Mol. Pain, 6 (2010) 66). The present study tested the hypothesis that pharmacological modulation of TRPV1 in the lateral(L) PAG would differentially regulate formalin-evoked nociceptive behaviour in SD versus WKY rats.

Adult male WKY and SD rats(n=10-12 per group; 260-290g) received intra-LPAG injections of either vehicle (100% DMSO), the TRPV1 agonist capsaicin (6nmoles/0.2µL), the TRPV1 antagonist 5'-IRTX (0.5nmoles/0.2µL) or co-administration of capsaicin and 5'-IRTX via bilaterally implanted stainless steel guide cannulae, 10 minutes prior to intra-plantar formalin injection (2.5%, 50µl). Nociceptive behaviour was assessed for 60 minutes using EthoVision XT. In a separate experiment, we used qRT-PCR to compare levels of TRPV1 mRNA in the LPAG of SD and WKY rats. Data were analysed by two-way ANOVA (with or without repeated measures) followed by Fisher's LSD post-hoc test. $P<0.05$ was considered statistically significant.

In SD rats, intra-LPAG administration of 5'-IRTX significantly reduced formalin-evoked nociceptive behaviour in the early part of the trial and at the peak of the second phase, compared with vehicle-treated rats. These effects of 5'-IRTX were not observed in WKY rats. WKY rats receiving either intra-LPAG vehicle or 5'-IRTX injection, but not capsaicin, exhibited higher nociceptive behaviour over the entire formalin trial compared with SD counterparts. Treatment with either capsaicin alone, or in combination with 5'-IRTX, had no effect on formalin-evoked nociceptive behaviour when compared with vehicle treatment in either SD or WKY rats. TRPV1 mRNA levels were significantly higher in the LPAG of WKY rats compared with SD rats.

In conclusion, pharmacological blockade of TRPV1 in the LPAG results in discrete and transient reductions in formalin-evoked nociceptive behaviour in SD but not WKY rats. These data, together with evidence for higher expression of TRPV1 mRNA in the LPAG of WKY rats, suggest a differential role of TRPV1 in the LPAG in nociceptive responding between the two rat strains.

Acknowledgement: This work was funded by grants from Science Foundation Ireland (10/IN.1/B2976) and College of Science, National University of Ireland, Galway.

ROLE OF CB1 AND CB2 CANNABINOID RECEPTORS IN THE EMOTIONAL AND COGNITIVE ALTERATIONS ASSOCIATED WITH OSTEOARTHRITIS PAIN

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Osteoarthritis is a degenerative joint disease of which chronic pain is the main symptom and the first reason of complaint in patients. Osteoarthritis pain is often associated with emotional and cognitive alterations that impair the quality of life of patients. Thus, an appropriate treatment able to improve not only pain manifestations, but also the emotional and cognitive symptoms is essential for an effective management of osteoarthritis. The purpose of this study was to investigate the role of the cannabinoid receptor 1 (CB1R) and 2 (CB2R) in the nociceptive, affective and cognitive alterations associated with osteoarthritis pain. The intra-articular injection of monosodium iodoacetate (MIA) was used to induce osteoarthritis pain in wild-type and knockout mice for CB1R (CB1KO) and CB2R (CB2KO). We first analysed the affective and cognitive consequences of this chronic pain exposure by evaluating the anxiety-like behaviour (elevated plus-maze test) and memory functions (object recognition test) at different time points after the intra-articular injection of MIA. Then, we also evaluated the ability of the selective CB1R agonist, ACEA (1 and 5 mg/Kg, i.p.), and the selective CB2R agonist, JWH133 (1 and 5 mg/Kg, i.p.), to improve the nociceptive manifestations (von Frey model) as well as the emotional (elevated plus-maze test) and cognitive deficits (object recognition test) observed in this mouse model of osteoarthritis. Mice that received the intra-articular injection of MIA showed an alteration of the anxiety-like behavior, as revealed by a decrease in the percentage of entries and time spent in the open arm of the elevated plus-maze. Interestingly, these alterations induced by MIA appeared more pronounced in CB1KO when compared with wild-type mice. The presence of osteoarthritis pain also produced memory impairment, since mice injected with MIA showed a decrease of the discrimination index in the object recognition task. No significant differences were revealed between wild-type and knockout mice for CB1R or CB2R in the memory deficits produced by MIA. The systemic administration of ACEA or JWH133 produced an improvement of the nociceptive responses, and the emotional and cognitive alterations observed in the MIA model of chronic joint pain.

These results revealed that CB1R, but not CB2R, is mainly involved in the control of the affective alterations associated with this osteoarthritis pain model. However, neither CB1R nor CB2R seem to play a major role in the memory deficits observed in this osteoarthritis model. Interestingly, both CB1R and CB2R agonists ameliorated the nociceptive manifestations as well as the affective and cognitive alterations associated with this chronic pain state, suggesting a potential interest of these compounds for osteoarthritis treatment.

Acknowledgement: Funded by RTA (RD12/0028/0023), Instituto de Salud Carlos III (Spain)

AGE OF EXPOSURE AFFECTS BEHAVIORAL RESPONSE TO DELTA-9-TETRAHYDROCANNABINOL DURING THE PERI-PUBERTAL PERIOD IN THE RAT

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Marijuana is the most commonly abused illicit substance in the United States. Additionally, in 2010, almost 60% of all first time marijuana users were under the age of 18. Research indicates that adolescents who use marijuana are at higher risk of developing anxiety, depression, and other mood disorders. Adolescence is a period during which both physical and behavioral maturations take place. Puberty occurs during adolescence; however, during a more narrowly defined time frame marked by the attainment of sexual maturity, which normally occurs around postnatal day 40 (P40) in male rats and P30 in females. The onset of puberty also coincides with peak cannabinoid receptor 1 (CB1) and endocannabinoid system (ECS) activity which suggests that puberty may be a particular time during development in which the subject is more sensitive to the effects of Δ-9-tetrahydrocannabinol (THC), the primary psychoactive substance in marijuana. Our study on the effects of early adolescent (P29-38) THC exposure revealed sex differences in measures of anxiety. Males showed less anxious behavior in the elevated plus maze (EPM) following THC administration whereas females did not exhibit behavioral differences in response to THC exposure. Although THC exposure in this study occurred within adolescence for all the rats, the males could be considered pre-pubertal while the females were pubertal during the administration period. THC was then administered to pre-pubertal (P21-30) females as well as pubertal (P39-48) males. Preliminary data suggest that pre-puberty is a critical time during which THC exposure elicits behavioral differences in measures of anxiety in both male and female animals. Reductions in anxious behavior, similar to those of pre-pubertal males, were observed in pre-pubertal females following THC treatment, whereas in pubertal males, these differences were no longer observed.

Acknowledgments: Supported by NIH P50 DA 025484-0001 and the Dean's Fund for Faculty Research, SUNY Downstate

LONG-TERM EFFECTS OF ADOLESCENT THC EXPOSURE ON ADULTHOOD PSYCHOPATHOLOGY

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Marijuana is the most widely used illicit drug among adolescents and young adults. Adolescence is a critical period of development between childhood and adulthood, encompassing cognitive, emotional and social maturation. This period is characterized by a brain in transition that differs anatomically (e.g. neuronal connections and morphology) and neurochemically (e.g. dopamine, GABA, Glutamate) from that of the adult. These modifications are thought to support the emergence of adults' cerebral processes and behaviors.

The endocannabinoid system is a central component of these neurodevelopmental changes. This is mainly due to its involvement in the maintenance of the synaptic plasticity. Delta-9-tetrahydrocanabinol (THC), the main psychoactive component of marijuana, acts as a cannabinoid receptor agonist. Therefore, an over activation of the cannabinoid system by THC exposure during adolescence may dramatically alter brain maturation, thereby adult cerebral functions.

In the present study, we hypothesize that long-term adolescent chronic THC exposure will disrupt adult rat behaviors and the underlying neuronal functioning related to dysfunctions observed in schizophrenia.

To achieve this investigation, our protocol consists to expose adolescent rats (postnatal day (PND) 35 to 45) to THC (i.p. injections) twice a day for 11 days. During adulthood (PND75), behavioral tasks, *in-vivo* electrophysiological recordings and molecular analyses are performed.

Our preliminary results show that adolescent THC exposure (1) reduces rats' social interactions/recognition; modifies (2) sensorimotor gating; (3) anxiety and (4) locomotor activity at adulthood. We are currently running electrophysiological recordings and molecular analyses in brain areas known to be disrupted in schizophrenia (e.g. the ventral tegmental area, nucleus accumbens) to correlate these behavioral changes with neuronal function alterations.

This animal model represents an interesting new framework for the study of the long-term consequences of marijuana exposure during adolescence. It might improve our understanding of the emergence of psychotic symptoms and lead to new therapeutics and perspectives in the prevention of schizophrenia.

A MOUSE MODEL SHOWS SYNTHETIC CANNABINOID TERATOGENICITY AND PROMISE FOR PRENATAL DRUG CO-EXPOSURE RESEARCH

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Previous research involving the teratogenicity of cannabinoids focused on low-potency compounds, namely Δ^9-THC. Currently, highly potent synthetic cannabinoids are being synthesized and used more frequently with little understanding of adverse health consequences, especially their teratogenic potential when used alone or in combination with alcohol (ethanol, a well-known teratogen). Researchers have recently identified numerous strong interactions between constituents of the cannabinoid system and ethanol during several stages of development, suggesting important roles for cannabinoid signaling in mediating ethanol-induced teratogenesis. Functional CB_1 receptors are present in the embryo at developmental stages that are present as early as week 3 of human gestation (corresponding to gestational day (GD) 7.0 in mouse). Previous work employing a chick model has shown this stage to be vulnerable to cannabinoid agonist O-2545 teratogenicity. As a first step toward characterizing the teratogenicity of prenatal co-exposure to high-potency cannabinoids and ethanol, we examined the teratogenic effects of early prenatal treatment with the synthetic cannabinoid agonist CP 55,940. For this, nulliparous female C57BL/6J mice were time-mated, and pregnancies confirmed by the presence of a copulation plug. GD0 was defined as the beginning of the breeding period in which the plug was found. Pregnant females were treated on either GD7.0 or GD8.0 with a drug mixture consisting of (1:1:18) 2mg/kg CP 55,940/100% ethanol: Alkamuls El 620: lactated Ringer's solution, or vehicle (1:1:18) 100% ethanol: Alkamuls El 620: lactated Ringer's. To control for potential teratogenic effects of drug-induced hypothermia, a cohort of dams was tested in a temperature-controlled environment. Pregnancies were allowed to mature to GD17, when fetuses were harvested, fixed, and examined for gross morphological abnormalities.

Results to date illustrate a range of CP 55,940 induced craniofacial malformations and ocular defects. Assessment of the latter employed a scoring system that revealed a much higher incidence of ocular dysmorphology in cannabinoid-treated versus vehicle control groups in both left and right eyes. Cannabinoid treatment at GD7.0 resulted in 1.79 and 2.02 fold increases in severity of left and right eye defects from vehicle treatment levels respectively. In GD8.0 treated embryos, cannabinoid treatment resulted in a 2.16 fold increase in severity of both left and right eye defects compared to vehicle-control group levels. Previous research employing a mouse fetal alcohol spectrum disorders model has shown comparable defects resulting from maternal ethanol treatment at these same early stages of development. These results underscore the importance of evaluating the potential dangers of synthetic cannabinoid exposure during early gestation, a time during which many women are not even aware of their pregnancies, and provide the basis for the testable hypothesis that co-exposure to cannabinoids and ethanol may be more damaging to a developing embryo than exposure to either drug alone.

Acknowledgements: Supported by NIAAA grants (U01AA021651 and U01AA021651-01)

MODULATORY INFLUENCE OF THE DEVELOPING ENDOCANNABINOID SYSTEM ON COGNITIVE ABILITIES DURING ADOLESCENT BRAIN DEVELOPMENT IN RATS

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Adolescence and puberty are highly susceptible developmental periods during which the neuronal organization and maturation of the brain is completed. It is exactly during these periods that many neuropsychiatric disorders (e.g. schizophrenia, substance use disorders, borderline personality disorder, etc.) have their onset. Adolescence is mainly characterized by impulsive behavior, reward seeking, and risky decisions, which have been suggested to derive mainly from the late maturation of inhibitory cortical control systems. A major aspect in cognitive development is the ability to suppress inappropriate thoughts and actions in favor of goal-directed behaviors, and these necessary impulse control mechanisms mature during adolescence. The endocannabinoid (ECB) system, which is well known to modulate cognitive processing, undergoes profound developmental changes during adolescence. With the present study we were aiming to examine the ontogeny of cognitive skills throughout adolescence in male rats and clarify the potential modulatory role of cannabinoid receptor signaling. Cognitive skills were assessed repeatedly every 10^{th} day in male rats from postnatal day (pd) 30 until adulthood (pd 130). All animals were tested for locomotor acitivity, object recognition memory and prepulse inhibition (PPI) of the acoustic startle reflex (ASR). Although cognitive performance in short-term memory for objects and sensorimotor gating abilities was lower during puberty compared to adulthood, both tasks were found to show different developmental trajectories throughout adolescence. A low dose of the CB1 receptor antagonist/inverse agonist SR141716 (SR; 0.3 mg/kg) was found to improve recognition memory and PPI in pubertal animals. The same dose of SR did not induce any changes in recognition memory in adult rats, although PPI was slightly decreased, indicating different pharmacological effects of SR in pubertal and adult rats. The present findings demonstrate that the developmental trajectory of cognitive abilities does not occur linearly for all cognitive processes and is strongly influenced by pubertal maturation. Developmental alterations within the ECB system at puberty onset may be involved in these changes in cognitive processing.

LYPLA2 IS A MAJOR PROSTAGLANDIN GLYCEROL ESTER HYDROLASE IN HUMAN CANCER CELLS

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Cyclooxygenase-2 (COX-2)-catalyzed oxygenation of 2-arachidonoylglycerol (2-AG) is the first step in the conversion of 2-AG to prostaglandin glycerol esters (PG-Gs). PG-Gs exert biological effects at low concentrations by activating receptors that have not yet been characterized. Hydrolysis of PG-Gs represents an important control point on their activities as well as a source of prostaglandins (PGs). Previous studies have revealed that PG-Gs are hydrolyzed by carboxylesterases 1 and 2 at rates comparable to those for the hydrolysis of 2-AG whereas they are hydrolyzed by monoacylglycerol lipase and fatty acid amide hydrolase at rates that are 30-100-fold lower than the rates of hydrolysis of 2-AG or arachidonoylethanolamide (AEA), respectively.

PGE_2-G hydrolases were detected in a range of human cancer cells. Most of the hydrolase activity was present in the cytosolic fraction and it was completely inhibited by fluorophosphonates suggesting it was due to a serine hydrolase. The levels of PGE_2-G hydrolase activity in four different cell lines were compared to published inventories of serine hydrolases identified by activity-based protein profiling. Among the nearly 30 different hydrolase activities quantified in these profiles, the best correlation to PGE_2-G hydrolase activity was with lysophospholipase A_2 (LYPLA2). siRNA knockdown of LYPLA2 in MDA-MB231 cells reduced the levels of PGE_2-G hydrolase by 60-80% compared to a scrambled control siRNA. Expression of LYPLA2 in HEK 293 cells or *E. coli* increased the activity of PGE_2-G in cellular extracts. A His-tagged LYPLA2 was expressed in *E. coli* and purified to homogeneity using a nickel affinity resin. The pure enzyme catalyzed hydrolysis of PG-Gs in the relative order, PGE_2-G > $PGF_{2\alpha}$-G > PGD_2-G. Interestingly, purified LYPLA2 did not catalyze hydrolysis of 2-AG or AEA at detectable rates. LYPLA2 is the major enzyme in several human cancer cells that catalyzes the hydrolysis of PG-Gs. In contrast to previously described PG-G hydrolases, it does not catalyze hydrolysis of 2-AG or AEA. LYPLA2 may play an important role in the control of the physiological and pathophysiological activities of PG-Gs in human cells.

Acknowledgements: Supported by research grants from the National Institutes of Health (GM15431 and GM65086).

ROLE OF THE ENDOCANNABINOID SYSTEM IN AUTOPHAGY

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The endocannabinoid system (ECS) and autophagy are implicated in many functions and pathological conditions. Increasing evidence suggests a role for the ECS in the control of cell survival and death, that are both involved in tumor progression, and a major role in CNS patterning of structures relevant for mood, cognition and reward. In this context, autophagy is involved in several physiological and pathological processes such development and embryogenesis, cancer, and neurodegeneration. Recently, the pro-autophagic action of exogenous cannabinoids has been documented, but the actual physiological role of the ECS in the autophagic process remains unclear.

The aim of the present study was to investigate the possible link between ECS and autophagy. We demonstrate that autophagy determines a modulation of the expression of distinct elements of the ECS. Conversely, selective activation of CB_2 receptor was able not only to alter the time course of the autophagic process, but also to decrease the expression of specific markers of endoplasmic reticulum and mitochondrial stress.

Furthermore, the analysis of intracellular signalling pathways activated by CB_2 receptor allowed to demonstrate that the regulation of autophagy involves the phosphorylation of JNK and the concomitant dephosphorylation of Akt, two kinases directly involved in the autophagic cascade.

These new acquisitions could help to design new therapeutic strategies for the management of those pathological conditions in which autophagy is defective, including tumors, neurodegeneration and development.

ANALYSIS OF ENDOCANNABINOID SYSTEM IN A 3D "CELL-ON-CHIP" MODEL OF HUMAN TUMORS

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Triple-negative breast cancer (TNBC), characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2), represents an important clinical challenge. TNBC does not respond to endocrine or HER2-targeted therapies, making cytotoxic chemotherapy the only systemic treatment available. Cancer stem cells (CSCs), a rare tumor cell population with stem cell activities, survive to chemotherapy agents and through a self-renewal mechanism probably due to high invasive capacity, clonal evolution and dormancy, promote blood vessel formation, trigger cell motility and primarily contribute to tumor progression and metastasis.

Here, we investigate the endocannabinoid system in MDA-MD-231 triple-negative breast cancer cells as a model of invasive human cancer in tumorspheres condition. Indeed, tumorspheres represent an established model for the proliferation of a subpopulation with CSC-like features.
Flow cytometric analysis of ALDH1-positive cells, used as CSC markers, significantly increased in tumorspheres compared with adherent MDA-MB-231. Moreover, qRT-PCR highlighted a significant increase in the expression of stemness genes (NANOG, OCT4, SOX2) and modulation of the expression of ECS in tumorspheres, compared with MDA-MB-231 adherent controls. In particular, tumorsphere induce an up-regulation of the expression of endocannabinoid biosynthetic enzymes, NAPE-PLD and DAGL-α.

Additionally, we developed a methodology for the performance of cell culture experiments in time-lapse taking advantage of a microfluidic cell-on-chip platform. Here, we report the design and fabrication of a microfluidic devices that allow 3D tumor culture. Our chip is characterized by a chamber hosting tumorsphere that are micro-encapsulated in a hydrogel, mimicking the tumor microenvironment. Moreover, our tumor-on-chip is full compatible with state-of-the-art live cell imaging and high-content analysis technologies, enabling the high throughput extraction of space- and time-resolved morphological data regarding cell interactions both at the population and at the single cell level.

We suggest that our 3D tumor-on-chip device fills the gap between conventional *in vitro* models, which are often scarcely predictive of an *in vivo* condition, and animal models, thus representing an innovative model to study ECS and its pharmacological modulation in cancer and other pathological conditions.

CYCLOOXYGENASE-2-DEPENDENT GROWTH INHIBITION OF ARACHIDONOYL ETHANOLAMIDE ON LARYNGEAL CANCER CELLS

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It has been suggested that endocannabinoids, anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG), might be the promising anti-cancer agents in clinical fields of cancer treatment. In this study, we tried to check if cyclooxygenase-2 (COX-2) might be related to the anti-cancer effects of AEA and 2-AG in laryngeal cancer cells.

Using XTT assay, we identified that AEA inhibited the cell proliferation of laryngeal cancer cells (SNU-1066 and SNU-1076) but 2-AG did not. Cannabinoid receptor-1 (CB1) was expressed in SNU-1066 and expression of VR1 was observed in both cells (no CB2 was detected). However, the anti-cancer effect of AEA seemed to be mediated by their receptors-independent actions since the antagonist of CB1 and VR1 (AM251, cay10448 and capsazepine) did not reverse the cell proliferation inhibited by AEA. Next, we checked the possibility of COX-2-mediated anti-cancer effect of AEA in laryngeal cancer cells (with high expression of COX-2) and identified that PGE_2-like products (PGE_2-ethanolamide, PGE_2-EA) were produced from AEA by COX-2. Finally, we observed that COX-2 inhibition (by its inhibitors and siRNA) reversed the anti-cancer effect of AEA partially. Also, we found that exogenous PGE_2-EA showed the cytotoxic effect on laryngeal cancer cells. These findings suggest that AEA might have the anti-cancer effect by their receptors-independent actions such as COX-2-mediated PGs-EA production in laryngeal cancer cells. However, the COX-2-mediated growth-inhibitory effect of AEA was not observed in pharyngeal cancer cells (with moderate expression of COX-2) and oral cancer cells (with low expression of COX-2). These mean the possibility that we might utilize COX-2 activity for effective cancer-killing modality in patients of some cancers with high COX-2 expression.

DEVELOPMENT OF A Δ^9-THC PRODRUG TRANSDERMAL DELIVERY SYSTEM FOR PREVENTION OF ACUTE AND DELAYED NAUSEA AND VOMITING ASSOCIATED WITH INITIAL AND REPEAT COURSES OF EMETOGENIC CANCER CHEMOTHERAPY

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Each year in the US there are approximately 1.5 million new cases of cancer (American Cancer Society). Chemotherapy induced nausea and vomiting (CINV) is consistently rated by patients as among the most feared and severe in frequency and duration of all side effects. The role of Δ^9-THC as an antiemetic is well established and it is an effective agent in controlling nausea. Cannabinoids are the only compounds that can stop vomiting, decrease nausea, and increase appetite simultaneously, as all other classes of compounds merely treat one aspect of these chemotherapy adverse effects. AllTranz is currently developing their product targeting this large and growing market utilizing proprietary dermal delivery technology. Our unique transdermal system is expected to prevent CINV and potentially eliminate the state of euphoria associated with the high dose needed for currently available cannabinoids like medical marijuana and Marinol®.

The hairless guinea pig (GP) model was used for the *in vitro* and *in vivo* skin permeation analysis. Dermatomed (250 μm) GP skin was used for the *in vitro* experiments. A PermeGear flow-through (In-Line, Hellertown, PA) diffusion cell system was used for the skin permeation studies. Diffusion cells were kept at 32°C (skin surface temperature) with a circulating water bath. Permeation area of the skin was 0.95 cm^2 and in vitro samples were analyzed by high pressure liquid chromatography (HPLC). For *in vivo* testing, patches were applied to the guinea pig and remained in place for 72 hours and blood samples were obtained until 103 hours. Plasma samples were collected, extracted and analyzed by LC/MS/MS. Plasma samples were analyzed for prodrug ALL00144, Δ^9-THC, (±)-11-Hydroxy-Δ^9-THC and (-)-11-Nor-9-carboxy-Δ^9-THC. (-)-11-Nor-9-carboxy-Δ^9-THC was not detected in the guinea pig plasma samples. (±)-11-Hydroxy-Δ^9-THC was detected in trace amounts in the guinea pig plasma samples.

Plasma hydrolysis of ALL00144 in GP plasma showed greater than 95% conversion to THC within 3 h, as compared to 90% conversion to THC in human plasma within 1 h. The *in vitro* flux of ALL00144 through dermatomed GP skin from an ALL00144 transdermal delivery system was found to be 10.0 ± 1.58 nmol/cm^2/h. The ALL00144 patch applied to the guinea pig gave a total THC equivalent steady state plasma concentration (C_{ss}) of 6.90 ± 1.47 ng/mL. THC clearance**,** 8.3 L/h, in GP was previously determined from an intravenous bolus dose of 1 mg/kg. The predicted steady state flux can be calculated using the following equation: $C_{ss} = \frac{J_{ss} A}{CL}$ Where C_{ss} is the steady state concentration (ng/mL), J_{ss} is the predicted steady state flux (nmol/cm^2/h), A is the application area (cm^2) and CL is clearance (L/h). Solving for J_{ss} gives a predicted flux of 29.1 nmol/cm^2/h from a transdermal dose of an ALL00144 formulation. The *in vitro/in vivo* correlation in GP was determined to be 34% using systemic clearance of THC. The underestimate of flux observed during *in vitro* GP permeation studies indicates that data obtained from *in vitro* human skin studies should translate into a significantly higher delivery rate in humans.

This research was funded by the National Institutes of Health: (Grant: RC2DA028984)

ENDOCANNABINOID-INDUCED GROWTH INHIBITION OF HUMAN COLON CANCER: INVOLVEMENT OF WNT/β-CATENIN PATHWAY

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Colorectal cancer (CRC) arises through a multistep process involving a series of pathological alteration ranging from microscopic mucosal lesion, like aberrant crypt foci (ACF), to malignant tumors. In the recent years the endocannabinoid system has been found to possess antitumoral effects both *in vitro* and *in vivo* studies. Indeed, endocannabinoids control tumor growth and progression acting at several levels, as antiproliferative, antiangiogenetic and antimetastatic compounds. Moreover, in colon cancer cells cannabinoids modulate CB1 and estrogen receptors (ERs). We previously demonstrated that 17β-estradiol, Met-F-AEA (a stable AEA-analogous) and URB597 (a selective FAAH inhibitor) significantly reduced the proliferation of DLD1 and SW620 cell lines. Both 17β-estradiol and Met-F-AEA induced upregulation of the CB1 receptor by triggering its promoter activation. Finally, increased availability of exogenous and endogenous AEA, induced the expression of estrogen receptor beta.

In a high percentage (≥ 85%) of both sporadic and familial adenomatous polyposis (FAP) forms of colorectal cancer, inactivation of the APC tumor suppressor gene initiates tumor formation. APC negatively regulates the levels of β-catenin, a multifunctional protein that transduces Wnt signals, mediates cell-cell adherents junctions through its interaction with E-cadherin, and stimulates cell proliferation.

Moreover, WNT5A is frequently silenced in human CRC cell lines and in primary tumors due to its promoter methylation. In this study we tested the hypothesis of a potential direct effect of cannabinoids on the WNT/β-catenin pathway in CRC.

We found that Met-F-AEA and SR141716 were able to modulate the expression of WNT5 protein in both DLD1 and SW620 cell lines. Time course experiments performed in CRC cell lines treated with Met-F-AEA (10μM) or with SR141716 (10μM), showed a cycling modulation of WNT5 in both SW620 and DLD1.

Moreover, a Luciferase assay, in colon cancer cells transfected with an inducible transcription factor responsive construct and constitutively expressing Renilla luciferase construct, was performed. In particular, the cells were transfected with a reporter construct containing the TRE for TCF/Lef and treated for 24hours with both Met-F-AEA and SR141716. In DLD1 cells, both compounds, Met-F-AEA and SR141716, increase the luciferase activity expressed under the control of TCF/LEF. Similar data were obtained in SW620 cells. Moreover, the cannabinoids significantly increase the luciferase activity controlled by both the TRE for Serum Response Element (SRE) and for AP1.

In conclusion, the preliminary results support the hypothesis that cannabinoids could modulate the activation of the WNT signaling pathway mainly through a non canonical mechanism WNT5-mediated.

Acknowledgements: MCP was supported by a fellowship from FIRC (Italian Foundation for Cancer Research).

A REVIEW OF THE USE OF HERBAL CANNABIS AND CANNABIS EXTRACTS FOR THE TREATMENT OF CANCER AND CANCER-RELATED SYMPTOMS

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Background: A large body of preclinical research has demonstrated the potential mechanisms whereby external cannabinoids interact with cannabinoid receptors to address cancer and cancer-related symptoms, with the focus of most clinical trials on single synthetic or isolated cannabinoids. These finding have lead to an increasing interest in herbal cannabis as a potential treatment option for cancer and cancer-related symptoms.

Objective: To review observational studies and clinical trials that have been conducted on herbal cannabis and cannabis extracts, in the treatment of cancer and cancer-associated pain and chemotherapy-induced nausea and vomiting (CINV).

Design: The review focused on human observational and randomized clinical trials of herbal cannabis and cannabis extracts for the treatment of cancer and cancer-related symptoms. The inclusion criteria were extended to the use of herbal cannabis in the treatment of pain and nausea/appetite loss in non-cancer populations. A systematic search was performed of articles published since 1988 in PubMed, Embase, CINAHL, Cochrane, Natural Standards, Web of Science, ProQuest, PubMed Health. For each study, the country where the project was held, the number of patients assessed, the type of study and comparisons made, the products and the dosages and dose-titration strategy used, efficacy and adverse effects were identified

Results: The search resulted in 15 studies that met the inclusion criteria. The sample sizes ranged from 7 to 263, with half the studies having sample sizes below 50. Ten studies were double blind randomized controlled trials. Seven studies directly examined cannabis in the treatment of cancer-related symptoms, including managing nausea and vomiting, stimulating appetite and treating pain. Eight studies involved herbal cannabis use in the treatment of pain, nausea and vomiting, and appetite stimulation in conditions other than cancer. No studies were found for the anti-cancer effects of either herbal cannabis or extracts. Overall, herbal cannabis and extracts were shown to be effective and well-tolerated for nausea/appetite loss and pain in patients with cancer and other conditions.

Discussion: A limited number of studies exist that have examined the efficacy of herbal cannabis in the treatment of cancer-related symptoms in patients with cancer and other medical conditions. Those studies that have been conducted are well-designed clinical trials, some of which are limited by a small sample size. The studies reviewed highlight the importance of potency, dose-titration strategy and route of administration in both clinical practice and research design and demonstrate beginning evidence of the role of herbal cannabis and cannabis extracts in the treatment of cancer-related nausea/appetite loss and pain.

Conclusion: Given the rising rates of cancer worldwide, and the growing number of patients using herbal cannabis, it is important to understand the potential efficacy of cannabis in addressing cancer and cancer-related symptoms. This review suggests that a priority area for research is the use of herbal cannabis as a treatment for cancer and cancer-related symptoms.

AN *IN VITRO* EVALUATION OF COMBINATIONS OF PHYTOCANNABINOIDS IN PANCREATIC, GASTRIC, RENAL, BLADDER AND LIVER CANCER CELL LINES

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There is an increasing body of evidence that the phytocannabinoids (pCBs) cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) selectively reduce the viability in cancer cells. Here we evaluate the anti-cancer actions of pure CBD, cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA), Δ^9-tetrahydrocannabivarin (THCV) and THC, in isolation and combination, in a cell viability assay using a range of cancer cell lines.

Pancreatic (PANC1, Mia-Pa-Ca-2), gastric (MKN45, HGC-27), renal (ACHN), bladder (RT112) and liver (HepG2) cancer cell lines were suspended in assay media (RPMI 1640; 4 x10^4 cells/ml) and incubated overnight at 37 °C, in 5 % humidified CO_2. The pCBs were dissolved in DMSO, before being diluted into 1% (v/v) FBS media to produce final assay concentrations of each pCB ranging from 0.79 to 200µM with 1 % DMSO. The cells were then incubated in the assay solution for 72 hours CO_2, before being developed with Cell Titer-Blue™ for 3 hours and the fluorescence measured using a Flex II Station plate reader (570 nm excitation, 600 nm emission).

pCBs in isolation produced a robust ability to reduce the viability of the different cancer cell lines in the 72 hour incubation protocol (Table 1). Additionally, CBD in combination with the other pCBs were investigated to test whether there were interactions in their abilities to reduce the viability of cells (Table 2).

Table 1 IC_{50} values of phytocannabinoids in isolation

	Cell line IC_{50} value (µM)						
	Pancreatic		Gastric		Renal	Bladder	Liver
pCB	PANC-1	Mia-Pa-Ca-2	MKN 45	HGC-27	ACHN	RT112	HepG2
CBD	3.0	2.6	3.4	1.5	3.3	2.0	3.3
CBGA	8.5	8.9	6.5	7.8	11.6	9.8	9.3
CBG	3.1	2.2	4.3	1.6	3.0	2.5	3.7
CBDA	13.1	7.9	8.8	1.3	10.7	8.2	7.4
THCV	6.5	5.2	6.5	4.1	6.8	4.7	7.2
THC	3.0	2.9	4.2	2.8	3.6	3.6	NT

NT= not tested

Table 2 IC_{50} values of phytocannabinoids in combination with CBD

	Cell line IC_{50} value (µM)						
	Pancreatic		Gastric		Renal	Bladder	Liver
pCB	PANC-1	Mia-Pa-Ca-2	MKN 45	HGC-27	ACHN	RT112	HepG2
CBGA	2.9	2.3	2.6	2.4	3.0	2.4	3.4
CBG	1.2*	0.8*	1.3	0.6	1.4	0.8*	1.6†
CBDA	3.0	2.0	2.6	2.1	3.0	2.4	4.0†
THCV	1.5	1.5*	1.7	1.5	1.5	1.4*	2.9†
THC	1.5	1.0*	1.7	1.4	1.3	1.4*	NT

*=significantly different ($p \leq 0.05$) from CBD or other pCB in isolation (Chou-Talalay method)
†= significantly different ($p \leq 0.05$) from CBD
NT= not tested

From these data we can conclude that combinations of CBD with other pCBs show the potential to treat a variety of different cancer types, with some of the combinations of pCBs and CBD demonstrating significant synergistic effects.

EFFECT OF *N*-ARACHIDONOYH GLYCIN (NAGly) AND Δ^9TETRAHYDROCANNABINOL (Δ^9THC) ON ERK1/2 PHOSPHORYLATION IN NATIVE HEK293 CELLS

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Identifying the pharmacology and signalling mechanisms of GPR18 will advance our-understanding of the complexity of the endocannabinoid system and may have important implications in identifying novel molecular mechanisms of action of phytocannabinoids. Δ^9THC is a phytocannabinoid that activates the classical cannabinoid receptors CB_1 and CB_2 as well as GPR18. NAGly is an endocannabinoid-like molecule, which activates GPR18 with no effect on CB_1 and CB_2 receptors. Both of these agents have also been reported to act through other molecular targets, which may confound the interpretation of receptor studies. In anticipation of an investigation of GPR18 pharmacology and signalling using HEK293 cells as hosts for recombinant expression, we have conducted some preliminary experiments to establish the host cell responses to putative GPR18 agonists. We have initially focussed on the ERK pathway as a robust signalling cascade that can be activated by a variety of biological stimuli.

HEK293 cells were maintained in cell culture and passaged at regular intervals. Carbachol and NECA were used as positive controls, being agonists of endogenous M3 muscarinic and A_{2B} adenosine receptors, respectively. Incubations were performed in 24 well plates, conducting Western blotting on the harvested samples using antibodies directed against ERK1/2 and phosERK 1/2.

5min incubation in the presence of 10μM THC or NAGly for 5min failed to alter phosphorylation of ERK 1/2 in native HEK293 cells. Treatment of native HEK293 cells in the presence of 100μM carbachol or 10μM NECA for 5 minutes elicited an ERK phosphorylation up to 5 fold basal levels. Combinations of carbachol or NECA with THC or NAGly failed to alter levels of ERK phosphorylation after 5 minute co-incubation.

These data suggest that HEK293 cells are suitable hosts for transfection of GPR18, which is our next step for assessment of the pharmacology and signalling characteristics of this novel cannabinoid receptor-like GPCR.

Acknowledgment: Funded by The Higher Committee For Education Development in Iraq (HCED)

ALLOSTERIC MODULATION AND BIASED SIGNALLING AT CB1 CANNABINOID RECEPTORS

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CB1 cannabinoid receptors are the most abundant G protein-coupled receptors in the brain and mediate the majority of the central nervous system effects of cannabis and other cannabinoid compounds (Devane et al., Mol Pharmacol. 34 (1988) 605-613). CB1 ligands can potentially be used to treat a variety of disorders; however their therapeutic applications are limited mainly due to their psychotropic effects. Selective allosterism is one strategy to obtain specific and efficient therapeutics. Recently, a small molecule named Org27569 has been reported to act as an allosteric inhibitor of agonist function, while being an enhancer of agonist binding at CB1 receptors (Price et al., Mol Pharmacol. 68 (2005) 1484-1495). A few endogenous ligands have also been suggested that allosterically act at CB1 receptors. The aim of the present study was to characterise the effects of CB1 allosteric modulators on receptor-mediated signalling pathways activated by various cannabinoid ligands. Flp-In CHO cells stably expressing hCB1 receptors were used to determine CB1-mediated signalling using AlphaScreen pERK1/2 phosphorylation and cAMP assays (Perkin Elmer).

In pERK1/2 assays, Org27569 had no efficacy by itself, and either did not significantly alter or only weakly modulated pERK1/2 activation induced by cannabinoid agonists Δ9-THC, methanadamide, anandamide and 2-AG. However Org27569 completely inhibited CP55940- and HU-210-mediated pERK1/2, indicating a probe dependent effect. Strikingly, in contrast to its weak interaction with Δ9-THC, methanadamide, anandamide and 2-AG in pERK1/2 assays, Org27569 significantly inhibited inhibition of adenylate cyclase by these ligands, highlighting pathway-specific allosteric effects. Whole-cell [3H]SR141716A binding studies are currently underway to further verify the allosteric nature of this compound. We demonstrate that Org27569 displays pathway-selective allosteric modulation and probe-dependence. Compounds such as Org27569 may therefore be used to gain selective therapeutic effects.

PROFILING THE ACTIVITY OF GPR55 ANTAGONISTS AGAINST RECOMBINANT AND ENDOGENOUS GPR55

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GPR55 is a putative novel cannabinoid receptor that is capable of being activated by a subset of cannabinoid ligands and the endogenous lipid, L-α-lysophosphatidylinositol (LPI). GPR55 mRNA is expressed widely throughout the body, particularly in the brain, bone and immune tissue, and is also expressed at high levels in certain types of tumour. Understanding the physiological and pathological role of GPR55 has been challenging due to the absence of selective pharmacological tools. However, recently novel antagonists have been developed, allowing for the determination of GPR55-specific effects.

The objective of the present study was to utilise molecular imaging techniques to evaluate the effectiveness of two previously published novel GPR55 antagonists (3-[1-[1-(4-methylphenyl)cyclopropanecarbonyl]piperidin-4-yl]-5-phenyl-1,3,4-oxadiazol-2-one and 4-[4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-5-yl]benzoic acid) on LPI-mediated GPR55 responses, in a HEK293 cell line stably expressing GPR55 (HEK293-GPR55) and also in a prostate cancer cell line that expresses endogenous GPR55 at high levels (DU145). The effects of the antagonists on LPI-mediated calcium responses and CREB phosphorylation were evaluated. In HEK293-GPR55 cells, treatment with antagonists of high nanomolar (100 nM-300 nM) and low micromolar (1 μM-10 μM) concentrations did not affect intracellular calcium levels or alter cAMP-response element-binding protein (CREB) phosphorylation when applied alone. However, they inhibited responses to LPI (1 μM) when applied at low micromolar concentrations (3 μM-10 μM). In DU145 prostate cancer cells, LPI-mediated CREB phosphorylation was also blocked.

This data suggests that the GPR55 antagonists are active in *in vitro* models that both over-express and endogenously express GPR55. Such pharmacological tools will help delineate the function of GPR55 in native cells and contribute to the validation of GPR55 as a therapeutic target.

ROLE OF INTRACELLULAR LOOPS IN THE HYDRATION OF GDP: RESULTS FROM MOLECULAR DYNAMICS SIMULATIONS OF THE 2-AG ACTIVATED CANNABINOID RECEPTOR SUBTYPE 2 / G_i PROTEIN COMPLEX

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Previously, we used molecular dynamics (MD) simulations to study the activation of the cannabinoid CB2 receptor, by its endogenous ligand, 2-arachidonoylglycerol (2-AG) via the lipid bilayer (*Hurst et al., 2010*). We then reported the CB2 / Gαi1β1γ2 complex formation using our 2-AG activated CB2 receptor model (ICRS 2013). In work presented here, we studied the next step in CB2 receptor signaling via Gi protein: G-protein activation via release of guanosine diphosphate (GDP). It has been hypothesized that GDP is released via the separation of the ras-like (also known as GTPase domain) and helical domains of Gαi (*Van Eps et al., 2011, Alexander et al., 2014*). For GDP to leave Gαi, it must swap its interactions with the Gαi ras-like and helical domains for interactions with water. By following the hydration of GDP, the progression of the complex towards G-protein activation can be monitored.

Our ongoing MD simulation of the CB2 / 2-AG/ Gαi1β1γ2 complex immersed in a fully hydrated POPC lipid bilayer is now at 4.5 μs. In work reported here, we probed the relationship between specific CB2 receptor interactions with the Gαi protein and the resultant progression of GDP hydration. We have seen the number of waters surrounding GDP increase from 16 (t= 0ns) to 36 waters (t=4.5 μs). Two important interactions between the receptor and G-protein appear to lead to the increased hydration of GDP. (1) A hydrophobic interaction occurs between the CB2 intracellular loop 2 (IC2) loop residue P139 and the Gαi hydrophobic pocket residues: V34 (N terminus; L194 (β1 sheet); F196 (β2 sheet); and, F336, T340, I343, I344 (α5 helix) multiple times in the 4.5 μs long trajectory. Each time this interaction occurs, an increase in GDP hydration is observed in our simulation. An analogous interaction between the IC2 loop and Gαs protein has been reported in the x-ray crystal structure of the β2-adrenergic receptor/Gαs complex (*Rasmussen et al., 2011*). (2) An IC3 loop interaction with the Gαi α4 helix also occurs between 1.4 to 1.6 μs in which the IC3 loop residue R229 reaches to interact with E297 and E298. This interaction also correlates with an increase in GDP hydration. Taken together, our recent results suggest how the CB2 receptor IC loops interacting with Gαi may cause increased hydration of GDP. This increase should ultimately lead to GDP dissociation. [Acknowledgements: Funding by grants RO1 DA003934 and KO5 DA021358 (PHR)]

CB1 RECEPTOR INTRACELLULAR LOOP 4 MUTATION MODULATES G PROTEIN ACTIVATION AND CAMP PRODUCTION IN HUMAN NEUROBLASTOMA CELLS

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The CB_1 receptor (CB_1R) carboxyl-terminus contributes critical domains that are important for CB_1 activity and regulation. The CB_1R intracellular loop 4 (IL4) deviates from the highly conserved NPXXY(X) 5,6F Class A G protein coupled receptor motif in that the CB_1R IL4 possesses a Leu instead of a Phe (Anavi-Goffer, et al., 2007). The aim of the present study is to investigate the ramifications of this deviation on signal transduction of CB_1R expressed in SHSY5Y human neuroblastoma cells. To do so, we compared the capabilities of the CB_1 wild-type (WT) with those of an L7.60F mutation, which mimics the conserved class A motif, and an L7.60I mutation which mimics the homologous CB_2 receptor sequence.

qPCR and Western blotting results show that the parental SHSY5Y cell expresses very limited CB_1R gene and protein levels, whereas the recombinant CB_1R WT, L7.60F and L7.60I mutants expressed significantly higher CB_1R levels relative to parental cells, but comparable receptor levels to each other. Immunoprecipitation studies using 40,000 x g membranes solubilized in NP40 detergent show that WT and both mutant receptors, co-immunoprecipitate with Gαi1, Gαi2, and Gαi3 proteins; however, both mutant CB_1Rs exhibit significantly greater coupling to Gi2, especially the L7.60I mutant. To investigate the effects of the IL4 mutation, we employed [^{35}S]-GTPγS assay using cell membranes. In parental SHSY5Y membranes, the agonists CP55940 and HU210 were not able to stimulate [^{35}S]-GTPγS binding. CP55940 and WIN5521-2 were able to stimulate [^{35}S]-GTPγS binding in SHSY5Y cells expressing CB_1R WT (Emax = 115±3.9, 116±8% of basal), or L7.60F mutant (111±4, 123±11%) or L7.60I mutant (140±2, 144±12 %), respectively. To investigate the functional changes in intact cells, we examined cAMP accumulation. In parental cells, 1μM CP55940 was poorly able to inhibit forskolin-stimulated cAMP accumulation, whereas CP55940 inhibited cAMP accumulation in SHSY5Y cells expressing CB_1R WT to 31±7.3%, L7.60F mutant to 33±12%, and L7.60I mutant to 10.9±7.7% of forskolin-stimulated levels. This inhibition in the L7.60I mutant shifted the curve to the left indicating higher potency of CP55940 when the receptor is mutated. The enhanced functionality of the L7.60 I mutant may be explained as the greater binding to Gi2 protein. These data show that the intracellular loop 4 mutations were able to modulate signal transduction in neuronal cells by promoting greater binding to Gi2 and thereby increasing the ability to inhibit cAMP accumulation in neuronal cells.

Acknowledgements: Support from NIGMS IRACDA grant 1K12GM102773 and NIDA grants R01-DA03690, P50-DA006634 is appreciated.

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CHRONIC NICOTINE EXPOSURE DIMINISHES INHIBITORY CONTROL OF VTA DA NEURONS THROUGH ENHANCED DIACYLGLYCEROL LIPASE-MEDIATED 2-ARACHIDONOYLGLYCEROL SIGNALING

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Chronic nicotine exposure (CNE) alters synaptic transmission in the VTA in a manner that results in enhanced dopaminergic signaling and nicotine reward that propels nicotine-seeking and use. The present experiments demonstrate that CNE results in enhanced nicotine-induced increases in VTA 2-arachidonoylglycerol (2-AG) formation, which subsequently blocks nicotine-induced GABA release through CB_1 receptor activation. Inhibition of 2-AG synthesis by novel, highly selective and efficacious DAGL inhibitors restored nicotine-induced GABA signaling in the VTA of CNE rats and reduced nicotine self-administration. Conversely, acute pharmacological attenuation of 2-AG clearance mechanisms in the VTA of nicotine-naive animals recapitulated the loss of nicotine-induced VTA GABA signaling present CNE animals. Collectively these observations demonstrate that excessive 2-AG signaling is necessary and sufficient for a loss of inhibitory GABAergic constraint of VTA dopamine excitability that contributes to enhanced incentive salience in nicotine-dependent animals.

Acknowledgements: This work was supported by NIH grants: F32 DA029994 (M.W.B.); F32 AA020430 (M.A.H); P01 DA017259 (L.H.P and B.F.C.); R01 AA020404 (L.H.P.); RO1 DA009789 (B.F.C.); RO1 DA033760 (B.F.C.); RO1 MH084512 (B.F.C.); P60 AA006420 (L.H.P. and M.R.); and the Hewitt Foundation Postdoctoral Fellowship (K.-L.H.).

A GPR119-BASED SIGNALING SYSTEM IN THE MURINE EYE REGULATES INTRAOCULAR PRESSURE IN A SEX-DEPENDENT MANNER

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GPR119 is a G protein-coupled receptor that may be the endogenous target for oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). These lipids are closely related to the endocannabinoid family of neurotransmitters that act on cannabinoid CB_1 and CB_2 receptors. Interest in GPR119 has centered on its role in regulating insulin secretion, with GPR119 agonists investigated in clinical trials relating to type II diabetes. However, the role of GPR119 has not been examined in the eye. We now report evidence that GPR119 regulates intraocular pressure in a murine normotensive model.

We have detected mRNA for GPR119 in several tissues of the mammalian eye. In addition using LC-MS we have tested for the presence of the likely endogenous ligands for GPR119, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), finding that both are present in anterior murine eye. Lastly, we have found that topical OEA (but not PEA) reduces intraocular pressure (IOP) in the eye relative to the untreated contralateral eye. This effect occurs in a concentration-dependent manner and is absent in $GPR119^{-/-}$ mice. Elevated IOP is associated with most forms of glaucoma, a major cause of blindness worldwide. Importantly this depression of IOP is seen in female but not male mice, revealing an element of sex-dependence in the function of this X-linked gene. The identification of a novel approach to reduce IOP is therefore of great therapeutic interest. We also compiled a cannabinoid-related lipid expression profile for the eyes of female GPR119-/- mice relative to WT females and also relative to males. Knockouts exhibit a broad elevation of oleoyl-based lipids while only 2-OG is altered relative to males. 2-OG has been proposed to be a GPR119 agonist but does not alter IOP in this model.

In summary, we offer evidence for a functional GPR119-based signaling system in the mammalian eye, with receptors, ligands and function in the form of a reduction in intraocular pressure. This offers the prospect of a novel mechanism to lower IOP while observed sex differences have implications for the desired use of GPR119 as a therapeutic target in diabetes.

CANNABINOID CB_1 RECEPTOR-MEDIATED ERK1/2 RESPONSES - EVIDENCE OF $G_{I/O}$ PROTEIN-INDEPENDENT SIGNALLING AND AGONIST BIAS

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The cannabinoid CB_1 receptor has been shown to activate several intracellular signalling pathways, including mitogen activated protein (MAP) kinases. Activation of these pathways can occur in an agonist-dependent manner. In the present study, we compare the activation of various MAP kinases by several structurally distinct cannabinoid receptor agonists in HEK293 cells stably transfected with the human CB_1 receptor.

The cannabinoid agonists HU-210, CP 55,940 (1 μM) and WIN 55,212-2 (10 μM) all produced an increase in phospho-ERK 1/2 levels over 60 min, with peak response at 4 min (% basal phospho-ERK; 531 ± 14, 488 ± 41 and 560 ± 13 % respectively). These compounds failed to alter phospho-p38 or phospho-JNK levels over the same period. At 5 min, HU, CP and WIN produced a concentration-dependent response (E_{max} - 363 ± 38, 387 ± 30 and 415 ± 27 % basal; pEC_{50} - 8.4 ± 0.1, 8.6 ± 0.1 and 7.7 ± 0.1, respectively). Overnight pre-treatment of cells with pertussis toxin (100 ng/ml) produced a partial reduction in agonist efficacy and potency (E_{max} - 179 ± 24, 169 ± 19, and 252 ± 15 % basal; pEC_{50} - 8.0 ± 0.2, 8.2 ± 0.1 and 7.1 ± 0.1, respectively) suggesting distinct $G_{i/o}$-dependent and -independent ERK phosphorylation. The addition of hypertonic sucrose concentrations (0.5 M), to inhibit receptor internalisation, caused a pronounced decrease in the WIN response, while almost completely abolishing the PTX-insensitive response (E_{max}, native – 198 ± 53, PTX – 34 ± 12 % basal). At 20 min, HU and CP again produced concentration-dependent responses (E_{max} - 185 ± 17 and 182 ± 14% basal; pEC_{50} - 9.1 ± 0.1 and 9.2 ± 0.1 respectively), the majority of which was PTX-insensitive. Interestingly, WIN produced a bell-shaped concentration response reaching a peak at ~100 nM (266 ± 10 % basal). Both high and low potency components of the WIN response were sensitive to the CB_1-selective antagonist AM 251. PTX appeared to inhibit the low potency response.

These results suggest that CB_1-mediated ERK phosphorylation can occur by at least two different and distinct pathways, while in addition, WIN exhibits agonist bias, providing a novel example of functional selectivity at the receptor.

PEROXISOMAL DYSFUNCTION BY PLA/AT FAMILY PROTEINS IS NOT RELATED TO THEIR NAPE-FORMING *N*-ACYLTRANSFERASE ACTIVITY

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N-Acylphosphatidylethanolamines (NAPEs) are a class of membrane glycerophospholipids and serve as precursors of bioactive *N*-acylethanolamines (NAEs), including *N*-arachidonoylethanolamine (anandamide). The HRAS-like suppressor (HRASLS) family, consisting of five proteins (HRASLS1–5), were originally isolated as tumor suppressors negatively regulating the activity of oncogene Ras. Our recent studies demonstrated that all HRASLS1–5 proteins possess NAPE-forming *N*-acyltransferase activity as well as phospholipase (PL)$A_{1/2}$ activity, and we proposed to rename these proteins phospholipase A/acyltransferase (PLA/AT)-1–5, respectively. The overexpression of PLA/AT-1 and PLA/AT-2 in animal cells significantly increased the endogenous levels of NAPE and NAE, while the overexpression of PLA/AT-3 (also known as H-rev107 or AdPLA) did not. Apart from *N*-acyltransferase activity, we found that the overexpression of PLA/AT-2 and PLA/AT-3 causes a remarkable decrease in endogenous levels of ether-type lipids, which are biosynthesized in peroxisomes, as well as abnormalities in the subcellular distribution of peroxisomal marker proteins, PMP70 and catalase. These results showed that the overexpression of PLA/AT-2 and PLA/AT-3 impairs the function of peroxisomes, suggesting the involvement of these proteins in the regulation of peroxisomes. However, such dysfunction of peroxisomes was not observed in PLA/AT-1-expressing cells. Thus, the peroxisomal dysfunction caused by PLA/AT family proteins (shown by PLA/AT-2 and PLA/AT-3) is not necessarily correlated with their ability to function as NAPE-forming *N*-acyltransferase (shown by PLA/AT-1 and PLA/AT-2).

LORATADINE ANALOGUES AS MAGL INHIBITORS

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The endocannabinoid 2-arachidonoylglycerol (2-AG) causes activation of cannabinoid receptors (CB1 and CB2) and is known to be involved in the regulation of numerous physiological and pathological processes. However, 2-AG has short duration of action due to the rapid hydrolysis by three different serine hydrolases. Among these, monoacylglycerol lipase (MAGL) is the main enzyme accounting for ~85% of the total 2-AG hydrolase activity in brain. In addition, MAGL is found to play important role in inflammation and cancer. At present, several structural classes of MAGL inhibitors are known among which some of the best examples are piperidine based urea/carbamate analogues JJKK-048[1] and KML29[2].

Loratadine (**1,** Fig. 1), a marketed H_1 histamine antagonist, possesses structural features of several MAGL inhibitors.[3] Hence, we decided to utilize the loratadine scaffold for further development of the MAGL inhibitors. Here we report our recent findings of loratadine analogues (general structure **2**, Fig. 1) as inhibitors of MAGL.

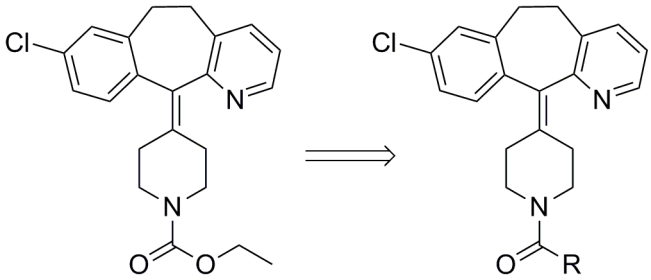

Fig. 1. Loratidine (1) and a general structure of loratadine-based MAGL inhibitors (2).

The best compound of the series inhibited MAGL at low nanomolar with an IC_{50} value of 45 nM. In addition, activity-based protein profiling using mouse brain membrane proteome showed good selectivity for MAGL among the metabolic serine hydrolases. Further structural modifications are in progress in order to achieve higher potency while retaining the achieved selectivity.

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MODELING THE ORG27569 INDUCED CB1/BETA-ARRESTIN 1 COMPLEX THAT ACTIVATES AN ARRESTIN BIASED PATHWAY

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The CB1 allosteric modulator, ORG27569, is an inverse agonist of the G-protein signaling pathway and an agonist of the beta-arrestin pathway (acting via beta-arrestin 1) (Ahn et.al JBC 288:9790 (2013)). The intracellular conformational change associated with arrestin-biased signaling is an outward movement of the TMH7/HX8 elbow region away from the intracellular end of TMH2 and also an increase in water accessibility to a previously inaccessible TMH7 residue 7.54 (Liu et.al Science 335:1106 (2012)). We report first here, a molecular dynamics study of the interaction of ORG27569 with the CB1 receptor via the lipid bilayer. Our CB1 inactive state model was suspended in a POPC bilayer, surrounded by 14 randomly oriented ORG molecules (7 per leaflet). The final system contained 177 POPC lipids, 14 ORG molecules, the CB1 protein, sufficient Na^+ and Cl^- ions to bring the ionic strength to 0.1M, and 20300 solvating water molecules. NPT MD simulations were performed at 300K using the CHARMM forcefield in the GPU accelerated PME (Particle Mesh Ewald) AMBER12 program with a Langevin bath collision frequency at 5 ps^{-1}, a long range PME electrostatic cutoff of 8Å, a 1.0 bar Berendson pressure control with relaxation time set to 8ps. Bonds to hydrogens were restrained with SHAKE, and a time step of 2 fs was employed. A 1.4μs simulation revealed a productive binding event in which ORG entered CB1 via the TMH6/7 interface interacting primarily with Y6.57 and F7.35. Subsequently, a direct interaction between the EC3 loop and ORG27569 induced a conformational change in the CB1 IC domains consistent with the production of beta-arrestin biased signaling. Here there was a large outward motion of the TMH7/HX8 elbow region, creating an intracellular binding crevice between TMH2/7. In addition, an increase in waters led to hydration of residue 7.54.

The resultant crevice opening in the IC domains (largest at t=356 ns) was large enough to dock beta-arrestin 1 with CB1. A recent activated rat beta-arrestin-1 crystal structure with the vasopressin V2R C-terminus bound was used as a basis for creating an active human beta-arrestin 1 model (Shukla et.al. Nature 497:137 (2013)). The critical arrestin finger loop region was modeled with residues 66-70 EDLDV as helical based on an NMR study of a peptide of visual arrestin bound to photoactivated rhodopsin (Feuerstein et.al. Biochemistry 48:10733 (2009)). To orient beta-arrestin 1 relative to the intracellular binding crevice of CB1, criteria from several studies mapping specific residues as important for arrestin binding to many different GPCRs, including an Alanine Scanning study of visual arrestin bound to photoactivated rhodopsin, were used (Gurevich et.al. Pharmacol Ther 110:465 (2006), Nakamura et.al. JBC 275:241 (2000), Ostermaier et.al. PNAS 111:1825 (2014), Vishnivetskiy et.al. JBC 286:24288 (2011), Zhuang et.al PNAS 110:942 (2013)). [Support: RO1 DA003934 and KO5 DA021358 (PHR)].

PHARMACOLOGICAL CHARACTERISATION OF A BINDING SITE FOR [^{3}H]LEELAMINE

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Previously we have described some of the behavioural effects of leelamine in mice (Devane et al, 22nd Annual Symposium of the ICRS, P2-10, 2012). Leelamine acts like a cannabinoid CB_1 receptor agonist in the tetrad tests, inducing hypolocomotion, hypothermia, catalepsy, and antinociception. Leelamine, however, is also behaviourally active in CB_1 receptor knockout mice. We therefore hypothesized that leelamine is acting at a site other than the CB_1 receptor. Here, we characterised the binding of [^{3}H]leelamine to brain membranes from CB_1 receptor knockout mice.

The binding assay used is described in the article (Devane et al, J Med Chem, 35(11)2065-9, 1992) except that the vehicle used was 1% ethanol and 0.05% cremophor instead of bovine serum albumin. The bound and the free ligand were separated by centrifugation.

The binding kinetics of [^{3}H]leelamine are unusual in that more radiolabeled leelamine is usually bound with 1 or 2 μM of unlabeled leelamine present compared with vehicle alone. Compounds which weakly inhibited binding include AM404 (a FAAH inhibitor, a TRPV1 agonist, and inhibitor of COX-1 and COX-2) by 4% at a concentration of 300 μM and ibuprofen (a non-selective COX inhibitor) by 23%. The more selective COX-2 inhibitor, nimesulide, inhibited specific binding by 62% at a concentration of 300 μM. Arachidonic acid and virodhamine (an endogenous CB_1 receptor agonist) were the most potent inhibitors yet tested, displacing specific binding by ~95% at a concentration of 300 μM. [^{3}H]Leelamine also bound appreciably to brain membranes from COX-1 and COX-2 knockout mice. .

Although arachidonic acid and virodhamine are potential ligands for the leelamine binding site, their downstream metabolites of COX-1 and COX-2 enzymatic activity do not appear to be involved.

Acknowledgment: Funded by the Wellcome Trust (095779/Z/11/Z).

DESIGN, SYNTHESIS AND BIOCHEMICAL EVALUATION OF NOVEL ELECTROPHILIC AND PHOTOAFFINITY COVALENT PROBES TO MAP THE CB1 RECEPTOR ALLOSTERIC SITE(S)

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The pathological endocannabinoid system has been identified in the etiology of various disorders like obesity, glaucoma, post-traumatic stress disorder, metabolic syndrome, inflammation and neuropathic pain, and the cannabinoid receptor 1 (CB1) has been recognized as a major target in treating these disorders. Orthosteric CB1 receptor antagonists/inverse agonists have met with limited success as medicines due to their "undesirable CNS side effects" and such drugs have either been withdrawn from the market or their clinical development has been halted. Negative allosteric modulation of the CB1 receptor represents an alternative approach with a potential to provide safer effective medications that lack the undesirable side effects of orthosteric CB1 receptor antagonists/inverse agonists.

We report here the design, synthesis and biochemical evaluation of the first CB1 allosteric site covalent probes. Either an electrophilic isothiocyanato or a photoactivatable azido group is incorporated at the judiciously selected positions of the two well-established CB1 allosteric modulators Org27569 and PSNCBAM-1. All the novel compounds were evaluated in cyclic AMP, β-arrestin and [^{35}S]GTPγS assays. Among these ligands, the most potent analog, 3-ethyl-5-isothiocyanato-*N*-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide **(GAT100)** labelled the receptor covalently, inducing a robust (200+%) increase in [^{3}H]CP55940's binding at both rCB1 and hCB1 receptors. Thus this covalent probe behaved as a positive modulator of binding and negative modulator of function. These paradoxical effects on binding and function are similar to those displayed by the parent compound. Unlike Org27569, **GAT100** did not dampen the constitutive activity of the CB1 receptor in the [^{35}S]GTPγS assay refuting the possibility that it is an inverse agonist. Additionally, this study also identified 5-chloro-3-(2-isothiocyanatoethyl)-*N*-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide (**GAT209)** as a potent and functionally selective covalent probe.

These allosteric covalent probes of the CB1 receptor open new avenues for identifying the allosteric binding motif responsible for their modulatory effects. We are now applying proteomics and mass spectrometry techniques to accurately tap these allosteric site(s). Such evidence is vital for understanding the molecular mechanism of allosteric action on the CB1 receptor and will guide structure-based design of potentially effective and safer drugs.

Supported by DA027113 to G.A.T.

REVIEW OF EVIDENCE FOR CANNABINOID RECEPTORS AND ENDOCANNABINOID SIGNALLING IN INVERTEBRATES WITH A SPECIFIC FOCUS ON ARACHNIDS

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The exact evolutionary origins of cannabinoid receptors (CBR) have yet to be established. To date CBRs have only been described in deuterostomian chordates (i.e. vertebrates, cephalochordates and urochordates). The present study was carried out with the dual aims of 1. providing an overview of the current published literature on endocannabinoid signalling in invertebrates and 2. investigating the presence of CBR orthologues encoded within the arachnid transcriptome – specifically within spiders of the genus *Stegodyphus*. Using the systematic framework of a literature review, Pubmed and Web of Knowledge databases were searched for studies involving CBR or endocannabinoid ligands in invertebrates. The results from 31 studies concerning 29 different invertebrate species were categorized by species, phylum and type of investigation carried out and entered into a table to highlight contradictory or corroborative results. In order to achieve the second aim, highly conserved regions and residues which confer cannabinoid ligand binding and selectivity between putative CBR orthologues in vertebrates and invertebrates were identified and selected using the ClustalX Multiple Sequence Alignment software program and a CBR functionality matrix constructed by McPartland *et al.* (Gene. 2003 Jul 17;312:297-303. PubMed PMID: 12909367). These highly conserved regions were then sent as nucleotide and protein queries for searching using the GenBank Basic Local Alignment Search Tool (BLAST). Sequence alignments between the queries and an unpublished spider transcriptome courtesy of Bechsgaard and colleagues (Aarhus university, Denmark) were identified using a database of putative G-Protein Coupled Receptor (GPCR) orthologues identified in the spider transcriptome.

BLAST searching with the CBR conserved region queries retrieved matches with evalues of 2e-17, 6e-14 and 3e-10, while fruit fly *Drosophila melanogaster* GPCR queries retrieved the same matches but with evalues 3 times more significant than those of the CBR queries. From this we concluded that the *Stegodyphus* spider transcriptome does not encode a CBR orthologue. This result combined with published negative *in silico* results in the sea urchin *Strongylocentrotus purpuratus* strongly suggests that the distribution of CBRs excludes the phylum *Echinodermata* and by extension all other non-chordate invertebrates and is thus exclusive to chordate vertebrates and invertebrates (i.e. cephalochordates and urochordates), leaving the effects of endocannabinoid ligands in non-chordate invertebrates to be mediated via different receptors and/or mechanisms.

Acknowledgements: Dr. Saoirse O'Sullivan, Dr. Sara Goodacre

ANALYSIS OF THE ENDOCANNABINOID SIGNALING SYSTEM IN BRAIN STRUCTURES OF SCA-3 TRANSGENIC MICE

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Spinocerebellar ataxia type 3 (SCA-3), the most frequent autosomal dominant hereditary ataxias, is caused by a polyglutamine expansion in the protein ataxin-3 that aggregates in intranuclear inclusions. The aggregates play a key role in eliciting different cytotoxic events in particular transcriptional dysregulation, which are ultimately responsible of the death of specific neuronal subpopulations in the cerebellum and the pons. Patients present progressive loss of motor coordination accompanied by other symptoms. The disease lacks of an effective treatment, but recent studies have proposed that the pharmacological manipulation of the endocannabinoid signaling system may be a promising option in SCA-3 and equivalent autosomal-dominant hereditary ataxias. These studies showed important changes in CB_1 and CB_2 receptors, as well as in FAAH and MAGL enzymes, in the postmortem cerebellum of patients affected by different types of ataxias, including SCA-3 (Rodríguez-Cueto et al., Brit J Pharmacol, 2014; Rodríguez-Cueto et al., Pathobiology, in press, 2014). However, there are no similar studies in experimental models of this disease, that could provide information on the changes in this system during disease progression. A transgenic mouse model of SCA-3 has been recently developed (Silva-Fernandes et al., Neurotherapeutics, in press, 2014) and a stable colony is presently available in our animal facilities. We used these mice for analysis of endocannabinoid ligands, receptors and enzymes in different CNS structures related to the symptoms observed in SCA-3. We first characterized the progression of neurological deficits in these mice using different behavioral tests related to motor coordination (e.g. rotarod, balance beam, hanging wire). With these neurological data, we defined three different stages for this study: (i) a presymptomatic/early symptomatic stage (16 weeks); (ii) a stable symptomatic stage (32 weeks); and (iii) an advanced stage (56 weeks). Next, we determined the concentrations of endocannabinoids, and related *N*-acylethanolamines and 2-acylglycerols, in CNS structures. The only differences found were in the brainstem; in particular, concentrations of anandamide and oleoylethanolamide were reduced in the stable symptomatic stage compared to control mice. However, these changes did not correlate with parallel alterations in the expression of endocannabinoid receptors and enzymes, changes that occurred in the striatum and the cerebellum associated with changes in the expression of calbindin and glutamate transporters. In summary, our results in SCA-3 mutant mice confirm that different elements of the endocannabinoid system are altered in the CNS structures affected in this type of ataxia, thus stressing the possibility to pharmacologically correct these alterations with beneficial effects in the disease progression.

Supported by MICINN (SAF2009-11847) and CIBERNED (CB06/05/0089) and by the Research and Education Component of the Advancing a Healthier Wisconsin Endowment.

THE N-ACYL AMINO ACIDS, N-ARACHIDONOYL-L-SERINE AND N-ARACHIDONOYL GLYCINE, ACTIVATE GPR55

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The orphan G-protein coupled receptor 55 (GPR55) is a novel lipid sensing receptor activated by the endogenous lipid, lysophosphatidylinositol (LPI) and is reported as a putative cannabinoid receptor. However GPR55 shares limited homology with the two cloned cannabinoid receptors (CB_1 and CB_2) but does exhibit some cannabinoid sensitivity. Recently a family of bioactive lipids, the N-acyl amino acids, are gaining interest due to their structural similarity to endocannabinoids (naturally occurring CB_1 and CB_2 agonists). Two members of the N-acyl amino acids family are N-arachidonoyl-L-serine (NAS) and N-arachidonoyl glycine (NAG). Previous studies provide conflicting evidence as to whether NAS can activate GPR55. Furthermore the structurally similar NAG is reported as a GPR18 agonist. Interestingly GPR55 and GPR18 exhibit some overlap in pharmacology.

In the present study we have used a previously characterised HEK293 cell line stably over-expressing recombinant human GPR55 (hGPR55-HEK) to investigate the effects of NAS and NAG on GPR55-mediated Ca^{2+} signalling events. We find that in these cells both NAS and NAG are able to induce oscillatory Ca^{2+} transients that are characteristic of GPR55 receptor activation. These effects were concentration-dependent over the range 10 nM – 10 μM. In control HEK293 cells, treatment with either NAS or NAG induced no oscillatory activity.

This study highlights both NAS and NAG act as GPR55 agonists in hGPR55-HEK293 cells. However NAS is found to be more potent and efficacious than NAG in promoting GPR55-mediated Ca^{2+} mobilisation. Moreover these data confirms that GPR55 is a novel lipid sensing receptor.

SIMULTANEOUS DETERMINATION OF ENDOCANNABINOID AND PROSTAGLANDIN BIOMARKERS AND OF MONOACYLGLYCEROL LIPASE INHIBITOR EXPOSURE USING A NEW AND FAST LC-MS/MS-METHOD

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Recent findings suggest evidence for the 2-arachidonoylglycerol (2-AG) degrading enzyme Monoacylglycerollipase (MAGL) representing a link between the prostaglandin- and endocannabinoidsystem due to arachidonic acid as degrading product of endocannabinoids and educt of prostaglandin synthesis. Specific inhibitors of MAGL might therefore induce anti-inflammatory effects by two different pathways: first, by increasing 2-AG and stimulating anti-inflammatory pathways related to cannabinoid receptor stimulation and second, by decreasing pro-inflammatory prostaglandins.

To investigate if MAGL inhibition induces these anti-inflammatory effects, it is essential to determine endocannabinoid- and prostaglandin- as well as arachidonic acid levels in different tissues. Additionally, plasma and tissue exposure of selective MAGL-inhibitors such as KML-29, JZL-184 and MJN110 were determined by LC-MS/MS to establish a pharmacokinetic-pharmacodynamic relationship in C57BL/6J mice.

Here we describe the development of a novel LC-MS/MS method for the determination of prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGJ_2, TXB_2), endocannabinoids (2-AG, AEA), arachidonic acid (AA) and three MAGL inhibitors (KML-29, JZL-184, MJN-110) within less than 9 min. Method validation was conducted according to FDA and EMA guidelines: The LLOQ ranges from 100 pM to 7.5 nM and Linearity is given up to > 10000 nM. Furthermore the method is characterized by high accurracy (94 – 106 %) and precision (1.3 – 15 %) (between run). All measurements were ensured by use of internal standards.

Applicability of this novel biomarker method is shown by determining the pharmacokinetic profile of KML-29 and subsequent changes in endocannabinoid as well as prostglandin levels in vivo.

This new LC-MS/MS method has the advantage to measure reliable different endocannabinoids and prostaglandins from biological samples in a very fast processing time.

Acknowledgements: funded by the Boehringer Ingelheim Ulm University BioCenter (BIU)

EFFECTS OF NOVEL SEMI-SYNTHETIC CANNABINOIDS ON CB1 AND CB2 RECEPTORS THROUGH BINDING AND SIGNALLING

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Background: CB1 receptors are expressed mainly by neurons of the central and peripheral nervous system whereas CB2 receptors occur centrally and peripherally in certain non-neuronal tissues, particularly in immune cells. Selective interaction with CB1 or CB2 may lead to targeted agonistic or antagonistic effects on the Cannabinoid receptor affected pathways and thus can be used in selective therapeutic concepts. Semisynthetic Cannabinoids, partially derived from natural compounds, and specifically modified, can be an approach to selectively interact with CB1 and/ or CB2 as agonists or antagonist.

Objective: Three novel semi-synthetic Cannabinoids (KG-SY-1310275, KG-SY-1310495, KG-SY-1310485) were tested for their effects on CB1 and CB2 binding and signaling.

Methods: The three compounds with an expected profile as CB1 and/ or CB2 receptor ligands were evaluated by competition studies that allowed to determine the affinity of these compounds (Ki values) for both receptors against a classical cannabinoid ligand ([3H]-CP55940). The competition studies were conducted with membranes transfected with either CB1 or CB2 receptors. Cytotoxicity of the compounds was determined by MTT assay. Subsequently the signalling profile of the three novel compounds was examined in CHO cells transfected with CB1 and CB2 receptors in a cAMP luciferase assay.

Results: We identified all 3 compounds to bind to CB receptors in nM doses and such in a physiological range. The three tested compounds have a higher selectivity for CB2 receptors compared to CB1 receptors. The selectivity values in favour of CB2 were KG-SY-1310275 10.4 (fold), KG-SY-1310495 7.8 (fold) and KG-SY-1310485 8.1 (fold). From the signalling assay, KG-SY-1310485 was found to be a dual and potent agonist on CB1 and CB2 receptor with a preference on CB2. KG-SY-1310495 and KG-SY-1310275 are CB2 agonists and CB1 antagonists.

Conclusion: We demonstrated, that semi-synthetic Cannabinoids reveal diverse patterns of CB1/ CB2 agonsism/ antagonism depending on the modification of the molecules. The three novel structures can potentially be used to trigger CB2 mediated effects and concomitantly could have a low potential to trigger CB1 related side effects. Nevertheless more research on structure-activity relationship is needed.

Acknowledgement: The study was sponsored by Symrise AG, Mühlenfeldstr. 1, D-37603 Holzminden, Germany

REVIEW OF THE EVIDENCE FOR CANNABINOID RECEPTORS IN INVERTEBRATES WITH A SPECIFIC FOCUS ON ARACHNIDS

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The exact evolutionary origins of cannabinoid receptors (CBR) have yet to be established. To date, CBRs have only been described in deuterostomian chordates (i.e. vertebrates, cephalochordates and urochordates). The present study was carried out with the dual aims of 1. providing an overview of the current published literature on endocannabinoid signalling in invertebrates and 2. investigating the presence of CBR orthologues encoded within the arachnid transcriptome (specifically within spiders of the genus *Stegodyphus)*.

Using the systematic framework of a literature review, Pubmed and Web of Knowledge databases were searched for studies involving CBR or endocannabinoid ligands in invertebrates. The results from 31 studies concerning 29 different invertebrate species were categorized by species, phylum and type of investigation carried out and entered into a table to highlight contradictory or corroborative results. In order to achieve the second aim, highly conserved regions and amino acid residues which confer cannabinoid ligand binding and selectivity between putative CBR orthologues in vertebrates and invertebrates were identified and selected using the ClustalX Multiple Sequence Alignment software program and a CBR functionality matrix constructed by McPartland *et al.* (Gene. 2003 Jul 17;312:297-303.). Highly conserved regions were then searched using the GenBank Basic Local Alignment Search Tool (BLAST) against the transcriptome of an eresid spider courtesy of Bechsgaard and colleagues (Aarhus University, Denmark).

Tabulation of the results gathered from our literature review revealed that a total of 11 *in silico* studies investigating CBRs had been reported concerning 6 species of invertebrates. Of these species, only urochordates and cephalochordates (*Ciona intestinalis* and *Branchiostoma floridae*) were shown to possess genes encoding CBR orthologues. *Strongylocentrotus purpuratus*, a member of the phylum *Echinodermata*, shares a phylogenetically immediate common ancestor with the aforementioned deuterostomian chordates. 3 separate *in silico* investigations in this species reported a lack of genes which encode CBR orthologues. BLAST searching of the spider genome with the CBR conserved region queries retrieved matches which shared high percentage sequence identity with a range of arthropod G-Protein Coupled Receptors (GPCRs). Fruit fly *Drosophila melanogaster* GPCR queries retrieved the same matches but with evalue scores 3 times more significant than those of the CBR queries. From this we concluded that the spider transcriptome does not encode a CBR orthologue. This result, combined with the negative *in silico* results in the sea urchin *Strongylocentrotus purpuratus,* strongly suggests that the distribution of CBRs excludes the phylum *Echinodermata* and by extension all other non-chordate invertebrates and is thus exclusive to chordate vertebrates and invertebrates (i.e. cephalochordates and urochordates).

CANNABIS OIL – CHEMICAL EVALUATION OF AN UPCOMING CANNABIS-BASED MEDICINE

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Introduction: Cannabis derived compounds are used by years for their palliative effects in cancer patients especially to inhibit chemotherapy-induced nausea and vomiting, stimulate appetite and inhibit pain [Guzmán, Nat. Rev. Cancer. 2003; 3(10):745-755]. In addition, preclinical evidence has shown cannabinoids to be capable, under some conditions, of inhibiting the development of cancer cells by various mechanisms of action, including apoptosis, inhibition of angiogenesis, and arresting the cell cycle [Calvaruso et al, Int. J. Oncol. 2012; 41(2):407-413; Valesco et al, Nat. Rev. Cancer 2012; 12(6):436-444.]. In recent years the captivating story of a former patient called Rick Simpson, who claims to have cured his skin cancer through repeated topical application of a concentrated cannabis extract also known as "Cannabis oil", has received increasing attention. According to his own recipe, hundreds of patients have been using the Cannabis oil for their selfmedication cancer-cure and describe the effects of the oil on websites dedicated to medicinal cannabis use, and through popular magazines, Youtube videos and other media. On this basis, the aim of this small study is to better understand the extraction methods and the composition of the new claimed anti-cancer treatment also known as Cannabis Oil.

Materials and methods: Cannabis plant material used in this study was of the variety "Bedrocan®" (19% THC w/w). Five different extraction protocols for the production of concentrates were assessed, these included: a naphtha and a petroleum-ether extraction according to instructions by Rick Simpson [Simpson 2008, Simpson 2013. An ethanol extraction based on an authoritative Dutch website on Cannabis oil [Bruining 2013]; and two olive oil extractions using different degrees of heating based on popular Youtube videos [Dr. Diane 2013].
All preparation methods consisted of a few simple steps: one or two extraction steps, separating plant material from solvent, and finally (in case of organic solvents) an evaporation step to produce a concentrate. For the ethanol extraction we also tested the effects of preheating (decarboxylation), treatment with activated charcoal and "winterization" (data not reported). After the extraction all the samples were diluted and analyzed through GC/FID, HPLC and ^{1}H-NMR analysis in order to detect the composition, cannabinoids and terpenes, and the residual solvent traces.

Results: When comparing five methods of Cannabis oil preparation, some interesting differences were observed between the resulting extracts. The most relevant differences were noted in the terpenes profiles Not so great differences were observed in the cannabinoids profile except for the two olive oil preparations that show a relevant increase in the cannabinoids peaks area.
Based on GC/FID and ^{1}H-NMR analysis, residual solvent traces still remain in the extracts especially for naphthalic extraction. We also performed a GC/FID analysis of a sample provided by a patient who makes the extraction following the Rick Simpson's method and the resulting data, in agreement with the ours, show a considerable amount of naphtha traces in the extract analyzed.

Conclusions: As extraction solvents for the production of Cannabis oil, ethanol and olive oil were shown to perform much better, extracting all terpenes and cannabinoids tested very efficiently and, additionally, these solvents are safe for consumption. Olive oil is cheap, not flammable or toxic and ethanol can be easily removed through evaporation. As a trade-off, however, olive oil extract cannot be concentrated by evaporation, which means patients will need to consume a larger volume of it in order to get the same therapeutic results. In a follow-up study on the use of Cannabis oils, there should be more focus on the characteristics and motivations of those who use it for self-medication.

LITHIUM CARBONATE IN THE MANAGEMENT OF CANNABIS WITHDRAWAL: A RANDOMIZED PLACEBO-CONTROLLED TRIAL IN AN INPATIENT SETTING

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Rationale: Preclinical studies suggest that lithium carbonate (lithium) can reduce precipitated cannabinoid withdrawal in rats by stimulating release of the neuropeptide oxytocin, while two open label studies indicate lithium may ameliorate cannabis withdrawal symptoms in humans.

Objectives: To examine the efficacy and safety of lithium in the inpatient management of cannabis withdrawal, and whether lithium affects plasma oxytocin and the rate of elimination of plasma cannabinoids during abstinence.

Methods: Treatment-seeking cannabis-dependent adults (n=38) were admitted for eight days to an inpatient withdrawal unit and randomized to either oral lithium (500 mg) or placebo given twice a day under double-blind randomized controlled trial (RCT) conditions. Primary outcomes included withdrawal severity (Cannabis Withdrawal Scale (CWS)), rates of detoxification completion, and adverse events. Plasma cannabinoids, plasma oxytocin and serum lithium levels were measured repeatedly over admission. Follow-up research interviews were conducted at 14, 30 and 90 days post-discharge.

Results: Lithium did not significantly affect total CWS scores relative to placebo, although it significantly reduced individual symptoms of 'loss of appetite', 'stomach aches', and 'nightmares/strange dreams'. No significant group differences were found in treatment retention or adverse events. Lithium did not increase plasma oxytocin levels nor influence the rate of elimination of cannabinoids. Both placebo- and lithium-treated participants showed reduced levels of cannabis use (verified by urinalysis) and improved health and psychosocial outcomes at 30 and 90 day follow-up relative to pre-treatment baselines.

Conclusions: Despite the strong rationale for the present study, the efficacy of lithium over placebo in the management of cannabis withdrawal was not demonstrated.

Acknowledgements: Funded by the National Health and Medical Research Council (NHMRC Project Grant 556301)

CANNABIS USE AND PERPETRATION OF INTIMATE PARTNER VIOLENCE: THE MODERATING ROLE OF PROBLEMATIC ALCOHOL USE

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Research has identified a significant association between cannabis use and perpetration of intimate partner violence (IPV) (Moore et al., 2008; Moore & Stuart, 2005), making increased risk for partner violence a potentially important cannabis-related health concern. However, few studies of the association between cannabis and partner violence have concurrently examined the influences of alcohol use and antisocial personality. Because both alcohol use and antisocial personality have been associated with cannabis use and with IPV these factors may confound the observed relationship between cannabis and violence. Indeed, the *general deviance theory* suggests that the relationship between cannabis use and violence may be due to underlying characteristics associated with a deviant or antisocial personality. The purpose of this study was to simultaneously examine the relationships among cannabis use, alcohol use, antisocial personality characteristics, and perpetration of physical violence in intimate partner relationships. Participants were 689 undergraduate students who endorsed cannabis use in the past 6 months. As expected, bivariate analyses identified positive associations between problematic cannabis use, and perpetration of physical assault (r = .17, p <.001), problematic alcohol use (r = .20, p < .001), and psychopathy (r = .28, p <.001). However, multivariate analysis revealed that the association between cannabis use and violence was accounted for by alcohol use and psychopathy: with all three predictors in the equation, psychopathic personality and alcohol use remained significantly associated with IPV whereas the association between IPV and cannabis use was reduced to non-significance. Furthermore, we identified an interaction between cannabis and alcohol use, such that cannabis use was associated with violence among participants with higher levels of problematic alcohol use (r = .20, p < .001), but was unrelated among participants with lower levels of alcohol use (r = .01, p = ns).

The findings from this cross-sectional study are consistent with the general deviance theory, in that the direct effects of problematic cannabis use on the perpetration of intimate partner violence were accounted for by covariance with alcohol use and antisocial personality traits. Moreover, problematic alcohol use moderated the relationship between cannabis and violence, such that cannabis use was associated with violence only in the presence of concurrent alcohol use. The results highlight the importance of examining concurrent alcohol use when estimating cannabis use outcomes, and suggest that problematic polydrug use (i.e., cannabis and alcohol use in combination) presents a potentially more important target for interventions to prevent partner violence than does cannabis use per se.

Acknowledgement: Funded by a grant to Zach Walsh, PhD, from the Social Sciences and Health Humanities Research Council.

WHAT GOES UP IN SMOKE: THE IDENTIFICATION AND PHARMACOLOGICAL CHARACTERIZATION OF SYNTHETIC CANNABINOID PYROLYSIS PRODUCTS FORMED DURING SMOKING

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Illicit use of synthetic cannabinoids has continued despite legislation and law enforcement efforts to ban numerous chemicals, and even classes of compounds, as controlled and scheduled substances. Furthermore, manufacturers have continued to diversify the cannabinoids used to evade detection and prosecution. While research and enforcement efforts have focused on identifying the cannabinoids present in herbal formulations, the pyrolytic fate of these cannabinoids, and hence the potential for human exposure and pharmacological impact, has yet to be adequately determined. This is particularly important given the increasing number of adverse events and emergency-room related reports relating to synthetic cannabinoid/herbal product exposure. In several instances, smoking and pyrolysis studies have demonstrated that significant thermal degradation of the synthetic cannabinoid drug substance occurs during smoking. This is particularly true with synthetic cannabinoids containing a tetramethylcyclopropyl ring substituent (e.g., XLR-11, UR-144), as well as ester containing synthetic cannabinoids (e.g., PB-22 and 5-F-PB-22). Exposure to these chemicals would therefore be anticipated to be significant and to represent an important biochemical marker of use and exposure; a hypothesis that is supported by recent reports of these previously identified pyrolysis products and their metabolites in significant concentrations in human biological fluids taken from individuals who had smoked herbal synthetic cannabinoid-containing products. Therefore, our laboratory has extended our pyrolysis studies to include both identification and characterization of the pharmacological properties of the various pyrolytic, degradation, and metabolic products that are associated with smoked synthetic cannabinoid preparations. The characterization of the affinity and efficacy of these synthetic cannabinoid analogs at both CB1 and CB2 receptors, determined using radioligand and GTP-g-S binding studies in transfected cell lines, serves to further inform the forensic and pharmacological/toxicological sciences with respect to synthetic cannabinoid exposure profiles, pharmacological effects and risk.

Acknowledgments: This project was supported by Award No. 2012-R2-CX-K001, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice.

NEURONAL CIRCUITS UNDERLYING CANNABINOID WITHDRAWAL

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The mesolimbic dopaminergic (DA) system displays a reduced spontaneous activity after chronic cannabinoid intake and withdrawal (Diana et al., 1998), the critical phases of addiction. These changes in neuronal plasticity are thought to play a role into withdrawal-induced negative affective states that eventually lead to relapse into drug taking.

The rostromedial tegmental nucleus (RMTg), a GABA structure located just posterior to the DA ventral tegmental area (VTA), is a key site implicated in aversion processes (Jhou et al., 2009). The RMTg provides a major inhibitory projection to the VTA and is a substrate for cannabinoid action on DA cells. Indeed, acute administration of cannabinoids suppress RMTg inputs to the VTA, thus contributing to cannabinoid-induced DA neuronal excitation (Lecca et al., 2011; Lecca et al., 2012).

In the present study, we sought to verify whether RMTg GABA projections to VTA neurons are causally involved in the hypodopaminergic state that characterizes cannabinoid withdrawal. To this aim, we took advantage of single unit extracellular recordings from RMTg and DA neurons in anesthetized male Sprague–Dawley rats.

To induce Δ^9-tetrahydrocannabinol (Δ^9-THC) dependence, rats were chronically treated with this drug (15 mg/kg, i.p.) twice daily for 6.5 days. Administration of the cannabinoid antagonist SR141716A (5 mg/kg, i.p.) precipitated an intense behavioral withdrawal syndrome, whereas abrupt Δ^9-THC suspension produced only mild signs of abstinence.

Electrophysiological experiments confirmed that Δ^9-THC withdrawal produced a marked decrease in the firing rate and burst firing of VTA DA neurons. As expected, RMTg stimulation elicited a complete suppression of DA neuron discharge activity. In Δ^9-THC withdrawn rats the duration of RMTg-evoked inhibition was increased when compared with controls, suggesting an augmented GABA inhibitory input onto DA cells. We are currently investigating whether spontaneous activity of RMTg GABA neurons is altered in cannabinoid-withdrawn rats.

While preliminary, our results support the hypothesis that enhanced GABA inputs from the RMTg might contribute to the hypodopaminergia induced by cannabinoid withdrawal, and confirm that the RMTg takes part in the neuronal circuits underlying drug dependence and addiction.

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DELIVERY OF THE CB_1 ANTAGONIST, AM251, BILATERALLY TO THE CENTRAL NUCLEUS OF THE AMYGDALA AND ITS EFFECTS ON MORPHINE WITHDRAWAL

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Considerable research suggests involvement of the endocannabinoid system in the etiology and maintenance of opiate addiction. Until recently, however, the role of this system in mediating affective opiate withdrawal had not been investigated. One paradigm that provides a sensitive measure of affective morphine withdrawal is the conditioned place aversion paradigm. Administration of naloxone (1 mg/kg sc) in rats having been injected with a high dose of morphine (20 mg/kg sc) 24hr prior is able to produce a robust one cycle conditioned place aversion (CPA). Using this paradigm, we have shown that CB_1 antagonists and neutral CB_1 antagonists attenuate the establishment of a naloxone-precipitated morphine withdrawal induced CPA, suggesting the role for an endocannabinoid tone in the manifestation of affective opiate withdrawal. The present study sought to elaborate on our findings by determining the brain region responsible in mediating these effects.

The extended amygdala represents a group of brain regions identified in mediating affective drug withdrawal. Within this system, the central nucleus of the amygdala (CeA) in particular has been implicated in the establishment of a one trial naloxone-precipitated morphine withdrawal induced CPA. Consequently, we evaluated whether delivery of the CB1 antagonist, AM251, bilaterally to the CeA would interfere with establishment of the CPA.

Rats were surgically implanted with bilateral guide cannulas directly to the CeA. The naloxone-precipitated morphine withdrawal induced CPA was established using a three day conditioning cycle: Day 1) saline floor pairing, Day 2) morphine treatment, Day 3) naloxone floor pairing. To determine whether AM251 was able to interfere with establishment of the CPA, AM251 (1 ug) or VEH was microinfused bilaterally into the CeA prior to Day 1 and 3 of conditioning. Physical symptoms of withdrawal (wet dog shakes, body weight, and activity) were also measured. Several days later, rats received drug-free tests where they were re-exposed to both drug paired floors to determine whether an avoidance of the withdrawal paired floor was established. AM251 interfered with the establishment of the CPA, but did not modify physical symptoms of withdrawal, suggesting that it selectively prevented the negative affective symptoms of morphine withdrawal. Future studies will determine the effects of agonist treatment in the CeA and attempt to elucidate the mechanism of action.

Acknowledgements: Funded by the Natural Sciences and Engineering Council of Canada.

BDNF INTERACTS WITH ENDOCANNABINOIDS TO REGULATE COCAINE-INDUCED SYNAPTIC PLASTICITY IN MOUSE MIDBRAIN DOPAMINE NEURONS

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Brain-derived neurotrophic factor (BDNF) and endocannabinoids (eCBs) have been individually implicated in behavioral effects of cocaine. The present study examined how BDNF-eCB interaction in the ventral tegmental area (VTA) regulates cocaine-induced synaptic plasticity and rewarding effects. We report that BDNF facilitated two forms of eCB-mediated synaptic depression, depolarization-induced suppression of inhibition (DSI) and long-term depression (I-LTD) of inhibitory postsynaptic currents (IPSCs) in VTA dopamine neurons in mouse midbrain slices. This facilitation was mimicked by a selective tyrosine kinase receptor B (TrkB) agonist 7,8-dihydroxyflavone (DHF) and blocked by a TrkB antagonist. BDNF is known to be coupled to phospholipase Cγ (PLCγ) pathway. The facilitation of I-LTD by BDNF and DHF was blocked by the broad spectrum PLC inhibitor U-73122, but not the inactive analog U-73343. DHF did not significantly affect CB_1 agonist WIN55212-2-induced depression of IPSCs. It is thus likely that BDNF facilitates DSI and I-LTD via enhancement of eCB production rather than CB_1 receptor responsiveness. Using Cre-loxP technology to delete BDNF in midbrain dopamine neurons, we showed that eCB-mediated I-LTD, cocaine-induced reduction of GABAergic inhibition, and potentiation of excitatory synaptic strength were absent in BDNF conditional knockout mice. Thus, BDNF-eCB interaction is required for cocaine-induced synaptic plasticity in VTA dopamine neurons. Finally, we showed that cocaine-induced conditioned place preference (CPP) was attenuated in BDNF conditional knockout mice, and *in vivo* DHF treatments restored cocaine CPP in these mice. Taken together, these results suggest that BDNF in midbrain dopamine neurons regulates eCB responses, cocaine-induced synaptic plasticity and associative reward learning.

Acknowledgements: Funded by NIH (R21 MH095921 and R01 DA024741) and Neuroscience Research Center of Medical College of Wisconsin, USA.

INTEGRAL FRAMES: INTERDISCIPLINARY TOOLS SUPPORTING CANNABIS RESEARCH

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The medical cannabis movement, an ongoing and current health care controversy, provides us with a rich vein of study and demonstrates the need for Integral approaches in public healthcare practices. This presentation explores the suitability of Ken Wilber's AQAL model as an Integral Frame to guide the development of healthcare practices that address the true needs of the citizens they are designed to support. Delving into narrative research, this presentation demonstrates that when we come to understand the narratives of patients, including their medical histories, we gain an understanding of how socially, culturally, and politically failed drug policies and corresponding healthcare practices impact not only the lives of cannabis patients, but of all global citizens. This knowledge supports the use of interdisciplinary methodologies, in addition to clinical trials, to help us gain a better understanding of cannabis and cannabinoid therapies, as well as the patients using these therapies. This presenter suggests Integral approaches to healthcare build a collective capacity that support increased cannabis education and research, while impacting society positively on a large scale.

C57BL6/J DEVELOP TOLERANCE TO CP55,940 IN INTRACRANIAL SELF-STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE

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D9-Tetrahydrocannabinol (THC) has been used for thousands of years but only within the last 15 years have synthetic cannabinoids emerged as drugs of abuse. While THC is well-researched in terms of both abuse liability and chronic treatment paradigms in a variety of assays, relatively little is known regarding the acute and chronic effects of synthetic cannabinoids on reward processes. These compounds may vary widely in structure and the prevalence of individual synthetics cycle with time. Therefore, selecting an individual abused synthetic is challenging as it may no longer be relevant upon completion of the study. Here, we chose CP55,940 to probe the effects of acute and chronic administration in C57BL6/J mice. Although not currently abused, CP55,940 possesses many similarities to abused non-classical cannabinoids such as CP47,497 and cannabicyclohexanol thus it serves as an archetypal drug for study. Intracranial self-stimulation (ICSS) of the medial forebrain bundle provides a useful operant task to study the potential reinforcing and withdrawal effects of cannabinoids. Acutely, CP55,940 (0.3-1.0 mg/kg) significant suppressed ICSS in a rate-frequency paradigm. Reversal of the rate decreasing effects of with rimonabant (3.0-10.0 mg/kg) indicated CP55,940 acted via a CB1 mechanism. In a chronic treatment paradigm, mice displayed significant tolerance to once daily injections of 0.3 mg/kg CP55,940 however the acute rate-decreasing effects did not return to vehicle-treated levels. There was no effect of repeated injections in the control group, indicating the recovery of responding is due to repeated CP55,940 treatment. Throughout treatment and for 7 days beyond, baselines for both vehicle and drug-treated groups were stable indicating there was no measurable withdrawal under these conditions. These data indicate no evidence for abuse liability of CP55,940 in this strain of mice, but it retained acute effects despite tolerance.

CANNABINOID CB1 RECEPTOR TRANSMISSION IN THE BASOLATERAL AMYGDALA BI-DIRECTIONALLY CONTROLS THE MOTIVATIONAL PROPERTIES OF OPIATES VIA FUNCTIONAL EXCITATORY INPUTS TO THE NUCLEUS ACCUMBENS SHELL

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The basolateral amygdala (BLA) and nucleus accumbens (NAc) are involved critically in opiate-reward processing. The cannabinoid CB1 receptor is highly expressed in the BLA, and studies have shown its involvement in associative learning processes and memory during the drug addiction process. In the BLA, inhibitory GABAergic neurons are inhibited by CB1 receptor activation, removing inhibition on BLA efferents. Indeed, activation of CB1 receptors within the BLA has shown to reinforce the motivational effects of opiates. Furthermore, opiates activate mesolimbic DAergic inputs to the NAc, and modulate the motivational effects of opiates. Using an unbiased conditioned place preference (CPP) procedure, we administered either a CB1 agonist (WIN 55,212-2) or antagonist (AM 251) into the BLA of Sprague-Dawley rats, and examined how intra-BLA modulation of CB1 transmission within these neural regions may influence opiate reward CPP, using either a sub-reward threshold (0.05 mg/kg; i.p.) or supra-reward threshold (5 mg/kg; i.p.) conditioning doses of morphine. Surprisingly, we found that CB1 receptor activation in the BLA made a normally sub-reward threshold dose of morphine, highly aversive, as rats demonstrated a strong aversion to morphine environments during recall testing. In contrast, intra-BLA blockade of CB1 transmission potentiated the rewarding properties of sub-reward threshold morphine, with rats demonstrating robust CPP. Thus, activation of CB1 transmission in the BLA produces bidirectional effects on opiate reward memory acquisition, switching morphine reward signaling into aversion, or potentiating normally non-rewarding doses of morphine. Our previous research has identified critical functional connections between the BLA and NAc during opiate reward memory processing (Lintas et al., 2012). Accordingly, we next examined if intra-BLA CB1 modulation of opiate reward signaling depends upon functional BLA>NAc projections by reversibly blocking excitatory BLA>NAc projections with the NMDA receptor antagonist, AP-5. We performed bilateral microinfusions of AP-5 (1 μg/0.5 μl) directly into the NAc shell or NAc core, prior to intra-BLA administration of either AM 251 or WIN-55. Interestingly, blockade of NMDA transmission in the NAc shell, but not core, prevented both intra-BLA CB1 blockade mediated opiate reward potentiation and CB1 activation-mediated aversion effects, demonstrating that intra-BLA CB1 receptor modulation controls opiate reward processing via functional inputs to the NAc shell. We are currently examining how intra-BLA CB1 transmission modulates in vivo neuronal network dynamics within the NAc.

Acknowledgements: Funded by Ontario Graduate Scholarship (OGS) and Natural Sciences and Engineering Research Council of Canada (NSERC).

RIMONABANT-PRECIPITATED Δ^9-THC WITHDRAWAL ALTERS MARBLE BURYING AND STRUGGLING BEHAVIORS IN MICE

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Chronic cannabinoid users develop tolerance and are susceptible to withdrawal. Reflecting the increased incidence of these phenomena, the DSM-V includes *Cannabis* Use Disorder and *Cannabis* Withdrawal Syndrome, the primary symptoms of which are increased cravings, anxiety, and depressed mood. Extant rodent models of cannabinoid withdrawal include somatic withdrawal symptoms, such as paw tremors and head twitches. One limitation of these models is that they do not measure changes in emotionality or arousal, such as anxiety-like and depressive-like behaviors. The goal of the present study was to test the hypothesis that rimonabant-precipitated THC withdrawal alters behaviors related to emotionality in mice. Male C57BL/6 mice were administered vehicle or Δ^9-tetrahydrocannabinol (THC) (50mg/kg, s.c.) for 6 days, and then withdrawal was precipitated by acute administration of the CB_1 cannabinoid receptor antagonist, rimonabant (SR141716A) (3 mg/kg, i.p.). The Tail Suspension and Marble Burying tests were performed to quantify depressive-like and anxiety-like behaviors, respectively. Mice subjected to precipitated THC withdrawal exhibited a significant increase in time struggling in the Tail Suspension Test (t (18) = 9.2, $p<0.01$), and decreased marble burying (t (19) = 6.1, $p<0.01$) with no concomitant decrease in locomotor activity. These data lead us to conclude that precipitated THC withdrawal alters behavior in mouse models of emotionality and motivation.

FATTY ACID AMIDE HYDROLASE (FAAH) ACTIVITY IN DIFFERENT RAT BRAIN REGIONS AFTER TWO WEEKS TREATMENT WITH ETHANOL

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The ECS is most likely involved in rewarding effects of ethanol, though less is evident of the importance of ECS in withdrawal and relapse. The aim of the present study was to measure FAAH activity in different rat brain regions after administration of ethanol for two weeks. Sixty male Wistar rats were given ethanol (2g/kg) by gavage twice daily during two weeks. The rats were decapitated 1h, 24h and 10 days after the last dose of ethanol. The brains were rapidly taken out and microdissected on ice. Ten brain structures and the pituitary was collected. FAAH activity was measured by following a protocol by Boldrup et al. (2004). In short, the brain region was weighed and homogenized in 10 volumes of Tris/HCl buffer (50 mM, pH 7,4 containing 3mM $MgCl_2$ and 1 mM EDTA), centrifuged and the pellet was resuspended and kept in

-80^{0}C until use. The FAAH activity was measured by incubating 175µl of homogenate (8µg of protein) with 25 µl substrate (^{3}H-AEA 50000dpm, 4mM AEA, 0,6mg BSA) 20 min at 37^{0}C. A charcoal suspension (400µl) was added and the mixture was incubated for five minutes before centrifugation. Two hundred µl of the supernatant was counted in a liquid scintillation counter. The highest FAAH activity was measured in the hippocampus (1,55+/-0,05 pmol/min/µg protein) followed by the amygdala (1,34+/-0,03) and the medial prefrontal cortex (1,09+/-0,03) and the lowest activities were seen in ventral tegmental area (0,20+/-0,01) and neurointermediate lobe of the pituitary (0,15+/-0,01). The FAAH activities in other brain areas were within this range. Two weeks treatment with high doses of ethanol *per os* twice daily had no effect on the FAAH activity neither 1h nor 24 h after the last dose in any brain region studied. Furthermore, there were no signs of long-term withdrawal effects since no change in FAAH activity was measured 10 days after the last dose.

ACTIVATION OF ADIPOSE TISSUE CANNABINOID RECEPTORS 1 (CB1R) ALTERS ANTILIPOLYTIC ACTION OF INSULIN AND INCREASES LIPOLYSIS IN MICE

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Recent data indicate that activation of peripheral endocannabinoid system (ECS) in tissues such as liver, muscle or adipose tissue may directly influence carbohydrate and lipid metabolism. The existence of cannabinoid receptors 1 (CB1R) and ECS enzymatic machinery has been demonstrated in mature adipocytes, nevertheless studies regarding their role in lipogenesis and lipolysis control are often conflicting. This study was designed to examine the consequence of ECS activation by anandamide on lipolysis activity and related regulation pathways.

Lipolysis activity was estimated measuring for 45 min plasma glycerol release in response to β3-adrenergic receptor agonist (BRL37344) in wild type, DIO or CB1R-/- mice after acute peripheral anandamide injection compared with vehicle. Additional in vitro experiments were conducted to test direct effects of anandamide on glycerol release and signaling pathways on adipose tissue explants exposed to various concentrations of insulin and norepinephrine.

While anandamide alone had no remarkable effects on basal lipolysis, ECS activation potentiated the effect of BRL37344 on glycerol release in wild type mice. The effect of anandamide on stimulated lipolysis was strongly reduced in CB1R-/- while it was amplified in obese animals whose adipose tissue CB1R mRNA expression was much higher than in lean mice. In control mice, the stimulatory effect of BRL37344 on glycerol release was totally counteracted by insulin injection (0,025UI/kg) while it was partially maintained in the presence of anandamide suggesting that ECS activation may be associated with an alteration of the inhibitory action of insulin on lipolysis. These findings were also observed in cultured explants exposed to anandamide in which inhibition of glycerol release by insulin was abrogated. Further, anandamide treatment increased protein levels of the active form of hormone-sensitive lipase and reduced level of Akt and Pi3K phosphorylation compared to control in accordance with a decrease in the activity of insulin-dependent signaling cascade.

All together, these data showed that activation of ECS in adipose tissue increases lipolysis by altering the antilipolytic action of insulin. This suggests that antagonism of CB1R may constitute a new strategy to limit ectopic fat deposition associated with obesity.

PERIPHERAL ENDOCANNABINOID SYSTEM ACTIVATION INHIBITS INTESTINAL GLUCOSE ABSORPTION AND IMPROVES POSTPRANDIAL GLYCEMIA IN LEAN AND OBESE MICE

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Recent convergent data indicate that endocannabinoid system (ECS) is associated with an alteration of glucose homeostasis dependent on cannabinoid receptor-1 (CB1R) activation. Nevertheless the role of ECS on intestinal glucose absorption has been poorly studied. To further explore this notion, we tested the effect of acute intraperitoneal anandamide administration on plasma glucose appearance after oral load in mice. Data indicated that anandamide attenuates hyperglycemia whereas glucose clearance and insulin sensitivity were impaired pointing out the implication of some gastro-intestinal events. Anandamide effect appeared not to be dependent on incretin production since GLP-1 receptor antagonist exendin-(9-39) did not blunt it. An inhibitory action of ECS activation on intestinal absorption was evidenced by oral D-xylose loading test which revealed a strong reduction of plasma xylose appearance during the first 45 min in response to anandamide injection versus controls. Notably, an effect of anandamide on postprandial glycemia was also detectable in $CB1R^{-/-}$ mice and reduced by the specific CB2R inhibitor AM630 suggesting that anandamide could decrease postprandial glycemia both slowing down gastric emptying through CB2R and inhibiting intestinal glucose absorption in a CB1R-dependent manner. In line with hypothesis, we observed that anandamide-induced inhibition of glucose absorption was maintained during duodenal glucose tolerance test (i.e. in condition abolishing gastric emptying) performed in wild-type mice while it was totally abrogated in CB1R-/- mice. Data also indicated that anandamide was more potent in reducing glucose absorption in obese than in lean mice in association with higher CB1R mRNA levels in the small intestine. Interestingly, CB1R antagonists administered alone to obese mice did not modified absorption profile after oral glucose or xylose loading suggesting that endogenous ECS activity was not increased by the high fat diet.

In conclusion, our findings demonstrated that gastro-intestinal ECS activation is associated with improvement of postprandial glycemia. This effect was due, at least in part, to a direct CB1R-dependent inhibition of glucose intestinal absorption. Nevertheless, the beneficial consequences of intestinal CBR activation likely not occur in obese mice whose gastro-intestinal ECS tone appeared not to be endogenously increased.

PHARMACOLOGICAL MODULATION OF THE ENDOCANNABINOID SYSTEM IN A RODENT MODEL OF "ACTIVITY-BASED ANOREXIA"

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Anorexia Nervosa (AN) is a chronic and disabling psychiatric pathology that is characterized by excessive loss of body weight and intense disturbed perception of body shape and size. Furthermore, AN is often accompanied by hyperactivity and by psychological illnesses such as depression, anxiety, or compulsive disorder (APA, 2013). The etiology of AN is complex and not yet completely understood and it is likely that genetic, biological and environmental factors are involved in the onset and maintenance of this disorder. The endocannabinoid system is documented to participate in both the homeostatic and the hedonic regulation of eating behaviour through central and peripheral mechanisms (Di Marzo and Matias, Nat Neurosci 8 (2005) 585-589). In recent years, several reports have led to hypothesise a link between a defect in the endocannabinoid system and AN. For example, a polymorphism of the CNR1 gene (encoding human CB1 receptor) is thought to contribute to the vulnerability to AN (Siegfried et al., Neuropsychiatr Genet 125 (2004) 126-130). Moreover, women with AN have elevated plasma levels of anandamide (AEA) (Monteleone et al., Neuropsychopharm 30 (2005) 1216-1221). Recently, Gèrard and colleagues (Biol Psychiatry 70 (2011) 777-784) using a CB1R positron emission tomography (PET) imaging study, showed an increased number of CB1 receptors in cortical and subcortical brain areas in AN patients in comparison with healthy volunteers. The "activity-based anorexia" (ABA) is the most robust animal model of AN that reproduces some of the key aspects observed in the human condition, especially hyperactivity, reduced food intake and a massive decline in body weight. In this paradigm, rats have free access to a running wheel situated in their home cage in combination with a restriction feeding schedules (90 minutes per day). Under this condition, animals engage excessive wheel running, and undergo reduced body weight. Using the rodent model of ABA, we performed a study to determinate whether the pharmacological manipulation of the endocannabinoid system could be effective in attenuating weight loss in rats exposed to the ABA regime.

We found that subchronic (6 days) treatment with the natural CB1/CB2 receptor agonist Δ9-tetrahydrocannabinol (THC) at both doses tested (0.5 and 0.75 mg/kg) transiently reduced body weight loss in ABA rats with a moderate effect on running wheel activity (RWA). However, subchronic treatment with the synthetic CB1 receptor agonist CP 55,940 at the higher dose of 0.06 mg/kg significantly reduced body weight loss and attenuated the RWA. On the contrary, subchronic treatment with the CB1 receptor inverse agonist/antagonist rimonabant at dose of 0.15 mg/kg did not affect body weight loss nor RWA. Taken together, these data suggest that therapeutic strategies based on drugs that increase the endocannabinoid signalling might be useful in the treatment of AN.

Acknowledgements: The research was supported in part by Regione Autonoma della Sardegna LR7 2007.

SYNTHESIS AND CHARACTERIZATION OF A NANOMICELLE CANNABINOID FORMULATION

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Cannabinoid receptor agonists are moderately effective at reducing neuropathic pain in rodent models. In humans, doses are limited by cannabinoid psychoactivity. In this investigation we developed a water soluble nanoparticle containing the potent cannabinoid WIN 55,212-2 (WIN), and tested the formulation in rats in the chronic constriction injury (CCI) model of neuropathic pain and with the rotarod test. WIN was encapsulated inside a styrene maleic acid (SMA) based micelle. Of the micelles produced, a loading of 21 % WIN to SMA was decided on for the behavioural studies, due to this loading of micelle having slow release properties and a large diameter. We hypothesised that the large size ensured that the micelle would remain in the circulation and not pass fenestrations of the kidney and reduce movement across blood brain barrier.

Using the chronic constriction injury model of sciatic neuropathy, the SMA-WIN micelles were efficacious in the treatment of neuropathic pain for a prolonged period compared to control (base WIN). Pain relief occurred for up to 8 hours at a dose of 11.5 mg/kg of SMA-WIN micelle. To evaluate cognitive impairment the rotarod assessment was utilised. Results showed initial impairment caused by SMA-WIN micelles to be identical to WIN control for up to 1.5 hours. Despite this, the SMA-WIN micelle formulation was able to produce prolonged analgesia over a time when there was decreased impairment in the rotarod test compared with base WIN.

INHIBITION OF ENDOCANNABINOID METABOLISM BY THE METABOLITES OF IBUPROFEN AND FLURBIPROFEN

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Introduction: NSAIDs mediate their effect via inhibition of cyclooxygenase (COX) enzymes, although data is accumulating that show involvement of the endocannabinoid system (review, see Fowler, *Trends Pharmacol Sci* 33 [2012] 468-73). The (*R*)-enantiomer of flurbiprofen, for example, reduce pain by restoring anandamide levels, due primarily to a substrate-selective inhibition of COX-2 (Bishay *et al., PLoS ONE* 5 [2010] e10628; Duggan et al., *Nat Chem Biol* 7 [2011] 803-9). However, flurbiprofen and ibuprofen also inhibit fatty acid amide hydrolase (FAAH) particularly under acidic conditions, such as are seen in inflamed tissue (Holt & Fowler, *Naunyn-Schmiedeberg's Arch Pharmacol* 367 [2003] 237-44), and this may also contribute to the endocannabinoid component of these compounds in such conditions. Investigations into the effects of NSAIDs on FAAH and the substrate-selective inhibition of COX-2 have focused on the compounds themselves, and the actions of their primary metabolites has not been considered. It is possible, for example, that ibuprofen and flurbiprofen have active metabolites with respect to FAAH and that these contribute to the endocannabinoid profiles of the parent compounds. In this study we investigated FAAH and COX inhibitory properties of ibuprofen and flurbiprofen metabolites to elucidate possible contribution to the effect of NSAIDs.

Methods. Ibuprofen, flurbiprofen and their main metabolites were assayed for their inhibitory properties towards rat brain FAAH and the cyclooxygenation of both arachidonic acid (for COX-1 and -2) and 2-arachidonoylglycerol (2-AG, for COX-2).

Results. The metabolites shared with the parent compounds the property of being more potent inhibitors of FAAH at pH 6.0 than at pH 7.3. At pH 6.0, the IC_{50} values for inhibition of anandamide hydrolysis were 70, 340, 380, 200 and 410 μM for ibuprofen and its 1'-OH-, 2'-OH, 3'-OH and carboxy- metabolites, respectively. The corresponding values for flurbiprofen and its 4'-OH-metabolite were 28 and 84 μM, respectively. At a concentration of 300 μM, ibuprofen and its 1'-OH-, 2'-OH-, 3'-OH- and carboxy- metabolites inhibit COX-1 activity by 98, 19, 9, 3 and -4% respectively. Concentrations of 100, 300 and 1000 μM of 4'-OH-flurbiprofen produce 3, 28 and 94% inhibition, respectively, whilst a complete inhibition is seen with 30 μM flurbiprofen. Preliminary results of COX-2 inhibition indicate that ibuprofen metabolites have little effect upon either arachidonic acid or 2-AG metabolism.

Conclusions. The findings so far accrued show that the metabolites of ibuprofen and flurbiprofen retain the ability to inhibit FAAH in a pH-dependent manner, although the potencies of the compounds are lower than the parent compounds.

Supported by a Grant from the Swedish Medical Research Council

DIETARY EFFECTS ON CIRCULATING OXYLIPINS AND ENDOCANNABINOIDS

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Lipids comprise a multitude of structurally diverse molecules, which are directly derived from the diet or synthesized *de novo* from simple precursors. The study of the complex network of dietary lipids and the effect of diets on the lipidome may shed new light on the biological responses of nutritional interventions, unique to each individual. Of special interest are the bioactive lipid-related signaling compounds, endocannabinoids and oxylipins, derived from polyunsaturated fatty acids, thus representing a subset of the lipidome. Endocannabinoids include *N*-acylethanolamines and glycerol derivatives such as AEA and 2-AG, while oxylipins include classical eicosanoids derived *via* the cyclooxygenase and lipoxygenase pathways, as well as non-classical fatty acid epoxides and diols produced by cytochrome P450. Increasing evidence suggest an interplay between the oxylipin and endocannabinoid pathways.

There is good evidence that diet affects the lipidome, but most work has focused upon comparisons of different individuals or short-term interventions. In this work, a single subject who changed diet from vegan to vegetarian was followed over a long period of time. On three non-consecutive days after 5 years on vegan diet, as well as after 1.5 years on vegetarian diet, plasma samples were collected at four different time points (fasting state, 0.5h, 1h and 2h after a well-defined meal) to assess the postprandial response (n=12 for each diet). Metabolite profiling using solid phase extraction followed by UPLC-ESI-MS/MS was applied to analyze 15 endocannabinoids and 38 oxylipins.

A total of 12 endocannabinoids and related *N*-acylethanolamine and monoacylglycerol homologues were detected with levels ranging from 0.1 to 211 nM. POEA was found in the highest levels followed by 2-LG, 2-AG, SEA and LEA. Thirty-five oxylipins were detected with levels ranging from 0.01 to 76 nM. Two-tailed and unpaired Student's t-test ($p<0.05$) was performed to detect diet-dependent differences in baseline levels, as well as at different time points after the well-defined meal. One compound (TXB2) was found to have a significantly decreased baseline level in vegetarian samples, whereas six compounds (9-HODE, 13-oxo-ODE, 9,10-EpOME, 12,13-EpOME, 20-HETE and 11,12-DHET) showed significantly lower levels in vegetarian samples at different time points during the postprandial response. Two-way ANOVA ($p<0.05$) was conducted to detect differences between time points in the postprandial response within the respective diet. Significantly different levels during the postprandial response were shown by six compounds (POEA, 9,10-DiHOME, 12,13-DiHOME, 13-oxo-ODE, 13-HODE, and 9-HODE) in vegan samples and by three compounds (POEA, 13-HODE and 9-HODE) in vegetarian samples.

OREXIN-A ENHANCES 2-AG BIOSYNTHESIS VIA CB_1/OX-1R HETEROMERS IN THE NEURONS OF THE MOUSE HYPOTHALAMIC ARCUATE NUCLEUS

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The hypothalamus is the brain region crucial for the regulation of food intake, body mass and energy balance. The cannabinoid receptor type 1 (CB_1R) is widely expressed in the hypothalamus suggesting its involvement in appetite and in metabolic functions (Tam et al., 2012). The orexinergic peptide hypocretin-1/orexin-A (OX-A) was given its name for its ability to stimulate feeding responses and function via the G protein-coupled orexin-A receptor (OX-1R) (Sakurai, et al., 1998). OX-1R distribution overlaps with that of CB_1R in the brain and increasing evidence exists on the functional/physical interactions between the two receptors and the physiological role of orexin/endocannabinoid signaling. *In vitro* data show that CB_1R and OX-1R form heteromeric complexes in which OX-1R stimulation by OX-A affects both the synthesis of the endocannabinoid 2-AG (Turunen et al., 2012), and the trafficking of CB_1R (Ward et al., 2011). This latter mechanism is regulated by the G-protein coupled receptor kinase (GRK)/arrestin pathway (Pitcher et al., 1998) which promotes CB_1R phosphorylation, subsequent binding to β-arrestin-2 and final desensitization by internalization into the membrane (Smith et al., 2010). In a previous study, we found a significant increase of OX-A release into the arcuate nucleus (ARC) of obese, leptin deficient (*ob/ob*) mice (Cristino et al., 2013). Here, we investigated the possible effect of OX-A both on OX-1R/CB_1R receptor heteromerization and synthesis of 2-AG via PLC/DAGLα –activation (Kukkonen and Leonard, 2014 for review).

Using immunofluorescence, immunoblots and co-immunoprecipitation methods we analyzed CB_1R/OX-1R co-expression in different subsets of hypothalamic neurons of lean (wt) and obese (*ob/ob*) mice. We found strong CB_1R OX-1R co-expression in neurons of the ARC. This was due, at least in part, to the formation of a CB_1R/OX-1R heteromers, as confirmed by the highly significant efficiency of the fluorescent resonance energy of transfer (FRET) in specific experiments. This effect was stronger in obese compared to lean mice, and in lean mice injected with OX-A, and was reversed by pretreatment with the OX-1R antagonist, SB-334867. Moreover, by co-immunoprecipitation of the CB_1R/β-arrestin-2 complex, we demonstrated that a peak of CB_1R internalization occurs when the CB_1R/OX-1R complex is most abundant. In order to test the functional consequences of CB_1R and OX-1R interaction on the modulation of intracellular signaling, we looked at intracellular Ca^{2+} mobilization in ARC primary neuronal cells by means of Fluo-4-based Ca^{2+} imaging experiments.We observed that the CB_1R agonist, ACEA (0.5 μM), or OX-A (0.5 μM) alone cause an increase of intracellular Ca^{2+}, whereas the lower concentration of 0.25 μM was ineffective for both compounds. Interestingly, we observed a significant increase of intracellular Ca^{2+} after treatment of neurons with combined ACEA (0.25μM) + OX-A (0.25μM), possibly indicating a synergistic action of the two agonists. Since, in the presence of even low tissue concentrations of endocannabinoids and OX-A, the formation of CB_1R/OX-1R heteromers might result in higher intracellular Ca^{2+} and increased 2-AG biosynthesis via the PLC-DAGLα pathway, we next examined the levels of 2-AG in the ARC nucleus of wild type (wt) and *ob/ob* mice, and in wt mice after i.p. OX-A injection, alone or after antagonism of OX-1R with SB-334867. We found a ~4 fold increase of 2-AG levels in *ob/ob* and OX-A-injected mice compared to wt mice, whereas 2-AG levels were decreased down to control values 2h after SB-334867 injection. All together, these data provide, for the first time in the brain, evidence of the interaction between CB_1R and OX-1R and for its potential functional impact on 2-AG biosynthesis and related effects on hypothalamic functions, such as appetite, reward and wakefulness.

INHIBITION OF RAT LIVER FAAH ACTIVITY USING THREE DIFFERENT FATTY ACID AMIDES AS SUBSTRATES

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Non-steroidal anti-inflammatory drugs are amongst the most widely used medicinal drugs, with the traditional mechanism of action involving inhibition of cyclooxygenases. NSAIDs have also been reported to inhibit FAAH activity (Fowler *et al*., BJP, 2000). More recently, the metabolism of endocannabinoids by cyclooxygenase-2 was suggested to be differentially regulated by NSAIDs (Duggan *et al.*, Nature Chem Biol, 2012). In the present study, we have investigated FAAH activity from rat liver using different endocannabinoid-like molecules (oleamide, arachidonamide and stearoylamide) as substrates in combination with a variety of NSAIDs to assess the potential for substrate-selective effects of these inhibitors.

FAAH activity from rat liver (Wistar males, $n \geq 3$) was assessed as previously described to the society (Garle *et al*., 2006) using a fluorescent OPA-based detection method for quantification of ammonia generated from the primary amide substrates.

FAAH activity from rat liver was able to hydrolyse oleamide, arachidonamide and stearoylamide with K_m values of 18 ± 11, 42 ± 9 and 4.6 ± 0.8 μM and V_{max} values of 8.3 ± 1.9, 10.4 ± 1.7 and 0.23 ± 0.03 nmol.min^{-1}.mg protein^{-1}, respectively. Inhibitor screening at substrate concentrations of 100 μM oleamide, 50 μM arachidonamide or 20 μM stearoylamide was conducted at 500 μM. Of the NSAIDs investigated, meclofenamate, carprofen, sulindac and diclofenac evoked an inhibition of FAAH activity to less than 50 % of control with all three substrates. Indomethacin and valdecoxib however, exhibited modest inhibition (50 - 80 % of control) with all three substrates. Whereas ibuprofen and sulindac sulphone were modest for oleamide, they were effective FAAH inhibitors to below 50 % of control when arachidonamide or stearoylamide were used as substrates. Diflunisal inhibited FAAH activity to below 50 % of control with oleamide but was less effective with arachidonamide and had no effect with stearoylamide. Ketorolac also inhibited FAAH activity to below 50 % of control with oleamide and stearolamide but was less effective (69 % of control) with arachidonamide. Dipyrone had a modest inhibitory effect with oleamide and evoked no inhibitory effect on FAAH activity when arachidonamide and stearoylamide were used as substrates. Meclofenamate (pIC_{50} = 3.57 ± 0.06), carprofen (3.58 ± 0.09) and sulindac (3.65 ± 0.08) exhibited concentration-dependent inhibition of oleamide hydrolase activity. In the presence of meclofenamate (100 μM) or indomethacin (200 μM), Michaelis-Menten analysis suggested a reduction in the V_{max} of oleamide and arachidonamide hydrolysis, without significant alteration in substrate affinity, indicative of a non-competitive action of these inhibitors.

Taken together, our results fail to indicate a selective action of NSAIDs using different primary amides as substrates of rat liver FAAH activity.

Acknowledgement: Funded by GETFund in collaboration with the University for Development Studies, Ghana and the University of Nottingham, UK.

EX-VIVO ENZYMATIC GENERATION OF 2-AG AND OTHER 2-MONOACYLGLYCEROLS IN PLASMA

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Endocannabinoid analysis in human plasma is a challenging issue. Their concentrations strongly depend on sample collection and processing conditions taking place in clinical and laboratory settings. It is already known that the endocannabinoid 2-AG and other 2-monoacyglycerols are chemically instable and isomerize to the isomer 1 under several processing conditions. Additionally, 2-monoacylglycerols are generated ex-vivo in plasma in the absence of cells. It is believed that this artifactual generation may be the cause of some of the discrepancies in endocannabinoids concentrations reported in clinical studies After testing with several inhibitors, we have found that the artifactual generation of 2-monoacylglycerols can be inhibited by the addition of the lipase inhibitor Orlistat to the plasma collection tube. This is an step that can easily be introduced in the sample collection protocol. Further, data suggest that the generation of 2-monoacylglycerols seems to be a mechanism independent of DAGL (diacylglycerol lipase) since specific DAGL inhibitors do not inhibit this enzymatic activity. The new methodological approach proposed will contribute to the harmonization of endocannabinoids measurement in clinical research.

Acknowledgements: This work was supported by DIUE de la Generalitat de Catalunya 2009 (Grant SGR 718) and CIBEROBN (Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition, CB06/03, CIBEROBN is an initiative of ISCIII).

EXPRESSION OF ENZYMES INVOLVED IN ENDOCANNABINOID METABOLISM BY HUMAN LEUKOCYTES

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AIM OF STUDY. The endocannabinoids 2-arachidonoyl-glycerol (2-AG) and arachidonoyl-ethanolamide (AEA) modulate immune cell functions. Their pro- and anti-inflammatory effects are explained, in part, by their metabolism. In this respect, we showed that 2-AG activates human neutrophils and eosinophils through its metabolites. Endocannabinoids are also oxidized into glyceryl-prostaglandins (PG-Gs) by the cyclooxygenase-2. Our data indicates that PGD_2-G and PGE_2-G inhibit neutrophil functions, in contrast to 2-AG. This suggests that endocannabinoids and their metabolites differentially modulate the inflammatory response. Therefore, we aimed to characterize the mechanisms by which endocannabinoid biosynthesis and hydrolysis occur in human leukocytes.

METHODOLOGY. Human leukocytes were isolated from the peripheral blood and bronchoalveolar lavage of healthy volunteers. The expression of enzymes involved in endocannabinoid biosynthesis and hydrolysis was assessed by qPCR and immunoblot.

RESULTS. Our qPCR data indicates that human neutrophils, eosinophils, lymphocytes, monocytes and alveolar macrophages express endocannabinoid biosynthetic enzymes (DAG lipase-β, and/or NAPE-PLD). Eosinophils, monocytes and alveolar macrophages, but not neutrophils, express MAG lipase, the main 2-AG-hydrolyzing enzyme. To identify additional endocannabinoid-hydrolyzing enzymes in leukocytes, we labelled serine hydrolases with the fluorescent probe TAMRA-FP, combined with the use of endocannabinoid hydrolysis inhibitors. TAMRA-FP labelling revealed a profile of inhibitor-sensitive enzymes that was different for each cell type. We are currently identifying the enzymes of interest by mass spectrometry.

CONCLUSIONS. Human leukocytes express several putative endocannabinoid-hydrolyzing enzymes and their expression pattern is distinct for each cell type. Our ongoing research will elucidate the involvement of these enzymes in the regulation of inflammation.

Acknowledgements*:* This work was supported by grants from the Canadian Consortium for the Investigation of Cannabinoids (CT), the Canadian Institutes of Health Research (NF), the Fonds de recherche du Québec-Santé (NF), and the *Natural Sciences and Engineering Research Council* of Canada (NF).

SR141716A-INDUCED HYPERACTIVITY IS NOT REVERSED BY RITALIN

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Previous studies have shown that SR141716A induces hyperactivity and we have also shown that SR141716A induces Attention-Deficit Hyperactive Disorder (ADHD)-like symptoms in mice. One of the most common drugs for treatment of hyperactivity and ADHD is methylphenidate. In this study we have tested the effect of Ritalin® (methylphenidate) on SR141716A-induced hyperactivity. SR141716A (20 mg/kg, s.c.) was injected to mice on postnatal day one and locomotor activity was tested in the open-field test when mice were 1 and 4 months old. At age 1 month, SR141716A induced a significant increase of ambulation and rearing behaviours in female but not in male mice ($p<0.005$; n=6 vs. vehicle n=5). MRI scan was performed in order to examine whether the effect of a single injection of SR141716A induced long term changes to the structural anatomy of the brain of hyperactive female mice. A quantitative MRI measurement (T2 analysis) at age 1 month revealed that compared with the vehicle-treated group the nucleus accumbens of the SR141716A-treated group was significantly affected ($p<0.005$). At age 4 months, the female mice were still hyperactive ($p<0.005$). Two days later, the response to methylphenidate was examined and Ritalin® (0.1 mg/kg, i.p.) was injected 60 min before the open-field test. The locomotor activity of the SR141716A group that had been treated with Ritalin® remained higher than that of the control group which received vehicle ($p<0.05$). These results further support the view that the endocannabinoid system is involved in hyperactive behaviour. Our results suggest that postnatal inhibition of the CB_1 receptor induces irreversible changes to the brain structure and highlight a role for the nucleus accumbens. As this brain area is associated with movement, addiction and impulsivity these results further support that SR141716A induces ADHD-like behaviour. The lack of response to methylphenidate suggests that dopamine transporter is not involved in the mechanism of SR141716A-induced hyperactivity. As thirty percent of the patients with ADHD do not respond to methylphenidate, one of the most common medications, these results further support the development of cannabinoid-based drugs for ADHD and hyperactivity.

Acknowledgments: The Young Investigator Award and The Charles E. Smith Fellowship in honour of Professor Joel Elkes, The National Institute for Psychobiology in Israel (SAG). The Irving and Cherna Moskowitz Foundation for supporting a scholarship to TH and the ICRS for Travel Award (TH). Funding for this conference was made possible in part by grant R13DA016280 from the National Institute on Drug Abuse. The views expressed in written conference materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention by trade names, commercial practices, or organizations imply endorsement by the U.S. Government.

CANNABINOID CB1 RECEPTOR CALIBRATES EXCITATORY SYNAPTIC BALANCE IN THE MOUSE HIPPOCAMPUS

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The endocannabinoid system negatively regulates the release of various neurotransmitters in an activity-dependent manner, thereby influencing the excitability of neuronal circuits. In the hippocampus, cannabinoid type 1 (CB1) receptor is present on both GABAergic and glutamatergic axon terminals. CB1 receptor-deficient mice were previously shown to have increased hippocampal long-term potentiation (LTP). In this study, we have investigated the consequences of cell-type specific deletion of the CB1 receptor on the induction of hippocampal LTP and on CA1 pyramidal cell morphology. Deletion of CB1 receptor in GABAergic neurons in GABA-CB1-KO mice leads to a significantly decreased hippocampal LTP as compared to wild-type controls. Concomitantly, CA1 pyramidal neurons have a significantly reduced dendritic branching both on the apical and on the basal dendrites. Moreover, the average spine density on the apical dendrites of CA1 pyramidal neurons is significantly diminished. In contrast, in mice lacking CB1 in glutamatergic cells (Glu-CB1-KO) hippocampal LTP is significantly stronger, and CA1 pyramidal neurons show an increased branching and an increased spine density in the apical dendritic region. Taken together, these results indicate that CB1 receptor both on inhibitory and excitatory neurons controls functional and structural synaptic plasticity of pyramidal neurons in the hippocampal CA1 region to maintain an appropriate homeostatic state upon neuronal activation.

THE THERAPEUTIC EFFICACY OF CANNABINOID RECEPTOR TYPE 1 (CB_1) LIGANDS IN HUNTINGTON'S DISEASE DEPENDS ON THEIR FUNCTIONAL SELECTIVITY

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There are no therapeutic strategies that effectively manage the cognitive, behavioral, and motor symptoms of Huntington's disease (HD). The type 1 cannabinoid receptor (CB_1) regulates neuronal activity in the regions on the brain that regulate cognition, mood, and motor coordination, such as the cortex, limbic system, and striatum. CB_1 levels decline in the neurons of HD patients prior to symptom onset. The decline in CB_1 levels may contribute to HD symptom manifestation. Consequently, strategies that maintain type CB_1 activity are being explored as a potential means of treating HD.

We have observed that cannabinoids differ in their functional selectivity and efficacy at CB_1 in a cell model of striatal neurons. Certain agonists, such as Δ^9-tetrahydrocannabinol (THC), are arrestin-biased ligands, while anandamide (AEA) is a $G\alpha_{i/o}$-biased ligand, and cannabidiol (CBD) is a $G\alpha_s$-biased ligand. The objective of this study was to determine which CB_1 agonists effectively enhanced neuroprotective signalling and whether these agonists promoted survival in a cell culture model of HD. We hypothesized that endocannabinoids, such as AEA and 2-arachidonylglycerol (2-AG), and the phytocannabinoid CBD, would promote neuronal survival in HD cells, whereas THC would exacerbate cell death.

We observed that the $G\alpha_{i/o}$-biased ligands, 2-AG and AEA, increased CB_1 levels and improved cell viability in HD cells. CBD was a $G\alpha_s$-biased ligand that increased neurotrophic factor levels and also improved cell viability. THC enhanced receptor internalization and downregulation and reduced cell viability. Titration of THC with increasing concentrations of CBD, as would be observed in different strains of marijuana, produced neuroprotective effects when the ratio of CBD to THC was 2:1 or greater.

AEA-, 2-AG-, and CBD-derived therapies may be useful in HD because of their ability to maintain CB_1 signaling and promote the expression of neurotrophic factors. In contrast, THC-like compounds may exacerbate CB_1 loss in HD. If HD patients are managing their disease with marijuana, then strains with high THC content may similarly affect CB_1 loss whereas strains with high CBD content may be beneficial in HD.

Acknowledgments: Funded by a partnership grant from CIHR, Nova Scotia Health Research Foundation (NSHRF), and the Huntington Society of Canada (HSC) (ROP-97185).

EVALUATION OF PHYTOCANNABINOID COMBINATIONS AS A DISEASE-MODIFYING THERAPY IN R6/2 MICE, A GENETIC MODEL OF HUNTINGTON'S DISEASE

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Different cannabinoid compounds, used alone or in combination, have provided neuroprotection in experimental models of Huntington's disease (HD). In the present study, we have investigated whether a 1:1 combination of botanical extracts enriched in either Δ^9-tetrahydrocannabinol (Δ^9-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex®, is neuroprotective in R6/2 mice, as it did in neurotoxin-based models of HD (Sagredo *et al*., J Neurosci Res, 2011; Valdeolivas *et al*., ACS Chem Neurosci, 2012). We recorded the progression of neurological deficits (e.g. rotarod performance) and the extent of the striatal damage, using different histological (immunostaining for neuronal and glial markers) and biochemical (CB_1 and CB_2 receptors, neurotrophins, glutamate transporters, cytokines) markers, in R6/2 mice daily treated, starting at the 4 weeks after birth, with Sativex®-like combination of phytocannabinoids (5 mg/kg weight for each phytocannabinoid) or vehicle, and their corresponding wild-type animals. We observed several alterations in neurological, histological and biochemical parameters in R6/2 compared to wild-type mice. However, none of these changes was reversed by the treatment with the Sativex®-like combination of phytocannabinoids. Given that the treatment with Δ^9-THC alone had been effective in R6/2 mice (Blázquez *et al*., Brain, 2011), we assumed that the lack of efficacy of the Sativex®-like combination of phytocannabinoids might be related to the presence of CBD in Sativex® which, in some cases, may antagonize some positive effects of Δ^9-THC. This prompted us to evaluate the effects of a botanical extract of this phytocannabinoid in absence of combination with CBD or with a lower proportion, whose results will be available soon. In conclusion, we were unable to find positive effects with the Sativex®-like combination of phytocannabinoids in the progression of striatal damage in R6/2 mice, despite the promising expectations generated by its beneficial effects found in neurotoxin-based models of HD. The possibility to use alternative combinations is presently under investigation.

Supported by MICINN (SAF2009-11847), CIBERNED (CB06/05/0089 and PI2010/06), MSPS (EC10-098) and GW Pharmaceuticals Ltd.

INHIBITION OF FATTY ACID AMIDE HYDROLASE (FAAH) PREVENTS COCAINE-INDUCED SEIZURE AND NEUROTOXICITY

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The present study was designed to test the hypothesis that inhibition of fatty acid amide hydrolase (FAAH) reduces seizures and neurotoxicity induced by cocaine. Male Swiss mice (n=9/10 per group) received intraperitoneal injections of vehicle or an inhibitor of anandamide hydrolysis (FAAH inhibitor, URB597; 0.3-3 mg/kg) followed by cocaine (75 mg/kg). In an independent experiment, the animals were pre-treated with the CB1 receptor antagonist, AM251, in order to evaluate the underlying mechanisms. Convulsive seizures and electroencephalographic activity were concomitantly monitored. Protection against cocaine-induced cell death in the hippocampus was evaluated by *ex vivo* and *in vitro* methods.

The statistical analyses (ANOVA followed by Newman-Keuls test) revealed that URB597 (1 mg/kg) increased the latency and reduced the duration of cocaine-induced electroencephalographic and behavioural seizures. These effects were reversed by pre-treatment with AM251. In addition, URB597 prevented the death of hippocampal neurons, which was also reversed by the CB1 receptor antagonist. It can be concluded that FAAH-inhibition confers protection against the deleterious effects of cocaine. This occurs through CB1 receptor activation, possibly resulting from an increase in the brain levels anandamide.

Financial support: FAPEMIG (APQ-01038-11)

REPEATED TREATMENT WITH THE FAAH INHIBITOR URB597 ATTENUATES LONG-LASTING CONSEQUENCES OF PREDATOR EXPOSURE IN MICE

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Long-lasting behavioural changes that follow predator exposure have been proposed as a post-traumatic stress disorder (PTSD) model. Enhancement of endocannabinoid signalling facilitates extinction of aversive memories, a process thought to be dysfunctional in PTSD. In the present work we investigated if facilitation of anandamide signalling with the FAHH inhibitor URB597 would attenuate the anxiogenic and fear extinction impairment induced by exposing a mice to a rat. Single-housed (10 days) C57Bl/6 male mice (9 weeks old) were exposed for ten min to a male Wistar rat (800g) in a 46x24x21-cm box. A wire-mesh wall separated the animals. Seven days later the mice were tested in the elevated plus maze (EPM) for 5 min. Immediately after they were placed in a conditioning box where they received 3 foot-shocks (0.75mA, 2 S). Twenty-four h later they were placed again in the same box and freezing time was evaluated during 20 min. Extinction consolidation and corticosterone plasmatic levels were measured 24 h later. Animals received a single or repeated injection of URB597 (0.3-1 mg/kg, i.p.), once a day, for 7 days (last injection performed 24-h before behavioural testing). This treatment started immediately after rat exposure and the tests were performed 24 h after the last injection.

Rat exposure induced an anxiogenic-like effect in the EPM, impaired fear extinction and decreased corticosterone plasma levels 1-week later. These effects were attenuated by repeated, but not single, treatment with URB597. The results suggest that enhancement of anandamide signalling could be useful in the treatment of PTSD.

Acknowledgements: Funded by grants from Fapesp and CNPq

CANNABINOID EFFECTS ON PREFRONTAL ACTIVATION DURING REGULATION OF NEGATIVE AFFECT

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Aversive emotional experiences can be regulated by antecedent-focused strategies, such as cognitive reappraisal, and response-focused strategies, such as extinction. Recent evidence suggests that activation of the cannabinoid (CB) system within brain structures important for extinction, such as the ventromedial prefrontal cortex (vmPFC), may regulate extinction learning and retention. Like extinction, cognitive reappraisal engages frontal brain regions encompassing anterior cingulate (ACC), ventro/dorsomedial prefrontal (v/dmPFC), and ventro/dorsolateral prefrontal cortex (v/dlPFC); however no studies have investigated cannabinoid system involvement during cognitive reappraisal of negative affect. We conducted a fMRI study using a randomized, double-blind, placebo-controlled, between-subjects design (N=14 /group) coupled with a reappraisal-based regulation of negative affect task and an acute pharmacological challenge with oral THC in healthy adult volunteers. We examined the effects of THC on amygdala-PFC brain function and connectivity during cognitive reappraisal (i.e., decrease negative affect) as compared to passive viewing (i.e., maintain negative affect) of emotionally-evocative aversive images.

Both groups engaged dlPFC, dmPFC, and vlPFC during attempts to regulate negative affect through reappraisal and there was no effect of drug. However, during passive viewing of aversive images the PBO group engaged the amygdala, whereas THC did not, and THC increased dlPFC activation. Moreover, THC decreased functional coupling between the amygdala and the vmPFC, specifically, and not any other PFC regions. This study is the first to look at the effect of cannabinoids on explicit regulation and suggests that cannabinoids may have very localized effects within the PFC regardless of implicit or explicit emotion regulation.

Acknowledgements: This study was funded by the Michigan Institute for Clinical and Health Research CTSA (UL1RR024986) and the National Institute of Mental Health (1R21MH093917-01A1).

DISSOCIATING THE PSYCHOACTIVE EFFECTS OF DISTINCT MARIJUANA COMPOUNDS IN THE MESOCORTICOLIMBIC CIRCUITRY

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The mesocorticolimbic system contains high levels of dopamine and cannabinoid CB1 receptors, which functionally interact with each other and are implicated in the etiological profile of schizophrenia. A growing body of etiological evidence supports the link between heavy marijuana exposure and an increased risk of developing schizophrenia-related psychoses. However, marijuana smoke represents a complex mixture of chemical components, possessing dissociable psychoactive properties. Indeed, emerging clinical evidence suggests a functional dissociation between the two main pharmacological components of cannabis, cannabidiol (CBD) and Δ9- tetrahydrocannabinol (THC). Clinical imaging evidence suggests that THC and CBD may exert differential psychoactive effects in distinct neural regions, with THC producing pro-psychotic effects and CBD producing anti-psychotic effects in different mesocorticolimbic substrates. In the prefrontal cortical and amygdalar regions, CBD has been shown to be a weak antagonist of CB1 receptors. In contrast, THC acts as a partial CB1 agonist. Our previous work has shown that modulation of CB1 transmission in the BLA>mPFC pathway can mediate the emotional valence of an associative fear memory. Activation of CB1 receptors in these areas results in the potentiation of normally non-salient emotional associative memories, while CB1 receptor blockade prevents the formation of fear memories. Our current objective is to extend these findings to other areas of the mesocorticolimbic system, including the shell of the nucleus accumbens (NASh) and the ventral tegmental area (VTA), and to examine the roles of CBD vs. THC in mediating emotional learning and memory formation.

Our results suggest that CBD has rewarding properties in the NASh and blocks the formation of fear memory to a normally highly salient footshock. These effects appear to be mediated by a serotonergic-dependent mechanism, specifically at 5-HT_{1A} receptors. In contrast, THC potentiates normally non-salient fear memory formation to a sub-threshold footshock through an apparent dopamine-dependent mechanism. We also report evidence of a rostrocaudal hedonic gradient in the NASh that is sensitive to THC, with rostral infusions producing rewarding behavioural properties, while caudal infusions produce an aversion. Preliminary *in vivo* electrophysiology results suggest a complex interplay between dopamaniergic and GABAergic signaling in the ventral tegmental area, which may account for our results.

DUAL FATTY ACID AMIDE HYDROLASE AND MONOACYLGLYCEROL LIPASE INHIBITION AFFECTS SOCIO-EMOTIONAL RESPONSES IN RATS

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The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are inactivated by enzymatic hydrolysis catalyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively.

Previous studies have shown that selective inhibition of FAAH or MAGL affects emotional reactivity in rats (Kathuria et al., Nat. Med. 9 (2003) 76-81; Gobbi et al., Proc. Natl. Acad. Sci. U S A. 102 (2005) 18620-5) and modulates social behavior at adolescence (Trezza et al., J. Neurosci. 32 (2012) 14899-908). Despite these findings, our understanding of the overlapping role of AEA and 2-AG in the regulation of brain functions and behavior remains quite limited. Therefore, the aim of this study was to investigate the effects of JZL195, a dual FAAH/MAGL inhibitor with high efficacy and selectivity in vivo (Long et al., Proc. Natl. Acad. Sci. U S A. 106 (2019) 20270-5), in the regulation of emotional behavior in adolescent and adult rats. To this aim, we tested the effects of JZL195 in the social interaction and elevated-plus maze tests, two tasks sensitive to environmental and physiological factors that can affect emotionality in rodents.

Our findings show that, in adolescent rats, a low dose of JZL195 (0.01 mg/kg; i.p.) increased social play behavior through activation of CB1 cannabinoid receptors, without affecting general social exploration. Conversely, a higher dose of JZL195 (1 mg/kg; i.p.) decreased general social exploration without affecting social play behavior. Under conditions of low environmental aversiveness, a low dose of JZL195 (0.01 mg/kg; i.p.) increased the frequency and the time spent by adult rats in social interaction through activation of CB1 cannabinoid receptors, while higher doses were ineffective. At the dose of 1 mg/kg, JZL195 induced anxiogenic-like effects in the elevated plus-maze test in both adolescent and adult rats. These effects were mediated by activation of CB1 cannabinoid receptors since pre-treatment with the CB1 cannabinoid receptor antagonist/inverse agonist SR141716 antagonized the anxiogenic-like effects induced by high doses of JZL195.

Collectively, our findings highlight the important role that the endocannabinoid system has in the modulation of social behavior and emotional reactivity at different developmental ages, and suggest that dual FAAH/MAGL inhibitors might be useful pharmacological tools to evaluate the behavioral impact of simultaneous elevations of brain AEA and 2-AG levels.

THERAPEUTIC EFFECT OF THE MONOACYLGLYCEROL LIPASE INHIBITOR KML29 IN THE SOD1^{G93A} MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of upper and lower motor neurons. Apart from the anti-glutamatergic drug riluzole which prolongs survival up to 3 months in ALS patients, no treatment is available for this devasting disease. Clinical and preclinical studies using cannabinoid receptor (CB) agonists suggest that the endocannabinoid system is a valuable therapeutic target in ALS. Previously, we reported an increase in 2-arachidonoylglycerol (2-AG) levels in an animal model of ALS and an induction of CB_2 expression on activated microglia (Witting *et al.*, J Neurochem 89 (2004) 1555-1557; Walter *et al.*, J Neurosci 23 (2003) 1398-1405). These disease-associated changes are thought to counteract the ongoing neuropathology by exerting neuroprotective and anti-inflammatory effects. Therefore, amplifying this 2-AG increase by inhibiting the 2-AG-degrading enzyme monoacylglycerol lipase (MAGL) might be beneficial – not only because of the high efficacy of 2-AG at cannabinoid receptors but also for the decrease in pro-inflammatory prostaglandins and arachidonic acid upon MAGL inhibition.

In our project, we examined the therapeutic effect of inhibiting MAGL in ALS by using the highly selective, second generation MAGL inhibitor KML29 in the low-copy SOD1^{G93A} (B6SJL-Tg(SOD1*G93A)^{dl}1Gur/J) mouse model of ALS. Therefore, we orally treated the mice with 10 mg/kg KML29 three times a week from postnatal day 150 until disease end stage. Furthermore, we also characterized the endocannabinoid system on a molecular level.

KML29 delayed the disease onset (35 days), the occurrence of first pareses (49.5 days) and prolonged the survival time (33 days) of SOD1^{G93A} mice. Furthermore, we could show that KML29 increased 2-AG levels mostly in the spinal cord, the diseased tissue in ALS.

In summary, our preclinical study evidences the MAGL as a promising target for the treatment of ALS. The concept of MAGL inhibition is of therapeutic value and should be taken into account for future preclinical and clinical investigations in the field of neurodegeneration and neuroinflammation.

Acknowledgements: funded by the Boehringer Ingelheim Ulm University BioCenter (BIU) and the International Graduate School in Molecular Medicine Ulm (IGradU).

ULTRA-LOW DOSES OF THC PROTECT FROM INFLAMMATION-INDUCED COGNITIVE DAMAGE WITHOUT SUPRESSING THE INFLAMMATORY RESPONSE

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In the present study we investigated the neuroprotective profile of ultra-low doses of tetrahydrocannabinol (THC) in an experimental model of neuroinflammation. The injection of bacterial lipopolysaccharide (LPS;10mg/kg) to mice induced acute (1-3 days) inflammation that was followed by long-lasting (at least 7 weeks) cognitive deficits. The application of THC (0.002 mg/kg, 3 orders of magnitude lower than the conventional doses) 2 days before LPS completely abolished these long-lasting cognitive deficits. The protective effect was blocked by SR141716A (but not by SR141528), indicating the involvement of CB1 (but not CB2) receptors. However, THC did not affect the acute inflammatory response to LPS and did not suppress the LPS-induced over-expression of CD11b, a marker for macrophage-related cells in the liver and for microglia in the hippocampus and frontal cortex, 3 days after the injection of LPS. Similarly, THC did not affect the LPS-induced over-expression of GFAP, a marker for astrocytic activation in the two brain regions. In addition, THC did not abolish the long-lasting neuroinflammatory response in the brain that was detected 7 weeks after LPS injection by the over-expression of CD11 and GFAP. Furthermore, treatment with THC 7 days after the injection of LPS, when the acute inflammation has already passed away, still protected the mice from the LPS-induced cognitive damage.

We suggest that the prevention of LPS-induced cognitive damage by the ultra-low dose of THC resulted from its direct neuroprotective effect and not from a putative anti-inflammatory activity. This is in accordance with our previous findings on the protective activity of the same ultra-low dose of THC against a wide range of non-inflammatory insults (including hypoxia, epileptic seizures, and neurotoxicity), and can be explained by its long-lasting (7 weeks) stimulatory effects on pERK, pCREB and BDNF, proteins that are involved in neuroprotection and neuroplasticity (Fishbein et al., Exp. Brain Res. 221 (2012) 437-448).

REPEATED LOW-DOSE Δ^9-THC PROMOTES LONG-TERM REDUCTIONS IN THE ACTUE NEUROBEHAVIOURAL EFFECTS OF THE ATYPICAL ANTIPSYCHOTIC RISPERIDONE

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Cannabis use is higher in schizophrenia patients than in the general population and is associated with poor treatment outcome. Few studies have examined whether cannabis reduces the effectiveness of antipsychotic drugs. The current study presents an animal model of cannabis-antipsychotic drug interactions by examining whether repeated pretreatment with the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), limits the acute effects of the widely prescribed atypical antipsychotic risperidone, long after withdrawal of THC. Male mice were repeatedly pre-treated with 14 daily injections (i.p.) of vehicle or a low dose of THC (0.5 mg/kg) typical of human consumption of the drug. After a 14 day washout period mice were challenged with vehicle or risperidone injection (0.3 or 1 mg/kg i.p.). Brain activation using c-Fos immunohistochemistry and behaviour in animal models of schizophrenia (prepulse inhibition of startle (PPI) and locomotor activity) was assessed. Further analysis using LC-MS/MS was performed to investigate differences in risperidone brain and plasma levels.

Risperidone-induced neuronal activation was attenuated by THC pre-treatment in a number of brain regions such as the ventral part of the lateral septum, the dorsomedial caudate putamen and the shell of the nucleus accumbens. Risperidone promoted PPI facilitation and locomotor suppression that was reversed by repeated pre-treatment with THC. Furthermore THC exposure lowered the brain concentrations of risperidone and its metabolite 9-OH risperidone compared to mice pretreated with control injections. THC pre-exposure reduced active moiety levels in the brain (i.e the sum of both the active parent and metabolite drugs) by 43% of control concentrations. These findings demonstrate that low, repeated doses of THC reduce the acute actions of risperidone on the brain long-after withdrawal of THC. The decrease in the brain concentrations of risperidone undermines its clinical efficacy and could lead to drug resistance. This provides a novel mechanism for increased rates of cannabis-induced psychotic relapse and that risperidone might not be the treatment of choice for schizophrenia patients with comorbid cannabis dependence issues.

Acknowledgements: Funded by the Brain and Behaviour Research Foundation.

GLUTAMATERGIC AND GABAERGIC COMPONENTS OF THE ENDOCANNABINOID SYSTEM AND THEIR INVOLVEMENT IN PROCESSING OF INNATE AND LEARNED FEAR

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The endocannabinoid system acts as a retrograde signaling system to inhibit synaptic transmission. Via the presynaptic cannabinoid type 1 (CB1) receptor, postsynaptically released endocannabinoids can suppress both excitatory and inhibitory neurotransmission. As the CB1 receptor is expressed in many different brain areas and cell types, it can modulate a large variety of functions. As a consequence, CB1 receptor null mutant mice show alterations in processes including extinction of fear memories, stress responses, seizure susceptibility, anxiety, and feeding behavior.

We applied the Cre/loxP system in mouse lines for conditional knockout ($CB1^{f/f}$) and rescue (Stop-CB1) to analyze the respective importance of CB1 receptor signaling in distinct cell populations for several endocannabinoid-dependent processes. Using Cre-expressing transgenic lines and/or stereotactic delivery via a viral vector, CB1 receptor expression in these mice can be modulated cell-type selectively and/or brain-region specifically.

In this way, we determined the respective contribution of the CB1 receptor in glutamatergic and GABAergic cell populations in several brain regions, including regions involved in e.g. processing of learned fear and the regulation of food intake. In addition, animals with the CB1 receptor present or absent on specific neuronal populations were tested in different behavioral assays. These included elevated-plus maze, open field, light-dark test and auditory fear conditioning to assess the subpopulation-specific importance of CB1 receptor-mediated modulation of synaptic transmission for the processing of innate and learned fear.

ENDOCANNABINOID AUGMENTATION THROUGH SUBSTRATE-SELECTIVE COX-2 INHIBITION: BEHAVIORAL EFFECTS IN AN ANIMAL MODEL OF STRESS-INDUCED ANXIETY

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Cannabinoid receptors have been examined as potential targets to alleviate the negative consequences of anxiety, trauma-related, and stress-related disorders. However, in preclinical animal studies, synthetic cannabinoid can produce adverse motoric and cognitive effects. Thus, pharmacological strategies that augment endocannabinoid levels in the brain, with the aim of enhancing signaling through cannabinoid receptors, are being investigated for their ability to modulate anxiety and stress responses. Previously, we have demonstrated that either genetic removal of the prostaglandin-endoperoxide synthase 2 (Ptgs2) gene, which codes for the cyclooxygenase-2 (COX-2) enzyme that degrades the endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonylglycerol (2-AG), or pharmacologically inhibiting COX-2 activity with a substrate-selective COX-2 inhibitor, LM-4131, can increase brain AEA levels. These elevations in eCB levels in the rodent brain resulted in enhanced endocannabinoid signaling through the cannabinoid type 1 receptor (CB_1R) and, subsequently, reduced anxiety-like behaviors in mice under basal conditions. Here we tested the hypothesis that endocannabinoid augmentation via substrate-selective COX-2 inhibition may have the potential to counteract stress-induced anxiety-like behaviors. We have found that the substrate-selective COX-2 inhibitors, LM-4131 (10 mg/kg), lumiracoxib (1 mg/kg), and the selective COX-2 inhibitor celecoxib (10 mg/kg), can reduce anxiety-like behaviors in juvenile and adult mice subjected to acute footshock stress in the novelty-induced food suppression (NIFS) assay. In contrast, these inhibitors had little effect in control, non-stressed mice. Ongoing studies will further elucidate the receptor mechanisms involved in anxiolytic effects of substrate-selective COX-2 inhibitors after stress exposure.

Acknowledgements: These studies were supported by NIH Grants MH090412 (S.P.), MH100096 (S.P.), DA031572 (D.J.H), GM15431 (L.J.M).

CHRONIC ADOLESCENT CANNABINOID EXPOSURE CAUSES LONG-TERM CHANGES IN ADULT SEXUAL BEHAVIOUR IN MALE RATS

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Adolescence appears to be a crucial sensitive period in the development of the endocannabinoid system. Perturbations of the endocannabinoid system during adolescence using exogenous CB_1 receptor ligands has been shown to produce long-lasting changes in specific adult behaviours (Lee & Gorzalka, Neuroscience. 204 (2012) 17-30). Exogenous exposure to cannabinoids may also cause persistent changes in male sexual behaviour, although this remains to be determined experimentally. It is hypothesized that excess CB_1 receptor activity during adolescence will cause a compensatory down-regulation of endogenous endocannabinoid signaling, which will subsequently manifest in a hypoactive state in adulthood. Given the inhibitory effects of CB_1 receptor signaling on male sexual behaviour in adulthood (Ferrari et al., Physiol. Behav. 69 (2000) 547-554), it is reasonable to predict that adolescent-exposed rats will exhibit increased sexual behaviour in adulthood. Similarly, given the facilitatory effects of CB_1 receptor antagonism on adult sexual behaviour (Gorzalka et al., Psychopharmacology. 198 (2008) 479-486), it is reasonable to predict an analogous but opposite process, namely inhibited adult sexual behaviour, following adolescent exposure to CB_1 receptor antagonists.

Fifty sexually adolescent male Sprague-Dawley rats were divided into five experimental conditions: 1) 25 ug/kg of CB_1 receptor agonist HU-210, 2) 75 ug/kg HU-210, 3) 5 mg/kg of CB_1 receptor antagonist AM-251, 4) vehicle control, and 5) non-injected control. Animals were injected once a day with the appropriate treatment during adolescence (post-natal day 35-45). Subsequently, the subjects received drug abstinence until adulthood (post-natal day 75). Then, they were tested for sexual behaviour during six consecutive sessions with hormone-primed female conspecifics. Sexual behaviour was scored by trained observers blind to the experimental condition.

Male rats exposed to AM-251 showed significantly decreased sexual behaviour compared to either control group ($p < .05$). Animals exposed to either dose of HU-210 showed notable but non-significant increases in sexual behaviour compared to either control group. No significant differences were seen between the two doses of HU-210 or between vehicle-exposed and non-injected rats; comparisons between the collapsed HU-210 group and the collapsed control group showed that HU-210 significantly increased sexual behaviour ($p < .05$). These results are consistent with the hypothesis and are in contrast to previous findings that showed opposite effects for adult HU-210 and AM-251 exposure on male sexual behaviour. Results from this study will contribute to an understanding of how changes in the endocannabinoid system during adolescence are involved in the development of adult male sexual behaviour, and how alterations to this system during adolescence can contribute to adult sexual dysfunctions.

Acknowledgements: Funded by the Natural Sciences and Engineering Research Council of Canada

ELEVATION OF KYNURENIC ACID LEVELS SUPPRESSES DELTA9-TETRAHYDROCANNABINOL-INDUCED EXCITATION OF MESOLIMBIC DOPAMINE AND PREFRONTAL CORTEX PYRAMIDAL NEURONS

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Delta-9-tetrahydrocannabinol (THC), the major psychoactive component of Cannabis extracts, like most drugs of abuse, enhances dopamine (DA) transmission by increasing both DA neuron firing rate and DA release in the nucleus accumbens shell (shNAc). This effect, that is mediated by cannabinoid CB_1 receptors, presumably underlies the rewarding and dependence-inducing effects of marijuana. Justinova *et al.* (*2013*), have very recently demonstrated that elevations of brain levels of kynurenic acid (KYNA), an endogenous product of the normal metabolism of amino acid L-tryptophan, suppresses THC-induced behavioral and neurochemical effects, in rats and monkeys.

On these bases, we carried out in vivo electrophysiological single cell recordings in anesthetized rats to investigate how KYNA modulates THC-induced electrophysiological actions on DA neurons in the ventral tegmental area (VTA) and pyramidal neurons in the medial prefrontal cortex (mPFC). Neurons were selected as projecting to the shNAc by antidromic stimulation. According with previous studies, we confirmed that intravenously administered THC (0.3 – 2.4 mg/kg), increased firing activity of DA (137.1 ± 4.1 %, n = 13, $p<0.05$) and mPFC (306.2 ± 75.6 %, n = 6, $p<0.01$) cells projecting to the shNAc. To enhance brain levels of KYNA, the kynurenine-3-monooxygenase inhibitor, Ro 61-8048 (Ro, 30 mg/kg, i.p.) was administered 40 minutes before recordings. Consistent with microdialysis and behavioral studies, THC-induced increase in firing activity was completely abolished in DA (103.6 ± 3.3 %, n = 7) as well in mPFC (119.4 ± 28.1 %, n = 6) cells recorded from rats pretreated with Ro ($p< 0.01$). KYNA was suggested to act as a negative allosteric modulator of α7 nicotinic acetylcholine receptors (α7-nAChRs), therefore, in the attempt to prevent Ro effects we administered positive allosteric modulators of α7-nAChRs. Galantamine (3 mg/kg, i.p.), non-specific modulator of α7-nAChRs, was unable to prevent the effects of Ro on VTA DA neurons, whereas PNU120596 (1 mg/kg, i.p. or i.v.), specific modulator of α7-nAChRs, partially prevented the effects of Ro on mPFC pyramidal neurons, suggesting that the electrophysiological effects of KYNA might be dependent on α7-nAChR.

Patients seeking help for Cannabis dependence are increasing worldwide but specific pharmacological treatments are lacking and urgently needed, especially after the failure of CB1 antagonists due to psychiatric side effects. Together with recent neurochemical and behavioral studies, our results support the hypothesis that specific modulation of KYNA levels might represent an innovative therapeutic approach to treat Cannabis dependence.

PROTEIN KINASE-C GAMMA INVOLVEMENT IN THE NEGATIVE EFFECTS PRODUCED BY DELTA9-TETRAHYDROCANNABINOL ON MEMORY CONSOLIDATION

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Delta9-tetrahydrocannabinol (THC), the main psychoactive component in *Cannabis sativa* plant, modulates several intracellular signaling pathways in the brain upon acute exposure. Some of these pathways are related to the memory impairing effect of THC. Using biochemical, pharmacological, genetic and behavioral approaches on mice, we found that acute administration of THC (3 mg/kg) promotes the phosphorylation of hippocampal protein kinase C (PKC) isoforms in their catalytic domain in a N-methyl-D-aspartate receptor (NMDAR)-dependent manner. Moreover, THC also modulates the phosphorylation neurogranin, a calmodulin regulating protein abundantly expressed in brain regions involved in cognitive function. In addition, THC modulated the phosphorylation of the myristoylated alanine-rich C-kinase substrate (MARCKS), involved in the regulation of cell shape and dendritic spine maintenance. Interestingly, both proteins are preferential targets for the PKC-gamma isoform. We then evaluated the relevance of PKC-gamma signaling in the cognitive deficits produced by acute and sub-chronic THC in mice lacking this PKC isoform (PKC-gamma KO mice). We used five memory tasks: object recognition, context recognition, delayed and trace cued fear conditioning, and active avoidance. PKC-gamma KO did not suffer the amnesic-like effects of THC in the cognitive paradigms mainly involving hippocampal function. However, THC was equally effective in PKC-gamma KO mice and wild type controls in an amygdala-dependent remembrance such as the delayed fear conditioning. Finally, object-recognition memory was evaluated after a sub-chronic THC treatment. PKC-gamma KO mice were only sensitive to the amnesic-like effects of THC after 4 days of treatment while the control mice were sensitive to the amnesic-like effects of THC from the first day of THC exposure. Other pharmacological effects of THC, such as hypothermia and analgesia were not modified in the PKC-gamma KO mice. All together, our results show a role of PKC-gamma signaling in the hippocampal cognitive deficits produced by THC. Thus, the modulation of calcium/calmodulin signaling by neurogranin phosphorylation, and the regulation of structural plasticity through MARCKS phosphorylation may underlie the role of PKC-gamma in the amnesic-effect produced by THC.

Acknowledgements: RTA (RD12/0028/0023), Instituto de Salud Carlos III (Spain)

AN INVESTIGATION OF PANX 1 MEMBRANE CHANNELS IN CANNABINOID MEDIATED NEUROPROTECTION

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Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by the progressive deterioration of cognition and memory resulting from synaptic loss and neuronal death. It is estimated that 10% of people over 65 years of age and 25% of people over 80 are affected by AD which equates to approximately 36 million people worldwide [1, 2]. The accumulation and aggregation of amyloid-β (Aβ) results in chronic activation of the immune response, excitotoxicity and oxidative damage leading to synaptic dysfunction and severe neurodegeneration. Modulation of the endocannabinoid system has been shown to confer neuroprotection against Aβ-induced neurotoxicity through the inhibition of Ca^{2+} permeable channels, apoptotic cascades and blocking the activation and subsequent release of pro-inflammatory cytokines from microglia [3]. A recently identified and ubiquitously expressed membrane channel, PanX1, has been implicated in the release of ATP from damaged cells as well as activation of the NLRP3 inflammasome and the resulting processing of IL-1β [4]. Regulation of PanX1 is governed by the $P2X_7$, intracellular Ca^{2+} flux and the activity of caspase-3 and -7 [5].

The aim of this study was to investigate the involvement of PanX1 in cannabinoid-mediated neuroprotection against Aβ. In primary cultured rat cortical neurons, no change in total PanX1 expression was found after 72 hrs treatment with Aβ (5 μM), URB597 (1 μM), which enhances the levels of anandamide, or the cannabinoid ligands, O-2545 (1 μM), a CB_1/CB_2 agonist, or the phytocannabinoid cannabidiol (1 μM). However, qualitative immunocytochemistry revealed increased somatic localisation of PanX1 in neurons treated with Aβ (5 μM; 48h). Using conditioned media from primary cultured neurons BV2 microglial cell migration was assessed using Boyden chamber assay. Increased BV2 cell migration was observed after 3 hrs in conditioned medium obtained from neurons treated with Aβ (10 μM) compared to control. Both the pharmacological inhibition of PanX1 by mimetic peptide ^{10}panx (200 μM; p=0.0415; n=5) and treatment with URB597 (5 μM; p=0.0028; n=5) significantly reduced the Aβ-mediated microglial migration. Interestingly, there was no additive effect from co-treating both ^{10}panx and URB597 on the Aβ-induced microglial migration indicating a possible overlap in the two pathways. The interaction between downstream cannabinoid signaling and PanX1 regulation may represent a novel mechanism for modulation of the neuroimmune response in AD. Ongoing research aims to map the localisation of membrane bound PanX1 after cannabinoid treatment and to investigate the effect of cannabinoid-mediated lysosomal stabilization and subsequent reduction in caspase-3 activity on PanX1 gating.

Acknowledgements: Funding received from the Higher Education Authority (HEA) under the Program for Research in Third Level Institutes (PRTLI) cycle 5 and co-funded by the Irish government and the European Union.

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DISTINCT MODULATION OF THE ENDOCANNABINOID SYSTEM UPON KAINIC ACID-INDUCED IN VIVO SEIZURES AND IN VITRO EPILEPTIFORM BURSTING

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There is clear evidence on the neuroprotective role of the endocannabinoid (eCB) signaling cascade in various models of epilepsy. In particular, increased levels of eCBs protect against kainic acid (KA)-induced seizures. However, the molecular mechanisms underlying this effect and its age-dependence are still unknown. To clarify this issue, we investigated which step of the biosynthetic and catabolic pathways of the eCBs may be responsible for the eCBs-mediated neuroprotection in the hippocampus of P14 and P56-70 KA-treated rats.

We found that both anandamide and *N*-palmitoylethanolamine, together with their biosynthetic enzyme significantly increased in the hippocampus of younger KA-treated rats, while decreasing in adults. In contrast, the levels of the other major eCB, 2-arachidonoylglycerol, similar to its biosynthetic enzyme, were higher in the hippocampus of P56-70 compared to P14 rats.
In line with these data, extracellular field recordings in CA1 hippocampus showed that enhancement of endogenous AEA and 2-AG significantly counteracted KA-induced epileptiform bursting in P56-70 and P14 rats, respectively. On the contrary, while the CB_1R antagonist SR141716 per se did not affect the population spike, it did worsen KA-induced bursts, confirming increased eCB tone upon KA treatment. Altogether these data indicate an age-specific alteration of the eCB system caused by KA and provide insights for the protective mechanism of the cannabinoid system against epileptiform discharges.

LONG-TERM EFFECTS OF ADOLESCENT CB1 RECEPTOR ANTAGONISM: RELEVANCE TO STRESS RESPONSIVITY AND EMOTIONAL BEHAVIOUR IN ADULT MALE RATS

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Previous work from our laboratory has reported dynamic and temporal-specific changes in anandamide (AEA) content and fatty acid amide hydrolase (FAAH) activity within corticolimbic structures throughout the peri-adolescent period. Moreover, CB1 receptor expression has been reported to peak during early to pre-adolescence, which then decreases to adult levels. Together, these findings suggest that normative adolescent endocannabinoid signaling within the corticolimbic stress circuit may be vulnerable to perturbations such as stress or exogenous cannabinoids. Therefore, we sought to examine the role of adolescent CB1 receptor activation in the development of HPA axis stress responsivity and emotionality behaviour in male rats. Between post-natal days (PND) 35-45, animals were administered daily IP injections of CB1 receptor antagonist, AM-251 (5 mg/kg), or vehicle. Upon reaching adulthood (PND 75), endocannabinoid content, HPA axis stress reactivity and emotional behaviour were assessed. AM-251 treated males exhibited greater anti-depressive-like behaviour in the forced swim test and greater risk assessment behaviour in the elevated plus maze, with no significant differences in general motor activity. Preliminary results suggest that adolescent AM-251 treatment had no significant effect on HPA axis stress reactivity to restraint. The relatively modest long term behavioural effects of adolescent CB1 receptor antagonism also resulted in moderate changes to the adult endocannabinoid system, with no significant changes to 2-AG content, and a small but significant increase and decrease in the amygdala and hypothalamus, respectively.

EARLY MODIFICATION OF THE ENDOCANNABINOID SYSTEM ACCOMPANIES THE LATE BEHAVIORAL PHENOTYPE OF A RODENT DEVELOPMENTAL MODEL OF SCHIZOPHRENIA

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Accumulating evidence suggests that a neurodevelopmental dysfunction could be one of the causes of schizophrenia (SCZ), which leads to severe personal and social dysfunctions. Early cannabis use has been suggestedto play a role in the genesis of SCZ in predisposed subjects. A variety of animal and human studies found a dysregulation of the endocannabinoid system (both in term of cannabinoid receptors and endocannabinoid ligands) in psychosis; thus the pharmacological manipulation of the ECS could be a novel approach for treating SCZ. In the present study, we aimed to investigate the potential effects of prenatal administration of the mitotoxin methylazoxymethanol acetate (MAM) on early neurophenotypic presentations using a battery of behavioral tests. We also measured the brain expression of endocannabinoid receptors and metabolic enzymes (such as FAAH and MAGL) and the levels of the endocannabinoids anandamide and 2-AG. Timed-pregnant Sprague Dawley rats were treated with MAM (22 mg/kg) or vehicle (VHC) intraperitoneally on gestational day 17 (GD17). To assess the development of neonatal behavior, starting on postnatal day 1, newborn pups were observed for neonatal reflexes (i.e.: righting, cliff aversion, forelimb placing, bar holding, forelimb grasping, negative geotaxis) as an index of brain maturation, until the maximal appearance of these signs was scored (i.e. 100% of the brood was found to exhibit the full repertoire of reflexes). During adolescence, the offspring was submitted to different behavioral tests such as the social interaction test or the novel object recognition and the Y-maze tests to assess negative-like symptoms or various cognitive aspects, respectively. At birth, neonatal reflexes had a delayed onset (i.e. percent of appearance) in prenatally MAM-exposed rats, as compared to the control group ($P<0.05$; $P<0.01$; $P<0.001$). At adolescent age, prenatally MAM exposed rats engaged in less social behavior as suggested by the reduced time of interaction ($P<0.05$). No difference in the number of episodes, as index of locomotor activity, was found. In the NOR test prenatally MAM-exposed rats showed an impaired cognitive performance, as described by the decreased discrimination index ($P<0.001$). By contrast, spatial recognition memory was not affected by prenatally MAM-exposure since no difference between the two groups of rats (MAM vs VHC) was found in the Y-Maze test. Interestingly, the behavioral alterations correlated with both decreased expression of FAAH and MAGL ($P<0.05$) and with enhancement of anandamide and 2-AG levels ($P<0.05$) in prenatally MAM-exposed rats. These results suggest that behavioral abnormalities resulting from a MAM environmental challenge, which resemble a SCZ-like phenotype, could be due to abnormalities in endocannabinoid tone.

Acknowledgments
This work was supported by the project financed from the SoMoPro II programme. The research leading to these results has acquired a financial grant from the People Programme (Marie Curie action) of the Seventh Framework Programme of EU according to the REA Grant Agreement No. 291782 (to V.M.). The research is further co-financed by the South-Moravian Region; by the project "CEITEC - Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund.

LOSS OF CB1 RECEPTORS LEADS TO DECREASED CATHEPSIN D LEVELS AND ACCELERATED LIPOFUSCIN ACCUMULATION IN THE HIPPOCAMPUS

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Early onset of age-related changes in the brain of cannabinoid 1 receptor knockout ($Cnr1^{-/-}$) mice suggests that cannabinoid 1 (CB1) receptor activity significantly influences the progression of brain aging. Now we showed that lack of CB1 receptors leads to a significant increase in lipofuscin accumulation and a reduced expression and activity of cathepsin D, lysosomal protease implicated in the degradation of damaged macromolecules, in the hippocampus of 12-month-old mice. The impaired clearance of damaged macromolecules due to the low cathepsin D levels and not enhanced oxidative stress may be responsible for the lipofuscin accumulation because macromolecule oxidation levels were comparable between the genotypes within the same age group. The altered levels of autophagy markers p62 and LC3-II suggest that autophagy is upregulated in CB1 knockout mice. Increased autophagic flux in the absence of CB1 receptors is probably a compensatory mechanism to partially counteract decreased lysosomal degradation capacity. Together, these results suggest that CB1 receptor activity affects lysosomal activity, degradation of damaged macromolecules and thus it may influence the course and onset of brain aging.

THE EFFECT OF CANNABIS ON DIVERGENT AND CONVERGENT THINKING: THE ROLE OF DOPAMINE

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Delta-9-tetrahydrocannabinol (THC), the compound responsible for most of the psychoactive effects of cannabis, has been shown to have an indirect impact on dopamine - a neurotransmitter involved in the control of goal-directed behavior, reward learning, reinforcement and addiction. However, not much is known about the effect of THC on specific dopamine-related cognitive functions, including creativity. In our study we aimed to address that issue by investigating a group of 60 healthy cannabis users, matched for a number of demographic variables. We administered two different doses of THC in the form of medicinal cannabis (Bedrocan®) using a Volcano® vaporizer, in order to assess the differential impact of a particular dose on tasks indicative of divergent and convergent thinking - two cognitive processes representative of creative performance . Since regular cannabis users display a specific tolerance to the impairing effects of THC, participants in our research were required to be regular cannabis users. Moreover, the current study was the first to use a placebo cannabis recently developed by Bedrocan - .The results of the study showed that a high dose (8 mg THC) of cannabis, as compared to a low dose (2 mg THC) and placebo , resulted in decreased scores for fluency, flexibility and originality - all critical elements of divergent thinking. No effects of cannabis were observed in the case of convergent thinking. In summary, the data suggests that cannabis selectively impairs aspects of creative performance. Additionally, the results support the notion that the generation of creative acts is composed of specific dissociable cognitive processes.

FAAH-MEDIATED MODULATION OF TLR3-INDUCED NEUROINFLAMMATION IN THE RAT HIPPOCAMPUS

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Toll-like receptors (TLRs) are important players in mediating and regulating neuroinflammatory processes associated with a host of CNS disorders. Several lines of evidence have demonstrated that (endo)cannabinoids regulate TLR-4 induced neuroinflammation, however, there is a paucity of studies investigating their effects on inflammation associated with the activation of other TLRs. *In vitro* data have demonstrated that the cannabinoid receptor agonist WIN55212-2 attenuates TLR-3-induced immune responses [1]. However, no studies to date have examined such responses *in vivo* or the effect of modulating endocannabinoid tone directly within the brain on TLR-3-induced neuroinflammation. The present study examined the effects of inhibiting fatty acid amide hydrolase (FAAH), both systemically and centrally, on the expression of various TLR-3-responsive genes in the rat hippocampus.

Male Sprague Dawley rats (250-300g; n = 6-10 per group) received URB597, systemically (1mg/kg i.p. in ethanol:cremophor:saline 1:1:18) or centrally(50µg, i.c.v in 100% DMSO), or corresponding vehicle, 30 minutes prior to systemic administration of the TLR-3 agonist poly inosinic:polycytidylic acid (poly I:C; 3mg/kg, i.p.) or sterile saline. Animals were killed 4 hours post poly I:C challenge, the hippocampus dissected out, snap-frozen and stored at -80°C. The expression of TLR-3-responsive genes, including the type 1 (IFNα and IFNβ) and type 2 (IFNγ) interferons, interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFNγ-inducible protein 10 (IP-10) were determined using qRT-PCR. Concentrations of the endocannabinoids, AEA and 2-arachidonylglycerol (2-AG), and the fatty acid ethanolamines, PEA and OEA, were determined using LC-MS-MS. Data were analysed using ANOVA followed by Fisher's LSD *post-hoc* test when appropriate. $P < 0.05$ was deemed significant.

Both systemic and central administration of URB597 increased hippocampal levels of PEA and OEA, but not AEA or 2-AG. Systemic administration of URB597 increased the hippocampal expression of IFNα, and IFNγ, in the presence of poly I:C. In addition, poly I:C-induced increases in the expression of the NFkB responsive pro-inflammatory genes, IL-1β and TNFα, were attenuated, while concurrently the expression of IL-6 was augmented by systemic URB597. In comparison, i.c.v administration of URB597 did not alter the expression of the type 1 interferons but attenuated the poly I:C-induced increase in IFNγ and its inducible chemokine IP-10. Furthermore, this treatment regime attenuated the poly I:C-induced increase in TNFα and IL-6 expression, and concurrently increased the expression of the anti-inflammatory cytokine IL-10.

Taken together, these data demonstrate that increasing FAAH substrates within the brain elicits anti-inflammatory effects which support an important role for FAAH-mediated regulation of TLR-3-induced neuroinflammatory responses.

Acknowledgements: Funding provided by Science Foundation Ireland Research Frontiers Project (Grant no. 11/RFP/NES/3175).

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TIME COURSE CHANGES OF CB1 AND CB2 RECEPTORS AFTER TRAUMATIC BRAIN INJURY IN MALE AND FEMALE MICE: STRUCTURAL AND FUNCTIONAL CORRELATES

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There are many animal studies demonstrating the existence of sex differences in the pathophysiology, recovery time and sequelae after a central nervous system lesion, such as traumatic brain injury (TBI). TBI is one of the main causes of death in young individuals and it triggers very heterogeneous signaling cascades that lead to the secondary damage. This includes brain edema, neuroinflammation, brain barrier disruption, changes in the endocannabinoid system and activation of neuroprotective pathways. We have recently provided the first evidence for the involvement of CB1 and CB2 cannabinoid receptors in the neuroprotective action of minocycline in a TBI murine model as well as a relevant role of CB2 receptor in the modulation of axonal damage (Lopez-Rodriguez et al. Cerebral Cortex, 2013 in press). The aim of this study was to evaluate in both, males and females, the time-related sequelae and recovery course, in relation to lesion severity, behavioral alterations, modifications in CB1 and CB2 receptors and vimentin and aquaporin4 expression changes.

Our results show that TBI induced, in both sexes, a neurological impairment 24h and 72h after lesion that recovered 2 weeks after lesion. TBI also produced an increase in the percentage of water content in males at 24h and 72h after TBI that disappeared 2 weeks after lesion whereas in females, a peak of brain water content was found exclusively at 72h after TBI. The expression of CB1 mRNA decreased at 24h and 72h after TBI in males, whereas in females a decrease in the expression of this receptor was found only 72h after injury. CB2 mRNA expression levels increased progressively in injured males whereas in females it increased just at 24h and 2weeks after lesion. mRNA levels of AQP4 augmented at 24h after lesion in males whereas females showed an increase at 24h and 2 weeks after trauma. In the case of vimentin, mRNA levels increased at 24h and 72h after TBI in both sexes and were recovered at 2 weeks after lesion. Ongoing Western blot analyses of these proteins, as well as possible correlation with neurological deficit and edema, will allow us to discuss further the functional significance of these findings.

Acknowledgements:

GRUPOS UCM-BSCH: 951579, Red de trastornos adictivos RD12/0028/0021.

THE SATIVA-INDICA DILEMMA

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In this study, we analyzed over 100 different cannabis accessions to elucidate relevant chemical and genetic differences between the putative *sativa* and *indica* types of cannabis.

Differentiation between *sativa* and *indica* types of cannabis is currently the most common way to classify different strains and their physiological effects among breeders, recreational users and patients. Although *Cannabis sativa* L. is a monotypic species according to current taxonomic consensus, many scientific discussions about the existence of multiple (sub)species have taken place over the centuries [1]. It is unclear whether this classification reflects any relevant differences in chemical composition [2].

Supposing there were originally two main gene pool centres of *sativa* and *indica*, some distinctive characteristics can be expected to have remained on the chemical and genetic level. In our study, we focused on chemical profiling of 8 main cannabinoids and 40 terpenoids by GC-MS. Additionally, samples were analyzed for genetic variations by the SNP-LINE method. Based on principal component analysis (PCA) of the chemical and genetic data, we were able to identify relevant markers for distinguishing *sativa* from *indica* types of cannabis. The study indicates the usefulness of a PCA approach for chemotaxonomic classification of Cannabis varieties.

Acknowledgements: Trimbos Institute; ERASMUS work placement scholarship

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EFFECTS OF EXTRA VIRGIN OIL PHENOLIC COMPOUNDS ON CB_1 GENE EXPRESSION: A ROLE FOR EPIGENETIC MECHANISMS

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Extra virgin olive oil (EVOO) represents the typical lipid source of the Mediterranean diet, a dietary habit that has been associated with a significant reduction of cancer risk in Mediterranean populations. The aim of our study was to investigate the ability of EVOO and its phenolic compounds to modulate gene expression and epigenetic regulation of elements of the endocannabinoid system (ECS), in colon cancer (Caco-2) cells, as well as to explore the underlying mechanisms responsible for its potential beneficial effects. Moreover, we investigated the short- and long-term influence of dietary EVOO on ECS components in rat colon mucosa.

Gene expression and promoter methylation was determined by qPCR analysis and pyrosequencing respectively, in colonic epithelial cells treated with EVOO or total phenolic extracts (TPE). Additionally, adult female Sprague Dawley rats were fed with either a standard diet or with EVOO supplement, and the expression of ECS elements was assessed in rat colonic epithelium after 2hr and 10 days.

We found a selective increase in CB_1 gene expression in Caco-2 cells exposed to EVOO (100ppm) or TPE (10-50 μM) for 24 hr, compared to untreated cells. The stimulatory effect of EVOO and TPE on CB_1 expression inversely correlated to levels of DNA methylation at *CNR1* promoter and was associated with DNA methyltransferase (DNMT) 3a down-regulation. We also found that EVOO and TPE inhibited proliferation of Caco-2 cells and arrested their cycle, and that animals receiving dietary EVOO supplementation for 10 days displayed a significant increase in CB_1 gene expression levels in colon. Finally, we found that CpG methylation of the rat *Cnr1* gene was reduced after acute EVOO administration, but remained unaltered after chronic EVOO administration compared to standard diet.

In conclusion, our findings that CB_1 gene expression can be modulated by EVOO or its phenolic compounds v*ia* epigenetic mechanisms, both *in vitro* and *in vivo,* may provide a new therapeutic strategy for treatment and/or prevention of colon cancer.

Acknowledgments: Funded by "Fondazione TERCAS-Progetto Speciale Assegni di Ricerca 2011–2013" fellowship to ADF and the Italian Ministry for University and Research (Futuro in Ricerca RBFR12DELS 001 to CDA and CC, and PRIN 2010-2011 to MM).

DEVELOPMENT OF A FLOW CYTOMETRY-BASED BRET ASSAY UTILISING GENETICALLY-ENCODED BIOSENSORS FOR MIXED CELL POPULATIONS: APPLICATION TO CANNABINOID CB1 RECEPTORS

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Genetically-encoded bioluminescent resonance energy transfer (BRET)-based biosensors have been invaluable tools in the quantitative and qualitative analysis of intracellular signalling processes. BRET biosensors are typically transiently transfected into cultured cells, and the resulting signal detected using a plate reader. These plate readers integrate the results from large samples of cells – typically 30,000-100,000 cells per 96-well. Here, we report the development process of a high throughput assay for BRET biosensors in single cells with the primary aim of developing a method to differentiate and selectively analyse subpopulations of cultured cells. Specifically, we are developing a flow cytometry (FCM) method for single-cell cAMP measurement, using the genetically-encoded BRET-based biosensor CAMYEL (Jiang, et al. JBC. 2007;282(14):10576-84).

FRET by FCM has previously been demonstrated (e.g. Banning, et al. PLoS ONE. 2010;5(2):e9344), but to date there have been no published examples of FCM BRET detection, although this approach would help overcome the spectral overlap of FRET-pair fluorophores. The BRET-based biosensor assay was optimised using a Becton Dickinson LSRII flow cytometer, with the 488 nm laser occluded to prevent laser-excitation of the BRET acceptor. As the 488 nm laser typically provides the forward and side-scatter data used to identify cells, an antibody-based cell-surface marker was utilised to identify and selectively gate for cells. We found that, while the Rluc and YFP BRET pair had insufficient energy for detection, with a sufficiently slow flow rate, the high emission of the variants Rluc8 and Venus provided satisfactory signal for detection of both wavelengths. Site directed mutagenesis was therefore employed to modify CAMYEL to optimise detection, and results with the optimised biosensor will be presented.

This technique is well-suited for mixed cell cultures, and can provide detailed data from as little as a few thousand cells. BRET by FCM is also useful for monitoring interactions from a range of interacting proteins, such as dimer partners. Here we present the proof-of-principle data from a transfected HEK cell population expressing the human cannabinoid CB1 receptor. In conclusion, while it is technically challenging to establish a suitable single cell detection method, such a high throughput approach would be a valuable to analysing cell signalling biology and for drug discovery.

ADOLESCENT THC EXPOSURE INDUCES EPIGENETIC CHANGES AND ALTERS GENE EXPRESSION IN THE RAT PREFRONTAL CORTEX

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We recently demonstrated that adolescent female rats treated with the psychoactive ingredient of marijuana delta-9-tetrahydrocannabinol (THC), develop a depressive/psychotic-like phenotype in adulthood. Interestingly, adolescent, but not adult, chronic THC exposure leads to this phenotype, suggesting that adolescence may represent a more vulnerable period for the adverse effect of THC exposure. However the neurobiology of this vulnerability is not clear. Recently, several papers support an involvement of epigenetic mechanisms in the pathogenesis of psychiatric illnesses. Thus we check for the presence of epigenetic alterations induced by adolescent THC exposure (e.g. H3 tri- and di-methylation or acetylation) in the prefrontal cortex (PFC) of adolescent THC-treated animals. To this aim, adolescent female rats were treated with increasing doses of THC twice a day from PND 35 to 45. Two and 24 hours after the last THC injection, epigenetic modifications were investigated by immunoblotting.

In the PFC of THC-treated animals we observed increased tri-methylation of Lysin27 on the histone H3 (H3K27me3, associated with transcriptional repression) 2 hours after the end of the THC treatment. Moreover, 24 hours later, this increase was still present together with increased di-methylation of Lysin9 and acetylation of Lysin14 on histone H3 (H3K9me2 and H3K14Ac, associated with transcriptional repression and activation respectively).

Since histone modifications impact transcriptional activity, the second aim of this work was to investigate the effect of adolescent THC exposure on gene expression in the PFC. Adolescence is characterized by intense processes of neuronal refinement in which the endocannabinoid system (ECS) seems to play a crucial role, thus we focused our attention on genes closely related to the ECS or involved in synaptic plasticity. To this aim, adolescent female rats were treated with increasing doses of THC twice a day from PND 35 to 45. Real-Time PCR array analysis was performed in the PFC 2, 24 and 48 hours after the last THC injection. Besides genes related to the ECS, we investigated 34 genes involved in synaptic plasticity (e.g. belonging to the glutamatergic and gabaergic system as well as coding for proteins or pathways related to plasticity).

Two and 24 hours after the THC treatment mRNA levels mainly decreased whereas they returned to control levels or even increased 48 hours after the end of the treatment. These alterations might play a role in the development of the depressive/psychotic-like disorder induced by adolescent THC exposure.

As a whole, these data suggest that adolescent THC treatment impairs the steady state expression of a set of genes involved in brain plasticity. These alterations might play a role in the development of the depressive/psychotic-like disorder induced by adolescent THC exposure.

Acknowledgements: this work was supported by Dipartimento delle Politiche Antidroga. M.G. has a predoctoral fellowships from D.P.A.

SELECTIVE ACTIVATION OF CB_2RS ELIMINATES AGONIST-INDUCED CA^{2+} SIGNALS IN PANCREATIC ACINAR CELLS: A NOVEL CELLULAR MECHANISM FOR ACUTE PANCREATITIS THERAPEUTICS

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Acute pancreatitis is an inflammatory disease of the pancreas, which has several causes and symptoms which require immediate medical attention. Acute pancreatitis occurs when pancreatic pro-enzymes (especially trypsin), secreted from pancreatic acinar cells, are activated in the pancreas instead of the small intestine, causing autodigestion. In clinical practice, there are still no efficient drugs that specifically treat acute pancreatitis. Emerging lines of evidence demonstrate that the primary event initiating the process of acute pancreatitis is the excessive release of Ca^{2+} from intracellular Ca^{2+} pools. This provides a promising therapeutic strategy, as the blockade of intracellular Ca^{2+} signals in pancreatic acinar cells may provide protection against Ca^{2+} overload, intracellular protease activation and necrosis, which are major triggers of acute pancreatitis. The cannabinoid receptor type 2 (CB_2R) is a G protein-coupled receptor from the cannabinoid receptor family. CB_2Rs are predominantly expressed in peripheral system, especially in immunal cells, suggesting that they are the major targets in mediating the effects of cannabinoids on the immune system. Thus, CB_2R agonists are commonly used as a therapeutic drug for the treatment of inflammation and pain.

A large body of evidence suggests that CB_1 and CB_2 receptors are expressed in the pancreatic gland and modulate pancreatic cell function, alter insulin release, and play important roles in the regulation of body metabolism. In addition, emerging data demonstrate that the activation of CB_2Rs in pancreatic acinar prevents acinar cell pathogenesis in acute pancreatitis animal models. Whether or not the activation of CB_2Rs modulates intracellular Ca^{2+} signals in pancreatic acinar cells is unknown. Also, whether the pancreatitis inducer L-arginine enhances Ca^{2+} oscillations, and if a CB_2R agonist eliminates L-arginine-induced enhancement of agonist-induced Ca^{2+} oscillations in pancreatic acinar cells remains elusive. In the present study, we address these important questions by using patch-clamp and confocal Ca^{2+} imaging approaches combined with qRT-PCR and immunohistochemical staining in wild type (WT), CB1 knockout (KO) and CB2 KO mice. We found that selective activation of CB_2Rs, by a selective agonist GW13542, significantly reduced ACh (10-30 nM)-induced Ca^{2+} oscillations in acutely-dissociated single pancreatic acinar cells. The inhibitory effect of GW13542 could be eliminated by a selective CB_2R antagonist, AM630, or was absent in CB_2R, but not CB_1R, KO mice. Next, we showed that intracellular application of GW13542 failed to inhibit Ca^{2+} oscillations and intracellular application of AM630 also failed to prevent bath-applied GW13542-induced inhibition in Ca^{2+} oscillations. Together, these results suggest that GW13542 eliminates ACh-induced intracellular Ca2+ signals through CB_2Rs on the acinar cell surface. Finally, we demonstrated that L-arginine enhanced ACh-induced Ca^{2+} oscillations, while GW13543 eliminated this enhanced effect. Collectively, our study provides a novel cellular mechanism that the CB_2R agonist GW13542 eliminates intracellular Ca^{2+} signaling in pancreatic acinar cells, which may provide a new therapeutic strategy for treating acute pancreatitis.

A NEW ANTI MOUSE CB2 ANTIBODY SENSITIVE TO RECEPTOR CONFORMATION

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A new anti mouse CB2 antibody was generated by immunizing mice with living HEK293 cells expressing the N-terminal 34 amino acids of mouse CB2 fused to mouse glycophorin A and directed to the cell surface with a bee venom melittin signal sequence. The AB 1/45 was identified by immunofluorescence using recombinant CHO cells to be selective for mouse CB2 since it did not cross react with either human CB2 nor CB1. Using the same recombinant mouse CB2 expressing CHO cells, the antibody exhibited a bright and specific signal detectable by flow cytometry and was useful to immunoprecipitate CB2. The CB2-specific signal observed via flow cytometry is lost however by the addition of CB2 ligands. Irrespective of the CB2 ligand function (Agonists: HU-910, WIN 55.212-2, 2AG; n=3; Inverse agonists: SR-144528, NE40), we found greater than 88% displacement of the fluorescent signal at an EC50 similar to the respective K_i ($r^2 = 0.93$).

A first hypothesis was that this ligand-mediated signal loss is a consequence of CB2 receptor internalization. However, ligand-mediated antibody displacement at 4°C was observed at comparable potency and kinetics (less than a minute) as compared to the initial experiment at 37°C, thus excluding the involvement of receptor internalization. We thus rather hypothesized, that AB1/45 is able to bind in a conformation-sensitive manner distinguishing bound and unbound CB2. This was confirmed by immunohistochemistry using a directly fluorophore labeled AB 1/45. The ligands from above were fully efficacious in displacing AB 1/45 with the exception of AM630 which only partly displaced AB 1/45 by 47% while the EC50 of 14nM still resembled the K_i. In contrast, Anandamide (AEA) increased rather than decreased AB1/45 binding up to 10μM. As so far the only anomalous ligand identified was AEA, it can be assumed that AEA results in a different conformational change to CB2.

We then determined the effect of AB 1/45 on CB2 function in a cAMP assay. After forskolin-induced activation, AB1/45 was able to act as an inverse agonist with an EC50 of 218nM. It remains to be shown if AB1/45 is displaced by the high affinity ligands or is rather recognizing a specific bound CB2 conformation. Interestingly, AB 1/45 is unable to detect CB2 on leukocytes isolated from mouse blood. This could be a result of a ligand bound state, or a masked epitope by glycosylation or interacting proteins of native endogenous mouse CB2 in leukocytes compared toCB2 expressed in CHO cells. In summary by using a whole cell immunization protocol based on a hybrid N-terminal CB2 antigen, we discovered a mouse CB2 selective antibody able to detect ligand free CB2 receptors in transfected CHO cells serving as a tool to unravel the difference between endogenous CB2 versus recombinant mouse CB2 receptors.

2-ARACHIDONOYLGLYCEROL PROMOTES LEUKOCYTE RECRUITMENT THROUGH CB_1 AND CB_2 RECEPTORS

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Accumulated evidence points to a key role for endocannabinoids in cell migration, and here we sought to characterize the role of these substances in early events that modulate communication between endothelial cells and leukocytes.

We found that 2-arachidonoylglycerol (2-AG) was able to initiate and complete the leukocyte adhesion cascade, by modulating the expression of selectins. A short exposure of primary human umbilical vein endothelial cells (HUVECs) to 2-AG was sufficient to prime them towards an activated state: within 1 hour of treatment, endothelial cells showed time-dependent plasma membrane expression of P- and E-selectins, which both trigger the initial steps (*i.e.*, capture and rolling) of leukocyte adhesion. The effect of 2-AG was mediated by CB_1 and CB_2 receptors and was long lasting, because endothelial cells incubated with 2-AG for 1 hour released the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) for up to 24 hours. Consistently, TNF-α-containing medium was able to promote leukocyte recruitment: human Jurkat T cells grown in conditioned medium derived from 2-AG-treated HUVECs showed enhanced L-selectin and P-selectin glycoprotein ligand-1 (PSGL1) expression, as well as increased efficiency of adhesion and trans-migration.

In conclusion, our *in vitro* data indicate that 2-AG, by acting on endothelial cells, might indirectly promote leukocyte recruitment, thus representing a potential therapeutic target for treatment of diseases where impaired endothelium/leukocyte interactions take place.

CANNABINOID 2 RECEPTOR MODULATION IN EXPERIMENTAL SEPSIS

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Introduction: Sepsis results from a dysregulated immune response to an infection. Currently available medical treatments are of limited efficacy with regard to improvement of survival. The endocannabinoid system (ECS) opens a novel avenue in sepsis therapy due to its unique characteristics. Cannabinoid 2 (CB_2) receptors expressed on the surface of immune cells provide a direct approach to the modulation of the systemic immune response in sepsis. The present study explored the effects of CB_2 receptor modulation in two mouse models of acute sepsis (endotoxemia and abdominal fecal peritonitis).

Methods: Endotoxemia was induced by intravenous administration of lipopolysaccharide (LPS, 5 mg/kg; from *E. coli,* serotype: O26:B6). Six groups of mice were used to assess leukocyte activation within the intestinal microcirculation as well as functional capillary density (FCD) by intravital microscopy. The treatment compounds tested were: a specific CB_2 receptor agonist, HU308, a CB_2 receptor antagonist/inverse agonist, AM630, and the cannabinoid degradation enzyme inhibitors URB597 (fatty acid amide hydrolase inhibitor), and JZL184 (monoacylglycerol lipase inhibitor). Abdominal fecal peritonitis was induced by implanting a stent into the ascending colon and allowing fecal matter to pour out into the abdominal cavity (CASP). Four groups of mice were used to asses the same microcirculatory parameters mentioned previously, with JZL184 as the treatment compound.

Results: In the endotoxemia model, administration of the CB_2 receptor agonist, HU308, reduced significantly the number of adhering leukocytes in submucosal venules, but did not restore muscular and mucosal villi FCD in endotoxemic mice. The CB_2 receptor antagonist, AM630, did not exacerbate leukocyte activation within the intestinal microcirculation, but further reduced muscular and mucosal FCD of the intestinal wall. The cannabinoid degradation enzyme inhibitors URB597 and JZL184 both significantly reduced the number of adhering leukocytes in submucosal venules. Furthermore, JZL184 administration completely restored muscular FCD. In the CASP model, JZL184 reduced significantly the number of adherent leukocytes in the submucosal venules. Rolling behaviour of leukocytes also showed a trend to normalization by JZL184 treatment.

Conclusions: CB_2 receptor activation by a specific agonist or cannabinoid degradation enzyme inhibition was effective in reduction of leukocyte activation within the intestinal microcirculation. The CB_2 receptor pathway seems to be involved in the inflammatory cascade elicited early during sepsis. Therefore, modulation of the CB_2 receptor pathway might offer new therapeutic options for the manipulation of the immune system in sepsis.

CB2R MODULATION OF LPS-INDUCED TOLL-LIKE RECEPTOR 4 SIGNALLING IN OCULAR INFLAMMATION

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Introduction: Uveitis is an ocular inflammatory disease that can lead to impaired vision. Experimental endotoxin-induced uveitis (EIU) can be generated with an intravitreal (IVT) injection of lipopolysaccharide (LPS), a component of gram-negative bacteria. LPS activates the Toll-like receptor 4 (TLR4) and induces the release of inflammatory mediators that may cause tissue damage. TLR4 can signal by both the MyD88-dependent pathway and the MyD88-independent pathway (TRAM/TRIF pathway). In the eye, cannabinoids acting at CB2 receptors (CB2R) are anti-inflammatory and can reduce pro-inflammatory cytokines, although the signaling mechanisms giving rise to these effects remain to be clarified. Therefore, this study examined the effects of CB2R modulation on TLR4 signaling in EIU.

Methods: Five groups of Lewis rats (n= 8-10 per group) were studied: control (saline, IVT), EIU (100 ng LPS, IVT), EIU + CB2R agonist (HU308; 1.5 μg/μL eye drop), EIU + CB2R antagonist (AM630; 2.5 mg/kg, i.v.), EIU + CB2R agonist + CB2R antagonist. Drug treatments were given 15 min after EIU induction. Tissue was harvested and RNA was extracted. Quantitative PCR was conducted using LightCyclerR system and software with SYBR Green I. All qRT-PCR data was normalised to the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA expression levels of different molecules in both downstream signalling pathways of the TLR4 were investigated: NF-κB (nuclear factor kappa B), activator protein-1 (AP-1), receptor-interacting protein 1 (RIP1), interferon regulatory transcription factor 3 (IRF3). The changes in mRNA levels were also investigated for the CB2R.

Results: A significant increase of NF-κB mRNA occurred in LPS-treated eyes compared to control ($P<0.001$). This increase in NF-κB mRNA was abrogated by administration of the CB2R agonist, HU308. The CB2R antagonist, AM630, significantly increased NF-κB mRNA compared to LPS ($P<0.05$), while the combination treatment of AM630 and HU308 decreased these levels to that comparable to control. AP-1 mRNA was significantly increased in LPS animals compared to control ($P<0.001$); this increase was attenuated by treatment of HU308. AM630 had no effect on altering AP-1 mRNA levels, although treatment with both AM630 and HU308 resulted in a significant decreased ($P<0.001$) of these levels. There were no significant changes in the mRNA levels of IRF3 and RIP1 amongst any of the experimental groups studied ($P>0.05$). CB2R mRNA expression was significantly increased in LPS in comparison to control ($P<0.05$).

Conclusion: These data demonstrate that CB2R are capable to modulate (ocular) inflammation via TLR4 signalling. CB2R agonist treatment decreases mRNA levels of NF-κB and AP-1. However, as CB2R activation does not modulate RIP-1 or IRF3 mRNA expression, these data suggest that the anti-inflammatory actions of CB2R activation may be primarily mediated by actions on the MYD88-dependent pathway and are independent of TRAM/TRIF signalling.

CANNABINOID RECEPTOR 2: A NOVEL IMMUNOSUPPRESIVE TARGET IN EXPERIMENTAL PROLIFERATIVE VITREORETINOPATHY

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Introduction: Proliferative vitreoretinopathy (PVR) is a sight- threatening complication of retinal detachment, ocular trauma or inflammation. Cannabinoid receptor 2 (CB_2) is expressed on immune cells and activation of this receptor is anti-inflammatory. In an experimental murine model of PVR, we previously showed that pathological changes in the retina of CB_2 receptor knockout animals were more pronounced than in control C57Bl/6 animals. These changes were characterized by the formation of retinal folds, inflammation of the choroid and a significant increase in activated microglia. We further examined the role of CB_2 in the pathology of PVR, including changes in the expression profiles of pro-inflammatory cytokines, prostaglandins and related lipids in both, $CB_2^{-/-}$ and C57BL/6 animals.

Methods: PVR was induced in $CB_2R^{-/-}$ and C57BL/6 mice by intravitreal dispase injection (0.1U; 2μl). At 7 days post-dispase injection, the morphology of the eyes was examined using light microscopy and scored using a clinical grading system. Animals were then sacrificed, eyes enucleated and the levels of prostaglandins and related lipids were analyzed by tandem mass spectrometry. In addition, the pro-inflammatory cytokines expression was evaluated by ELISA.

Results: Dispase injection (0.1U; 2 μl) resulted in pronounced damage to ocular tissues in $CB_2^{-/-}$ animals. As previously shown, there was a mild or absent pathology observed in C57BL/6 animals. The mass spectrometry analysis revealed elevated levels of prostaglandins and related lipids in $CB_2^{-/-}$ animals, as compared to controls. In addition, changes in the levels of pro-inflammatory cytokines were also observed in $CB_2^{-/-}$ animals.

Conclusion: Mice lacking CB_2 had increased susceptibility to dispase-induced retinal damage. The retinal pathology in $CB2^{-/-}$ mice was associated with elevated levels of prostaglandins and neurotoxic lipids, as well as selective cytokines. This data is supportive of an immunosuppressive role for CB_2 in inflammatory ocular conditions and suggests that drugs that activate CB_2 may be useful in the treatment of ocular inflammatory disease.

ASSESSMENT OF CB2 IN HUMAN BRAIN TISSUE USING *IN SITU* HYBRIDISATION

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Background and Aim. In the past 5 years, there has been increasing debate around the presence or absence of the cannabinoid receptor-2 (CB2) in the healthy central nervous system. Most of the pro-voice is from research using rodent brain tissue and antibody based applications. Prior to this, much of the cannabinoid field was in reasonable agreement that CB2 was largely absent from the CNS, but can be elevated during neuroinflammation (due to leukocyte infiltration). The aim of this research is to investigate CB2 expression at the mRNA level and protein level using a combination of in situ hybridisation using healthy or normal appearing brain tissue.

Methods. Complementary RNA probes (riboprobes) were synthesised for CB2 and markers of various cell types including endothelial cells (CD34), pericytes (SMA), microglia (IBA-1), neurons (NeuN) and CB1 as a control probe. Cell-lines were generated expressing the target gene for each probe set and grown as an artificial tissue for the purposes of ISH sectioning to create positive controls. Human brain tissue from the Neurological Foundation Brain Bank was used in this study.

Results. Our histological data indicates the absence of CB2 protein expression in neuronal populations and glial (astrocytes and microglia) cells. Some positive antibody staining has been observed within structures of the blood-brain barrier, at this time thought to be the endothelial cells. The specificity of *in situ* riboprobes were rigorously validated using positive control cells grown in 3D-Alvetex scaffolds to generate artificial tissue layers. *In situ* hybridisation for CB2 probes reveals a very low to negligible signal from the human brain tissue regions investigated to date. In contrast, signals from CB1 and NeuN probes were very strong, validating the effectiveness of the *in situ* method.

Conclusions and Future Developments- Based on our current histological and *in situ* data we conclude that CB2 is not abundantly expressed in the healthy human CNS tissue. Future experiments will involve a greater range of human brain regions and development of colorimetric *in situ* for anatomical localisation of CB2 signals. Extension of the approach using neurological tissue with pronounced inflammation is an important future goal.

Acknowledgements; Authors gratefully thank all of the contributions to this project from members of the CBR and Brain Bank. We acknowledge the in put from Dr Anower Jabed. Funding; Auckland Medical Research Foundation (ESG/MG) and Health Research Council of New Zealand (ESG).

DIFFERENTIAL EXPRESSION AND FUNCTION OF THE CB2 RECEPTOR IN DISTINCT SUBSETS OF HUMAN MONOCYTES AND MACROPHAGES FROM BLOOD

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Background and Aim. CB2 receptor agonists have anti-inflammatory properties in rodent models of stroke and multiple sclerosis, and appear to improve the neurological deficits caused in part by the detrimental neuroinflammation in these debilitating models. The aim of this research is to better understand the function of the CB2 receptor expressed by specific human immune cells circulating in blood. We are particularly interested in CB2 expression and function of human monocyte subsets as these cells have key roles in most forms of neuroinflammation.

Methods. Flow-cytometry was conducted to compare CB2 expression across the distinct monocyte subsets including the classical $CD14^{+}$ monocytes (represents ~80% of monocytes), intermediate $CD14^{+}/CD16^{+}$ (~10% of monocytes) and the non-classical $CD16^{++}/CD14^{low}$ monocytes (~10%) to reveal distinct expression patterns for CB2. Functional assays (including cytometric bead array and phagocytosis of fluorescent particles) were conducted to determine the role of the CB2 receptor in regulating secretion of a key panel of inflammatory cytokines and influence of CB2 on phagocytic activity of different monocyte subsets.

Results. All monocyte subsets expressed CB2, with the non-classical $CD16^{++}$ subset having 10-fold higher levels than the $CD14^{+}$ monocytes. However, CB2 agonists did not have a major effect on any of the major monocyte-secreted chemokines or cytokines measured in our CBA panel. In contrast, CB2 substantially suppressed the phagocytic activity of monocyte-derived macrophages at agonist concentrations within 10nM to 100nM.

Conclusions- The differential expression level of CB2 across human monocyte subsets is novel, but more importantly suggests functions associated with specific phenotypes or states of activation. Intriguingly, we observed pronounced effects of CB2 agonists on macrophage phagocytosis, but not on monocyte cytokine secretion. Cytokine/chemokine communication is a major component of the inflammatory response and is surprising that CB2 had no major influence. These data may suggest that CB2 is involved in regulating a more mature macrophage phenotype than the monocyte phenotypes observed in blood.

Funding; Auckland Medical Research Foundation (ESG/MG) and Health Research Council of New Zealand (ESG).

NEW MOUSE MODEL FOR THE STUDY OF CB_2 CANNABINOID RECEPTORS

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The study of cannabinoid CB_2 receptors has been limited by several factors. Among others, the questions on the specificity of anti-CB_2 antibodies and the expression of a truncated form of the protein in CB_2-knockout mice have been of most relevance. We here report the design and generation of a new transgenic mouse line that may help to unveil the precise pathophysiological roles of cannabinoid CB_2 receptors. The mouse model was generated by inserting an eGFP reporter gene preceded by an IRES sequence in the 3' UTR of the *Cb2* mouse gene. This approach results in the expression of the reporter gene under the control of the endogenous mouse *Cb2* promoter and transcript from the same bicistronic mRNA as the CB_2 protein. In addition, the whole exon 3, including the 3' UTR and the knocked-in reporter was flanked by *loxP* sites, allowing the conditional inactivation of the *Cb2* gene. The mouse model ($CB_2^{eGFP/f/f}$) was generated by homologous recombination in embryonic stem cells, in the C57BL/6J genetic background. The presence of GFP was determined in circulating cells by flow cytometry, revealing a relative abundance concordant with that expected for CB_2 receptors (CD4<CD8<macrophages<B cells). In addition, GFP was detected in cortical areas of the spleen and in the epithelial layer of the bladder. In the intact, healthy CNS, the expression of GFP was undetectable. We performed a hemilateral administration of lipopolysaccharide (LPS) in the striatum of $CB_2^{eGFP/f/f}$ mice. After one week of survival, mice were anesthetized, perfused and their brains used for morphohistological analysis. A dramatic increase in GFP expression was evident in the lesioned cortex, corpus callosum and striatum, but was unchanged in the non-lesioned side. In a second study, electric foot shock was used to produce pain. Twenty four hours later, significant GFP fluorescence was detected in the vicinity of blood vessels and in the amygdala. We present a novel mouse model that may be useful to characterize the role of cannabinoid CB_2 receptors in a wide variety of pathophysiological processes.

Acknowledgements: Funded by the Research and Education Component of the Advancing a Healthier Wisconsin Endowment at the Medical College of Wisconsin, the Boynton Fund of the Greater Milwaukee Foundation, and the Ministry of Education (PR2009-0169) and Ministry of Economy (SAF2010/16706) of Spain.

NON-PSYCHOACTIVE PHYTOCANNABINOIDS FOR THE TREATMENT OF CANCER ANOREXIA-CACHEXIA SYNDROME: EVIDENCE FOR THE THERAPEUTIC POTENTIAL OF CANNABIGEROL

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Cancer anorexia-cachexia syndrome (CACS) is a multifactorial wasting syndrome for which no standard of care treatment currently exists. Thus, there is an unmet clinical need for compounds which stimulate food intake, attenuate weight loss and improve physical performance. Clinical trials of Δ^9-THC, the main psychoactive phytocannabinoid (pCB), have shown limited clinical efficacy, likely due to dose-limiting psychoactive side-effects (Pacher & Kunos, FEBS J. 280 (2013) 1918-43). Recent pre-clinical studies indicate that non-psychoactive pCBs can stimulate feeding behaviours (Farrimond et al., Psychopharmacology. 223 (2012) 117-29) and may thus have therapeutic potential in the treatment of CACS. In the present study we investigated the non-psychoactive pCB cannabigerol (CBG), in both pure isolate and botanical drug substance (BDS) forms, to determine neuromotor tolerability profiles and ability to stimulate feeding.

Tolerability Profile: Male Lister-hooded rats (200-225g, n=12) were administered CBG pure or BDS (0, 30, 60, 120 mg/kg; *p.o.*) using a counterbalanced, repeated measures design. One hour later animals started a battery of neuromotor tolerability tests: (1) an open field to test gross locomotor activity, (2) a static beam to assess balance and motor control, and (3) grip strength to measure muscular strength. Neither pure CBG nor CBG-BDS elicited significant effects on line cross measures in the open field (p=0.716 & p=0.175) or pass rate on the static beam (p=0.30 & p=0.112). Pure CBG also had no effect on forelimb grip strength (p=0.643), however there was a significant effect of CBG-BDS (p=0.029) with attenuation of grip strength at both 30 and 120 mg/kg (planned comparisons: p=0.013 & p=0.02 respectively).

Feeding Behaviour: Male Lister-hooded rats (200-225g, n=16) were tested using a well-established pre-feed paradigm for the investigation of hyperphagia (Williams et al., Physiol Behav. 65 (1998) 343-6). Using a counterbalanced, repeated measures design, animals were administered CBG pure or BDS (0, 30, 60, 120, 240 mg/kg; *p.o.*) and feeding behaviour was assessed for 2 hours. Both CBG pure and BDS showed significant increases in total food consumed during the test (p=0.006 & p=0.028), resulting from highly significant reductions in the latency to begin feeding (p=0.021 & p=0.009) and increases in the amount of food consumed within meals (p=0.007 & p=0.044).

Both CBG pure and BDS demonstrated favourable tolerability profiles with no impairments in nearly all measures. However, CBG-BDS did attenuate performance in the grip strength test which may indicate a potential clinical concern and requires further investigation. Both CBG pure and BDS significantly stimulated both the appetitive (latency) and consummatory (meal size) components of feeding behaviour, with the more robust effect elicited by pure CBG. Based on these data pure CBG (120-240 mg/kg) will be investigated for efficacy in pre-clinical models of tumour-induced wasting and chemotherapy-induced nausea and vomiting.

Acknowledgements: Jointly funded by GW Pharmaceuticals and the University of Reading Faculty of Life Sciences

RELEASE OF *N*-ACYLETHANOLAMINES BY BIOMATERIALS OF CLINICAL RELEVANCE

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Tissue engineering is considered an attractive field of research, in virtue of its promising potential for recovery and replacement of organ subunits. Targeting tissue regeneration or replacement, a good deal of the literature currently focuses on developing 3D scaffolds using synthetic polymers to reproduce the extracellular matrix (ECM) and provide a good substrate for cell adhesion, proliferation and differentiation. Electrospinning is one of the approaches that allow the fabrication of several synthetic materials into fibrous and porous structures in the micro- and nanometer scale. Moreover, electrospun materials can be functionalized with drugs for localized delivery.

Here, we report the convenient incorporation of *N*-acylethanolamines (NAEs) into an electrospun polyesther scaffold for their sustained release in vitro.
Scaffolds were prepared starting from a 8 wt% poly(ε-caprolactone) (PCL) solution containing [^{14}C]-AEA (0.1% w/w vs. PCL), that was electrospun at 15 kV onto a grounded target placed at 15 cm, with a feed rate of 1.5 mL/h. Scaffold morphology were characterized by Field Emission Scanning Electron Microscopy (FE-SEM). A time-dependence of release of AEA by the scaffold was assessed by measuring the amount of radioactivity associated with the eluates for up to 14 days at 37°C in PBS. Additionally, FAAH enzymatic assay performed in rat brain membrane preparation confirmed that AEA released by the scaffold was still substrate for its main hydrolytic enzyme.

In conclusion, we reported data indicating that electrospun PLC scaffold might be functionalized with AEA without interfering with its chemical structure and might be a good platform for AEA release. Moreover, the reported data encourage the use of biodegradable electrospun polymers as suitable delivery system for AEA long-term administration on 3D cultured cells.

CANNABIDIOL ATTENUATES SCHIZOPHRENIA-LIKE EFFECTS AND CHANGES ON PARVALBUMIN EXPRESSION IN THE MEDIAL PREFRONTAL CORTEX INDUCED BY ANTAGONISM OF NMDA RECEPTORS IN MICE

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Preclinical and clinical data suggest that cannabidiol (CBD), a major non-psychotomimetic constituent of Cannabis sativa, has antipsychotic-like effects. However, the antipsychotic properties of repeated treatment with CBD in laboratory animals have been poorly investigated. Thus, we have evaluated if the repeated treatment with CBD would attenuate the behavioral changes induced by chronic administration of MK-801, an NMDA receptor antagonist, since this treatment is considered a valid animal model to induce psychotic-like signs. In addition, we also evaluated changes in parvalbumin (PV) expression, a calcium-binding protein expressed in a subclass of GABAergic interneurons. Abnormalities in PV(+) neurons are found in schizophrenic patientsand have been related to a hypofunction of NMDA receptor signaling. Methods: Male C57BL/6J mice (6 weeks of age when experiment began) received daily ip injections of MK-801 (0.1, 0.5 or 1 mg/kg) for 14, 21 or 28 days. Twenty-four h after the last injection, the animals were submitted to the prepulse inhibition test (PPI). After that, we investigated if repeated treatment with CBD (15, 30 and 60 mg/kg) would attenuate the impairment induced by chronic treatment (28 days) with MK-801 (1 mg/kg) in PPI. CBD treatment began on the 6th day after the start of MK-801 administration and continued until the end of the treatment. In a third experiment, we investigated if CBD would attenuate the MK-801-induced behavioral changes in the social interaction (SI) and object recognition (OR) tests. Immediately after the PPI, the animals were perfused to evaluate changes in PV expression in the medial prefrontal cortex (mPFC), striatum, nucleus accumbens and hippocampus by immunohistochemistry. MK-801 administration at the dose of 1 mg/kg for 28 days impaired PPI. CBD (30 and 60 mg/kg) attenuated PPI impairment. Repeated treatment with MK-801 also impaired social interaction and object recognition, effects also attenuated by CBD. MK-801 treatment induced a decrease in PV expression in the mPFC. This change was attenuated by CBD. Moreover, CBD by itself did not change the behavioral responses or PV expression. These results indicate that repeated treatment with CBD was able to reverse the psychotomimetic-like effects and to attenuate PV expression changes in the mPFC observed after chronic administration of a NMDA receptor antagonist. The data support the view that CBD may have antipsychotic properties.

Financial support: FAPESP, CNPq

CHARACTERIZATION OF STRUCTURAL REQUIREMENTS FOR POTENT MECHANISM-BASED INHIBITION OF HUMAN CYP1A1 BY CANNABIDIOL

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Our previous study has shown that cannabidiol (CBD) is a potent mechanism-based inhibitor of human cytochrome P450 1A1 (CYP1A1) (Yamaori et al., Biochem. Pharmacol. 79 (2010) 1691-1698). In this study, we investigated the characterization of structural requirements for the inactivating effect of CBD on CYP1A1 using nine CBD-related compounds. A preincubation of olivetol, which corresponds to pentylresorcinol moiety of CBD, exhibited time- and NADPH-dependent enhancement of the inhibition for ethoxyresorfin *O*-deethylase activity of human recombinant CYP1A1. The lack of both phenolic hydroxyl groups (pentylbenzene) or the pentyl side chain (resorcinol) in olivetol abolished the metabolism-dependent CYP1A1 inhibition. These results indicate that the pentylresorcinol structure in CBD plays an important role in CYP1A1 inactivation. In addition, orcinol (methylresorcinol) preserves the ability to inactivate CYP1A1 activity to some extent. Furthermore, the inactivation studies with CBD-2”-monomethylether (CBDM) and CBD-2”, 6”-dimethylether (CBDD) revealed that two free phenolic hydroxyl groups in CBD may be required for the potent CYP1A1 inhibition. The substitution of the pentyl side chain of CBD to a propyl group (cannabidivarin, CBDV) did not significantly affect the ability to inactivate CYP1A1 activity. The preincubation of CBD-hydroxyquinone marginally enhanced CYP1A1 inhibition. The kinetic studies demonstrated that olivetol, CBDM, CBDD, CBDV, and orcinol , as well as CBD (k_{inact} = 0.215 min^{-1}), inactivated CYP1A1 activity; their k_{inact} values were 0.154, 0.0638, 0.0643, 0.226, and 0.0353 min^{-1}, respectively. The present study has indicated that the CYP1A1 inhibition of CBD is chemically based primarily on the presence of the methylresorcinol partial structure.

Acknowledgements: CBDV was generously provided from Dr. Yukihiro Shoyama at Nagasaki International University (Sasebo, Japan). This work was funded by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [Grant-in-Aids for Scientific Research (C) and Young Scientists (B)].

IN VIVO CANNABIDIOL TREATMENT ENHANCES VASORELAXATION TO ACETYLCHOLINE AND SODIUM NITROPRUSSIDE IN ARTERIES FROM ZUCKER DIABETIC RATS

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In vitro incubation of aortae or femoral arteries with cannabidiol (CBD) enhances endothelial function in a model of type 2 diabetes (Zucker Diabetic Fatty (ZDF) rats) (Stanley & Wheal *et al*. 2013). The aim of the present study was to determine if chronic *in vivo* treatment with CBD would have the same results.

Male ZDF rats (n=12) were treated for 7 days (daily i.p. injection) with either 10mg/kg CBD or vehicle (3 ethanol: 1 Tween 80: 16 Saline). Following the treatment period, the rats were killed by a Schedule 1 method. Thoracic aortae and femoral arteries were dissected into rings and mounted on fixed hooks, and mesenteric arteries were mounted on wires in a multi-channel myograph. The arteries were bathed in warmed (37^{o}C) and gassed (95% O_2/5% CO_2) modified Krebs'-Henseleit solution in the myograph, with the femoral and mesenteric arteries set to a resting tension of 4.9mN, and thoracic aortic rings set to 9.81mN. After an equilibration period, the arteries were contracted with methoxamine, then cumulative concentration-response curves to the endothelium-dependent vasorelaxant acetylcholine (ACh, 1nM-30μM) or the nitric oxide donor sodium nitroprusside (SNP, 1nM-30μM) were constructed. Comparison of arterial vasorelaxation between CBD-treated and vehicle-treated groups was performed using two-way ANOVA, with data written as mean ± SEM, and $P<0.05$ taken as significant unless otherwise stated.

The weights and post mortem blood glucose levels of ZDF rats were not different after CBD- or vehicle-treatment ($P>0.05$, Students' unpaired t-test). ACh and SNP caused concentration-dependent vasorelaxations in all arteries. Treatment with CBD significantly enhanced vasorelaxation to ACh in femoral (R_{max}: CBD-treated ZDF = 72.4 ± 3.2 % (n=6); Vehicle-treated ZDF = 67.9 ± 4.0 % (n=6)) and mesenteric arteries (R_{max} CBD-treated ZDF = 98.8 ± 2.4 % (n=6); Vehicle-treated ZDF = 90.9 ± 4.3 % (n=6)), but not in aortae except when also in the presence of indomethacin or L-NAME. In mesenteric arteries, the enhanced vasorelaxation was abolished in the presence of indomethacin, but not by L-NAME treatment. CBD also enhanced sodium nitroprusside vasorelaxations in mesenteric arteries taken from ZDF rats (R_{max} CBD = 97.2 ± 4.3 % (n=5); Vehicle = 85.8 ± 14.2 % (n=6), $P<0.0001$).

In conclusion, *in vivo* treatment with CBD enhances endothelium-dependent and -independent vasorelaxations in ZDF rats. In mesenteric arteries, the augmented vasorelaxation to ACh is likely via a cyclooxygenase-mediated mechanism.

Acknowledgements: This research was funded by Diabetes UK. GW Pharmaceuticals provided the CBD. Thank you to Professor Victoria Chapman for providing us with the tissues.

Stanley C. & Wheal AJ. *et al*. (2013) Eur J Pharmacol 720: 376–382.

TEMPORARY THERAPEUTIC WINDOW OF CANNABIDIOL FOR NEUROPROTECTION AFTER BRAIN HYPOXIA-ISCHEMIA IN NEWBORN MICE

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Background: cannabidiol (CBD) administered to newborn rodents 15 min after a hypoxic-ischemic (HI) insult leads to significant and long-term sustained neuroprotection.
Aim: to determine the temporary therapeutic window (TTW) of CBD, that is how long CBD administration can be delayed after HI without losing its neuroprotective effect. Such TTW is stablished in 6 h for the standard therapy, hypothermia.
Methods: 9-day old C57BL6 mice underwent a HI insult by being exposed to 10% oxygen for 90 min after electrocoagulation under anaesthesia of the left carotid artery. Then, 0.1 mL of vehicle (ethanol:solutol:saline 2:1:17) (HV, n=25) or CBD (1 mg/kg) was administered s.c. 15 min, or 1, 3, 6, 12 or 24 h after the end of the HI insult (HC0.15 n=10; HC1, n=6; HC3, n=7; HC6, n=6; HC12, n=9; HC24, n=9, respectively). Seven days later pups were killed, transcardially perfused with formaline 10% and their brains removed and stored in formaline. Then, the ipsilateral hemisphere volume (IHV) loss was calculated from T2W sequences of brain MRI scan. Then, the brains were processed for conventional (Nissl) staining to assess necrotic by a neuropathological score (NPS, from 0=no damage to 5=massive tissue damage). In addition, TUNEL staining was done to assess apoptotic damage. Non-HI mice served as controls (SHM, n=15).
Results: post-HI administration of CBD induced a neuroprotective effect as observed in all items (Table). The best results were obtained with CBD being administered just after the end of the HI insult. However, the neuroprotective effects were still significant even when CBS administration was delayed up to 12 h after the HI insult. CBD-induced reduction of brain damage did not achieve statistical significance when post-HI CBD administration was delayed 24 h.

Item	***SHM***	***HV***	***HC0.15***	***HC1***	***HC3***	***HC6***	***HC12***	***HC24***
IHV (%)	0	*13.1(1.3)*	*5.1 (1)*	*6.4(1.8)*	*6.6(1.8)*	*6.4(0.9)*	*7.5(1.2)*	***10.2(1.7)***
NPS (pts)	0.5(0.2)	*2.8(0.2)*	1.0(0.4)	1.2(0.5)	*1.7(0.4)*	***1.8(0.5)***	***1.5(0.2)***	***2.2(0.5)***
TUNEL (n/field)	0.4(0.1)	*12.2(3.8)*	0.5(0.1)	*1.2(0.1)*	*2(0.3)*	***3(0.4)***	***3.4(0.6)***	***6.2(2.8)***

Italic: p<0.05 vs SHM. Underlined: p<0.05 vs. HV. **Bold: p<0.05 vs HC0.15**

Conclusions: CBD shows a TTW longer than usual for hypothermia. TTW for CBD seems to be between 12 and 24 h after the end of the HI insult. Supported by FIS PS09/01900, Health Trust South East Norway and GWCRI091190-2

THE EFFECT OF CBD, CBG, AND THCV ON UWB1.289, A HUMAN NON-HORMONE DEPENDANT OVARIAN CARCINOMA CELL LINE

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Our recent studies have shown that phytocannabinoids can induce cytotoxicity in hormone- dependant ovarian carcinoma cell lines (Daniel et al., 2013, 2012; Javid et al., 2013). The aim of the present study was to investigate the potential anti-tumour activity of cannabinoid extracts on non-hormone dependant ovarian tumour cells. UWB1.289 cells were grown and maintained in media containing equal proportions of RPMI 1640 and MEGM supplemented with 3% fetal bovine serum at 37^{o}C, 5% CO_2 .The cells were plated in 96-well culture plates at a density of $1x10^4$ cells/well and allowed to adhere at 37^{o}C for 24 hours. The following day, various doses of CBD, THCV, a combination of both CBG plus THCV (1nM-100 μM) or vehicle were added to the cells and further incubated for 24, 48, 72 and 96 hours. Then the supernatant was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added for 4 hours. The ability of cells to form formazan crystals by active mitochondrial respiration was determined by using a Microplate reader after dissolving the crystals in DMSO. Cytotoxicity was expressed as a relative percentage of the absorbance measured at 540 nm in the control and extract-treated cells. Data were presented as the mean±s.e.mean and analysed using ANOVA followed by Dunnet's t-test; n=4.

μM for CBD, 7.11 μM for THCV and 10.48 μM for CBG in 24 hours, IC50 of 5.68 μM for CBD, 7.5 μM for THCV, 8.86 μM for CBG and in 48 hours, IC50 of 8.767 μM for CBD, 10.11 μM for THCV and 10.34 μM for CBG in 72 hours. IC50 of 3.327 μM for CBD, 5.22 μM for THCV and 5.91 μM for CBG in 96 hours. The application of the vehicle alone did not affect the cells at any time. The data showed that the CBD, THCV and CBG seemed to be inducing a stronger anti-tumour activity in UWB1.289 as compared to the IC50 values of the above pure extracts in hormone-dependant carcinoma cell line, A2780 (Daniel, et al., 2012, 2013). Further experiments are required to investigate the receptor type/subtypes involvement and the mechanism of cell death.

Acknowledgement: We thank GW Pharmaceuticals for providing the extract and fund for consumables.

References: Daniel et al., (2012) Br J Pharmacology;10, issue 4. Daniel et al., (2013) Presentation at ICRS, Vancouver, Canada. Javid et al., (2013). Br J Pharmacology. PA2 in press.

REDUCES INFLAMMATORY AIRWAY IMMUNE CELL ACCUMULATION IN A MOUSE MODEL OF ALLERGIC ASTHMA

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Asthma is a chronic airway disorder characterized by inflammatory immune cell accumulation and airway remodeling leading to bronchial hyperresponsiveness and airway obstruction. Acute-challenge animal models such as the ovalbumin (OVA)-induced allergic asthma, and the staphylococcal enterotoxin B (SEB)–induced asthma, differentially reproduce characteristic immune cell infiltration profiles, a key feature of clinical asthma. In the present study we investigated the effects of CBD on immune cell accumulation and lipid production comparing these two mouse models of airway inflammation.

We detected significant immune cell accumulation in the airways obtained by bronchoalveolar lavage (BAL) of OVA-treated mice. OVA-treated animals showed increased immune cell accumulation (mainly eosinophils) in the BAL, which was significantly reduced by CBD treatment. In addition, CBD treatment modified the immune cell population profile in the BAL of OVA-treated mice, significantly reducing the percent of eosinophils. Analysis of selected lipids using LC/MS/MS revealed significant increases in lung tissue levels of PGE2/PGD2 and linoleoyl glycerol following OVA-induced airway inflammation. SEB-treated animals showed increased BAL immune cell accumulation (mainly lymphocytes). CBD treatment significantly modified the immune cell population profile in the BAL of SEB-treated mice, significantly reducing the percent of lymphocytes and eosinophils. Similarly, lipid analysis revealed significant increases in lung tissue levels of PGE2/PGD2 and linoleoyl glycerol. To summarize, CBD reduces inflammatory immune cell accumulation in a mouse model of allergic asthma.

ACTIVATES GLYCOGEN SYNTHASE KINASE IN NEURONS: EVIDENCE FOR MEDIATION BY CHANGES IN CELLULAR REACTIVE OXYGEN SPECIES

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Glycogen synthase kinase-3 (GSK3) is found throughout the CNS and its over-activity has been linked to bipolar disorder and schizophrenia. Epidemiological studies demonstrate a co-morbidity between *cannabis* use and both schizophrenia and bipolar disorder. The overall hypothesis of this project is that one of the most abundant cannabinoids in *cannabis*, cannabidiol (CBD) affects GSK3 activity and that this contributes to the co-morbidity of *cannabis* use and these disorders. The primary mechanism of regulation of GSK3 occurs via deactivating phosphorylation. Signaling cascades that activate protein kinases PKA, PKB (Akt) and PKC converge and phosphorylate GSK3. Phosphorylation is dynamic; removal of the phosphate occurs via constitutively active protein phosphatases (PP-1 and PP-2), so on-going kinase activity is required to maintain GSK3 in a phosphorylated (and inactive) state.

CBD concentration- and time- dependently decreased GSK3β phosphorylation in cultured rat cerebellar granule neurons (CGN). CBD did not affect either PP-1 or PP-2, suggesting that the mechanism of CBD does involve the removal of phosphates. Phosphorylation of CREB, Akt and ERK1/2 were used to assess the activities of PKA, Akt and PKC, respectively. Twenty-four hour treatment with CBD significantly decreased the phosphorylation of CREB (Ser 133), Akt (Ser 473) and ERK1/2. Thus, the site of action of CBD is up-stream of all of the kinases known to regulate GSK3 phosphorylation.

Previous studies have demonstrated increased production of reactive oxygen species (ROS) activates GSK3 in neuronal and non-neuronal cells; and CBD treatment evokes production of ROS in multiple cell types. Thus, we tested the hypothesis that increased ROS production is required for the effects of CBD to activate GSK3. CBD concentration-dependently produces superoxide measured by dihydroethidium (DHE) staining in CGNs. Pretreatment of the CGN with the antioxidant, N-acetyl-L-cysteine (NAC), significantly inhibits superoxide production and antagonizes CBD-decreased phosphorylation of GSK3, CREB, AKT and ERK1/2, suggesting the activation of these kinases by CBD requires superoxide production. We have begun to explore the molecular target for CBD and find that pertussis-toxin sensitive G proteins are required but that cannabinoid, adenosine and serotonergic receptors are not likely to be involved.

Given the important role of GSK3 in development and neuronal morphology, these data suggest that exposure of susceptible individuals to CBD could produce detrimental effects on the brain through increased neuronal ROS and GSK3 activity.

Supported by NIH grant DA26996 and the Research and Education Component of the Advancing a Healthier Wisconsin Endowment at the Medical College of Wisconsin.

PHARMACOLOGICAL PROPERTIES OF PURE CANNABICHROMENE AND CANNABICHROMENE BOTANICAL DRUG SUBSTANCE

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Cannabichromene (CBC) is produced by CBC synthase activity at cannabigerol and is one of the four major cannabinoids in *C.sativa*. Despite its relative abundance, the pharmacology of CBC has not been studied extensively. CBC has, however, been shown to be an inhibitor of endocannabinoid uptake and a potent activator of TRPA1 channels. In addition, it has been found to display anti-inflammatory, analgesic, antibiotic, anti-cancer and antifungal effects, and also antidepressant activity and the ability to reduce THC intoxication in rodents (Russo, 2011).

The tetrad test for pure CBC (1, 10 and 100 mg/kg) was conducted in mice, with pure THC (100 mg/kg) and WIN55,212-2 (10 mg/kg) as comparison and reference substances respectively (n = 10/group). Effects were compared to vehicle via one-way ANOVA with Dunnett's *post hoc* test. Pure CBC did not affect the number of crossings or the number of rears in the Activity Meter Test, rectal temperature, foot-licking in the Hot Plate Test, or the latency to remove forelimbs in the Bar Test. The plasma concentration of CBC was 592.7 ± 138.9 ng/ml in mice that were treated with 100 mg/kg pure CBC (n = 3).

The activity of CBC at CB_1 and CB_2 receptors was investigated with adult male MF1 mouse brain tissue or CHO cells transfected with human CB_1 or CB_2 receptors, using [^{3}H] CP55,940 radioligand displacement assays and [^{35}S] GTPγS binding assays. Pure CBC appears to bind to the CB_1 receptor in the micromolar range, but does not activate the receptor. Pure CBC also appears to bind to the CB_2 receptor in the micromolar range and causes a slight activation of this receptor (16.01% above basal at 10 μM).

The ability of CBC and CBC botanical drug substance (CBC BDS) to displace radioligands from a total of 76 receptors, channels and enzymes was evaluated. Only compounds that displaced a radioligand by ≥ 50% at 10 μM were further tested in order to obtain IC_{50} and K_i values. Pure CBC was found to bind to the following: opioid μ (MOP) receptor (70% displacement, IC_{50} = 68 μM, K_i = 28 μM) and Na^+ channel (site 2) (53% displacement, IC_{50} = 19 μM, K_i = 17 μM). CBC BDS was found to bind to the following: sigma-1 (σ_1) receptor (52% displacement, IC_{50} = 16 μM, K_i = 13 μM), Na^+ channel (site 2) (55% displacement, IC_{50} = 8.5 μM, K_i = 7.7 μM), noradrenaline transporter (54% displacement, IC_{50} = 7.5 μM, K_i = 2.5 μM) and L type Ca^{2+} channel (dihydropyridine site; 56% displacement, IC_{50} = 8.2 μM, K_i = 6.1 μM).

In summary, pure CBC did not induce THC-like responses in the tetrad test, an observation that is consistent with the data showing that CBC does not activate CB_1 receptors. Pure CBC appears to bind to, and behave as a very low efficacy partial agonist, at CB_2 receptors. Pure CBC also binds to and inhibits an opioid receptor and Na^+ channel, whilst CBC BDS binds to and inhibits a σ receptor, the noradrenaline transporter and calcium and sodium channels.

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EVALUATION OF THE GENOTOXICITY OF CANNABIDIOLIC ACID

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The toxicity of cannabinoids such as cannabidiol (CBD) and Δ^9-tetrahydrocannabivarin (THCV) are well studied, as both candidate cannabinoids are currently progressing through clinical development. However, the safety and toxicity of pure cannabidiolic acid (CBDA) and the corresponding CBDA botanical drug substance (BDS) is not well known. Therefore, we report the results of studies evaluating the potential genotoxicity of pure CBDA and its corresponding BDS in standard genotoxicity assays.

Two models of genotoxicity, as recommended by world-wide regulatory authorities, were employed: *in vitro* bacterial reverse mutation assay (Ames test) and *in vivo* rat micronucleus test (RMT). The Ames test is a rapid, reliable and economical method of evaluating the mutagenic potential of a test article by measuring genetic activity in one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system. Concentrations of Pure CBDA and its corresponding BDS were tested in the Ames assays up to a maximum recommended concentration of 5000 μg ACTIVE/plate, plus negative (vehicle) and positive controls.

The RMT is a test to evaluate the potential of chemicals to induce micronuclei (chromosome fragment which are unable to attach to spindles and are left behind in the erythocytes when the main nucleus is extruded) during chromosomal and cell replication. In two separate studies, pure CBDA and its corresponding BDS, formulated in ethanol, were administered to Wistar rats up to 500 mg/kg/day for pure CBDA and up to 350 mg ACTIVE/kg/day for CBDA BDS (over 2 days) at the maximum tolerated dose (MTD).

In the Ames test, there were no increases in the revertant colonies of *Salmonella typhi* following treatment with CBDA or CBDA BDS that was considered of biological significance. In the *in vivo* RMT study, the only clinical observations were transient salivation (pure CBDA) or ataxia and decreased activity (CBDA BDS) at the highest doses employed. These signs returned to normal by the third day. There were no increases in micronucleus frequencies following administration of CBDA or CBDA BDS over the concurrent vehicle control for any of the groups receiving the test articles. CBDA levels measured in plasma were high following treatment, thus confirming systemic exposure.

In conclusion, there was no evidence of genotoxicity of pure CBDA and its corresponding BDS noted in the standard Ames and RMT assays when tested up to the maximum doses. .

PHARMACOLOGICAL PROPERTIES OF PURE CANNABIGEROL AND CANNABIGEROL BOTANICAL DRUG SUBSTANCE

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Cannabigerol (CBG) is the direct precursor in cannabis of the cannabinoids cannabidiol (CBD), tetrahydrocannabinol (THC) and cannabichromene (CBC). Pure CBG has been shown to have weak activity at cannabinoid (CB) receptors and other components of the endocannabinoid system. CBG has also been reported to activate TRPA1, TRPV1 and TRPV2 and potently block TRPM8 channels.

The pharmacology of pure CBG and CBG botanical drug substance (BDS) at CB_1 and CB_2 receptors was compared using displacement and GTPγS binding assays. The cannabinoid composition of CBG BDS used in the binding studies was (w/w) CBGV 0.4%, CBG 0.1% & CBC 0.1%. The study found that both CBG and CBG BDS had very little affinity or activity at CB_1 and CB_2 receptors.

In further work, the ability of CBG and CBG BDS to displace radioligands from a range of receptors, channels and enzymes was evaluated. Only compounds that displaced a radioligand by at least 50% at 10μM were further tested in order to obtain IC_{50} and K_i values. Pure CBG was found to bind to the following: L type Ca^{2+} channels (dihydropyridine site: 75% displacement, IC_{50} = 3.8μM, K_i = 1.3μM; diltiazem site: 57% displacement, IC_{50} = 5.1μM, K_i = 4.7μM) and Na^+ channels (site 2; 97% displacement, IC_{50} = 500nM, K_i = 450nM). CBG BDS was found to be active at the following: melatonin MT_3 receptors (53% displacement; IC_{50} = 9.9μM, K_i = 9.7μM), L type Ca^{2+} channel (dihydropyridine site; 74% displacement, IC_{50} = 2.7μM, K_i = 910nM) and Na^+ channels (site 2; 87%, IC_{50} = 580nM, K_i = 520nM). Furthermore, pure CBG was able to inhibit uptake of dopamine and serotonin into mouse brain synaptosomes, with effects observed in the nanomolar range.

In a model of drug discrimination, rats were trained to discriminate between vehicle and THC prior to the main study. In this initial study, the vehicle percentage response was 17.0 ± 9.5%. When THC (1, 3 or 10 mg/kg p.o.) was administered 90 minutes before the test session, the THC-lever responding responses were 54.2 ± 13.5%, 99.5 ± 0.2% and 95.5 ± 2.6%, respectively (n=12/group). In the CBG study, the vehicle response was 18.3 ± 10.3%. When CBG (1, 3 and 10 mg/kg p.o.) was administered 90 minutes before the test session, no increase in THC-lever responding was observed compared with vehicle controls; 0.3 ± 0.2%, 1.0 ± 0.4% and 25.2 ± 12.2% respectively (n=11/CBG group).

CBG and CBG BDS (3-100 mg/kg) were evaluated in an Irwin assay to determine behavioral and physiological changes induced by active compound compared to vehicle. Parameters tested included spontaneous activity in home cage and novel cage, reactivity, restlessness, touch-escape response, positional passivity, grooming, startle response, body posture, abdominal tone, abnormal gait and piloerection. Animals were treated with CBG, CBG BDS or vehicle (n=4/group) and observed at the following time points: 0.17-1hr, 1-2hr, 2-3hr, 4-5hr & 24-25hr. Pure CBG caused a slight reduction in spontaneous activity and reactivity only at the 1-2 hr time point, which was not observed at the following 2-3hr time point. Pure CBG and CBG BDS did not alter any other measured parameters at doses of up to 100 mg/kg compared to vehicle group.

Overall CBG and CBG BDS do not appear to have affinity for CB receptors but do bind to calcium and sodium channels and do inhibit synaptosomal uptake of dopamine and 5-HT. Pure CBG has low potential for abuse at doses up to 10 mg/kg and has minimal effects on behaviour up to 100 mg/kg.

THE PHYTOCANNABINOID, Δ^9-TETRAHYDROCANNABIVARIN, CAN ACT THROUGH 5-HT_{1A} RECEPTORS TO PRODUCE ANTI-PSYCHOTIC EFFECTS

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We have reported previously that three phytocannabinoids, cannabigerol (CBG), cannabidiol (CBD) and cannabidiolic acid (CBDA) can interact with serotoninergic 5-HT_{1A} receptors both *in vitro* and *in vivo* [1, 2, 3, 4]. Here, for the first time, we present evidence that another phytocannabinoid, Δ^9-tetrahydrocannabivarin (THCV) shares the ability of CBD, CBDA and CBG to interact with 5-HT_{1A} receptors *in vitro* and also produces apparent anti-psychotic effects in rats that are, at least in part, 5-HT_{1A} receptor-mediated.

Our *in vitro* experiments consisted of (a) 8-[^{3}H]-OH-DPAT displacement and [^{35}S]GTPγS binding assays performed with MF1 mouse whole brain membranes or Sprague Dawley rat brainstem membranes, and (b) 8-[^{3}H]-OH-DPAT dissociation kinetic assays performed with MF1 mouse whole brain membranes. In our *in vivo* experiments, vehicle- and THCV-treated Sprague Dawley rats were subjected to a spontaneous locomotor activity assay as well as to novel object recognition, social interaction and forced swim tests. These rats were all first pre-treated once daily for 7 days with the non-competitive NMDA receptor antagonist, phencyclidine (PCP), and then subjected to a 7-day withdrawal period.

We found that in both mouse whole brain and rat brainstem membranes (a) like the well-established 5-HT_{1A} receptor agonist, 8-OH-DPAT, THCV potently displaced [^{3}H]-8-OH-DPAT from specific binding sites, (b) the displacement produced by THCV was partial in nature, and (c) THCV resembles CBD and CBDA by displaying both an ability, at 100 nM, to produce significant enhancement of 8-OH-DPAT-induced activation of 5-HT_{1A} receptors and a bell-shaped dose-response curve for the production of this effect. THCV (100 nM) did not significantly affect the rate of dissociation of [^{3}H]-8-OH-DPAT from specific binding sites in mouse brain membranes. In PCP-treated rats, THCV shared the ability of clozapine to (a) antagonize stereotyped behaviour, (b) reduce the amount of time spent immobile in the forced swim test and (c) normalize hyperlocomotor activity, social behaviour and cognitive performance. The 5-HT_{1A} receptor antagonist, WAY100635, abolished the ability of THCV to modify PCP-induced stereotyped and social behaviour and partially reduced the suppressant effect of THCV on PCP-induced cognitive deficiency in the novel object recognition test, suggesting that some of the above apparent anti-psychotic effects of THCV are 5-HT_{1A} receptor mediated. We conclude that THCV shows therapeutic potential for ameliorating some of the negative, cognitive and affective symptoms of schizophrenia.

Acknowledgements: Funded by GW Pharmaceuticals.

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THE PHYTOCANNABINOID CANNABIDIVARIN DEMONSTRATES NOTABLE ANTIEPILEPTIC PROPERTIES AND IS A GENUINE CANDIDATE FOR THE TREATMENT OF TEMPORAL LOBE EPILEPSY

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Epilepsy is a chronic and debilitating neurological condition characterised by recurrent seizures; 50 million people worldwide are affected. Temporal lobe epilepsy (TLE) is the most common epilepsy and one of the most refractory to existing medicines; ≥30% of patients are unresponsive to antiepileptic drugs (AEDs) while 20% have limited seizure control. Currently available AEDs have significant side-effects, adversely affecting alertness, motor control and cognition, making more efficacious and better tolerated AEDs an urgent requirement. Growing evidence from pre-clinical research on cannabis-derived compounds, and self-medication by epilepsy patients with cannabis, support the study of phytocannabinoids as new AEDs.

Here, the antiepileptic and tolerability profiles of a non-psychoactive phytocannabinoid, cannabidivarin (CBDV) were assessed. The repeated low dose lithium pilocarpine model of TLE was used to induce spontaneous recurrent seizures in male Wistar rats. Animals were treated with 200 mg/kg/day CBDV for 12 weeks and effects on seizure frequency and severity were behaviourally assessed daily for 3 weeks. CBDV significantly reduced both severe seizure incidence and seizure index (seizure severity x seizure frequency). Tolerability was assessed using static beam, grip strength and inclined screen tasks, where CBDV produced no motor function deficits or neurotoxicity. CBDV also attenuated motor function deficits observed in epileptic animals, further supporting its antiepileptic effects. Finally, electrocorticography (ECoG) recordings were used to assess the effects of acute CBDV administration (200 mg/kg) on baseline (non-seizure) neuronal activity of epileptic and non-epileptic animals. CBDV exerted few effects upon the power spectra of signals recorded from hippocampus and parietal cortex, further supporting CBDV's tolerability.

In conclusion, CBDV has significant antiepileptic properties and is extremely well tolerated, supporting CBDV as a genuine candidate for the treatment of human TLE.

PHARMACOLOGICAL PROPERTIES, PHARMACOKINETICS AND BIOAVAILABILITY OF PURE CANNABIDIOLIC ACID AND CANNABIDIOLIC ACID BOTANICAL DRUG SUBSTANCE

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Cannabidiolic acid (CBDA) has weak activity at CB_1 and CB_2 receptors, and components of the endocannabinoid system. CBDA has also been reported to desensitise TRPA1, TRPM8 and TRPV4 channels.

Here, the ability of pure CBDA and CBDA botanical drug substance (BDS) to displace radioligands from a range of receptors, channels and enzymes was evaluated; these were non-cannabinoid and non-TRP targets. Only compounds that displaced a radioligand at 10 μM were further investigated in order to obtain IC_{50} and K_i. Pure CBDA was found to bind to 5-HT_{5a} (IC_{50} 15 μM, K_i 750 nM). CBDA BDS was unable to displace any of the standard ligands.

In a separate investigation, plasma and brain samples were obtained from male Wistar rats to provide information on the concentrations of pure CBDA and CBDA BDS, derive pharmacokinetic (PK) data and evaluate absolute/relative cannabinoid bioavailabilities where possible. Pure CBDA was dosed by the intravenous (IV), oral (PO) and intraperitoneal (IP) routes and CBDA BDS by the PO and IP routes only. Pure CBDA and CBDA BDS were administered in a 1:1:18 ratio of ethanol, cremophor EL and physiological saline (0.9 % NaCl) as either a single bolus dose or repeatedly over a five day period at intervals of approximately 24 hours. In this study, the systemic bioavailability of CBDA following a single administration, ranked highest to lowest was as follows: pure CBDA, IP, 10 mg/kg (80 %); CBDA BDS, IP, 10 mg/kg (74 %*); pure CBDA, IP, 1 mg/kg (62 %); pure CBDA, IP, 0.1 mg/kg (36 %); CBDA BDS, PO, 10 mg/kg (36 %*); pure CBDA, PO, 10 mg/kg (19 %). Whereas, following repeated daily administration, the systemic bioavailability of CBDA, ranked highest to lowest was: pure CBDA, IP, 10 mg/kg (145 %); CBDA BDS, IP, 10 mg/kg (127 %*); pure CBDA, IP, 1 mg/kg (70 %); CBDA BDS, PO, 10 mg/kg (60 %*); pure CBDA, PO, 10 mg/kg (39 %); pure CBDA, IP, 0.1 mg/kg (7 %). The relative (*) and absolute oral bioavailability values were calculated using the IV dosed pure CBDA area under the curve (AUC_{0-t}) data, demonstrating high systemic exposure via the IP and PO routes.

In conclusion, neither pure CBDA nor CBDA BDS (at 10 μM) bind to many other traditional receptor targets with any great affinity in this screen. Also, PK and bioavailability data demonstrate good bioavailability for repeated and single doses of pure CBDA and CBDA BDS at 10 mg/kg.

Acknowledgements: Funded by GW Pharmaceuticals and Otsuka Pharmaceuticals

THE NON-PSYCHOTROPIC PLANT CANNABINOIDS, CANNABIDIVARIN (CBDV) AND CANNABIDIOL (CBD), ACTIVATE AND DESENSITIZE TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1) CHANNELS *IN VITRO*: POTENTIAL FOR THE TREATMENT OF NEURONAL HYPEREXCITABILITY

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Epilepsy is the most common neurological disorder in humans, with over 50 million people worldwide affected. Recent evidence suggests that the transient receptor potential cation channel subfamily V member 1 (TRPV1) may contribute to the onset and progression of some forms of epilepsy. Since the two non-psychotropic cannabinoids cannabidivarin (CBDV) and cannabidiol (CBD) exert anticonvulsant activity *in vivo* and produce TRPV1-mediated intracellular calcium elevation *in vitro*, we evaluated the effects of these two natural products on TRPV1 channel activation and desensitization and in an *in vitro* model of epileptiform activity.

Patch clamp analysis in transfected HEK293 cells demonstrated that CBD and CBDV dose-dependently (1-30 μM) activate and rapidly desensitize rat TRPV1, as well as rat TRP channels of subfamily V type 2 (TRPV2) and subfamily A type 1 (TRPA1). TRPV1 and TRPV2 transcripts were shown to be expressed in rat hippocampal slices by quantitative PCR, whereas TRPA1 was at the limit of detection.

When tested on epileptiform neuronal spike activity in such slices exposed to a Mg^{2+}-free solution using multi electrode arrays (MEAs), CBDV (10 μM) reduced both epileptiform burst amplitude and duration. The prototypical TRPV1 agonist, capsaicin (10 μM), produced similar, although not identical effects. CBDV effects were not always sensitive to IRTX (1 μM), a selective TRPV1 antagonist. These data suggest that CBDV anti-epileptiform effects in the Mg^{2+}-free model, are not uniquely mediated via activation of TRPV1.

As assessed by means of western blot analyses using a polyclonal antibody against its phosphorylated form, TRPV1 was strongly phosphorylated, and hence likely sensitized by capsaicin (10 μM) and CBDV (10 μM), but not IRTX (1 μM), in control hippocampal slices. An increase of TRPV1 phosphorylation was observed in hippocampal slices exposed to Mg^{2+} free solution. Interestingly, in this experimental condition, both capsaicin (10 μM) and CBDV (10 μM) caused instead dephosphorylation of TRPV1, consistent with its possible desensitization.

We propose that the fast desensitization of tonically activated TRPV1 determined by agonists and CBD or CBDV, could open a new therapeutic opportunity to treat neurological disorders caused by an excess of neuronal activity such as epilepsy, and that CBDV effects on TRP channels should be next assessed also in the context of *in vivo* models of epilepsy. **Acknowledgment**: Funded by GW Pharmaceuticals.

INFLUENCE OF HEPATIC METABOLISM OF Δ^9-TETRAHYDROCANNABIVARIN (THCV) AND CANNABIDIOL (CBD) ON THEIR ANTI-STEATOTIC ACTIONS *IN VITRO*

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The liver plays key roles in regulating total body energy homeostasis and its ability to do so is greatly affected by the occurrence of pathological conditions such as hepatosteatosis or non-alcoholic fatty liver disease (NAFLD), which contributes to hepatic insulin resistance, metabolic syndrome and potentially end-stage liver disease-related mortality. Triglyceride (TG) accumulation in hepatocytes of steatotic livers results from the incorporation of adipose-derived plasma free fatty acids as well as de novo biosynthesis. Recent reports indicate that the cannabinoids THCV and CBD can protect the liver in a various models of hepatosteatosis [1] [2]. Given that THCV and CBD are metabolized within the liver, resulting in the formation of the metabolites 11-OH-THCV and 7-OH-CBD (among others), respectively, we tested the ability of purified THCV, CBD, 11-OH-THCV and 7-OH-CBD to modulate intracellular TG levels directly in an *in vitro* hepatosteatosis model in hepatocytes, as well as in mature adipocytes. Additionally, given that TCHV is also able to inhibit the development of insulin-insensitivity in HHL5 hepatocytes [1], we determined if 11-OH-THCV has the same effect.

TG accumulation was induced in the HHL5 cell line of human hepatocytes by exposure to 100 μM oleic acid for 24 hours. Mature adipoctyes were derived from mouse 3T3-L1 cells. TG levels were quantified in each through the use us a commercially available Nile Red preparation to determine the effects of exposure to the cannabinoids at various concentrations. Assessment of insulin sensitivity was performed through Western blot analysis of insulin-induced AKT activation (phosphorylation) in HHL5 cells exposed to 200 μM palmitic acid to induce insulin resistance.

As shown previously [3], our results indicate that both THCV (5-20 μM) and CBD (2.5-20 μM) are able to markedly decrease intracellular TG levels in HHL5 cells exposed to 100 μM oleic acid for 24 hours. We found that while 11-OH-THCV (up to 20 μM) had no lipid lowering capability, the reverse was true for 7-OH-CBD (2.5-20 μM), which in fact was more potent than CBD at the higher concentrations tested. In mature 3T3-L1 adipocytes, as in hepatocytes, THCV (10 μM), CBD (5, 10 μM) and 7-OH-CBD (2.5, 5, 10 μM) all decreased intracellular TG stores upon 48 hr exposure, while, again, 11-OH-THCV (up to 10 μM) had no effect. Finally, in insulin-resistant HHL5 cells, while THCV (3 μM) re-sensitized cells to insulin, low concentrations of 11-OH-THCV (0.3, 1 μM) tended to slightly enhance insulin resistance and no effect was observed at the highest concentration (3 μM) tested.

These data highlight the differences that metabolism of THCV and CBD has on their lipid-lowering capacity and provide in vitro evidence that THCV, CBD and 7-OH-CBD hold promise as potential therapies for fatty liver disease and diabetes. The potential molecular targets through which these compounds exert their actions in hepatocytes do not include cannabinoid receptors and are currently under investigation.

Acknowledgment: This work was generously supported by GW Pharmaceuticals

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ADDITIVE NEUROPROTECTIVE EFFECT OF CANNABIDIOL AND HYPOTHERMIA IN HYPOXIC-ISCHEMIC PIGLETS

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Background: The proportion of newborns beneficed from hypothermia (HT) after brain hypoxia-ischemia (HI) is limited. Thus, looking for synergistic therapies is warranted. Cannabidiol (CBD) has shown neuroprotective effects in animal models of newborn HI brain damage.

Objective: To test the possible additive neuroprotective effects of CBD and HT.

Design/Methods: Sedated and ventilated piglets (1-2 day-old) underwent HI brain damage (hypoxia -FiO2 10%- + bilateral carotid artery compression for 30 min). Then, normothermic (NT) piglets were maintained at 37-38 C using a warmed air blanket; HT piglets were cooled by a cool water mattress to 33-34 ºC. Thirty min after HI piglets received i.v. vehicle (VEH) or CBD 1 mg/kg. Six hours after HI brains were obtained for histological studies quantifying the number of neurons (Nissl) and astrocytes (GFAP) in parietal cortex, for proton magnetic resonance spectroscopy (H-MRS) to quantify Lac/NAA (neuronal damage), Glu/NAA (excitotoxicity) and GSH/Cr (oxidative stress) ratios and for Westerblot studies quantifying protein nitrosylation (Oxyblot, oxidative stress) and caspase-3 (apoptosis) and TNFα (inflammation) expression. Similarly studied animals without HI insult served as controls (SHM).

Results: Neuroprotection was CBD+HT>VEH+HT=CBD+NT>VEH+NT in terms of reduction of neuronal and astroglial death, apoptosis and Lac/NAA ratio increase (Table). Either CBD or HT similarly modulated HI-induced excitotoxicity, inflammation and oxidative stress, with both therapies together showing additive effects.

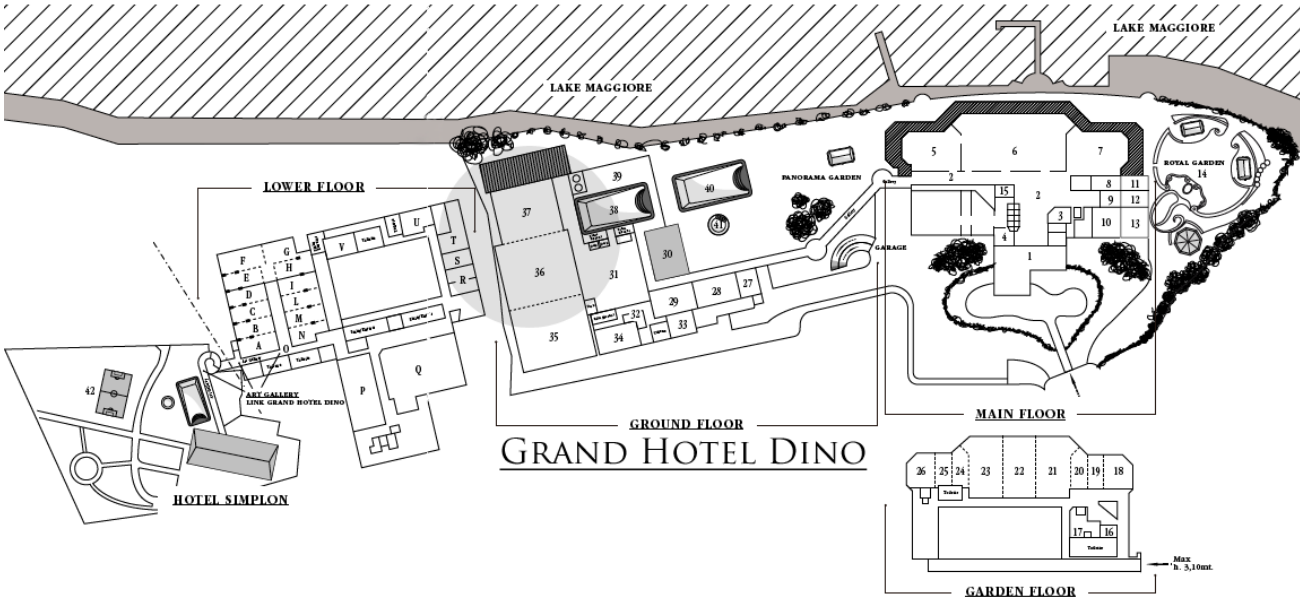
Parameter	SHM+NT	VEH+NT	CBD+NT	SHM+HT	VEH+HT	CBD+HT
Histology						
Necrotic neurons (%)	4.7(1.3)	*25.5(4.8)*	***11.8(3.2)***	1(0.1)	*13.6(1.3)*	**3.7(0.9)**
Astrocytes (n)	33(3.2)	31.1(2.2)	***39.4(3.8)***	33.4(1.3)	*23.8(0.6)*	**34.3(1.5)**
H-MRS						
Lac/NAA	2.4(0.2)	*6.6(2.3)*	**2.9(0.3)**	1.4(0.1)	*2.3(0.1)*	**1.6(0.1)**
Glu/NAA	0.51(.02)	*0.61(0.04)*	**0.49(0.03)**	0.42(0.02)	*0.45(0.02)*	**0.39(0.01)**
GSH/Cr	0.17(0.01)	*0.11(0.01)*	**0.17(0.01)**	0.16(0.01)	*0.14(0.01)*	**0.16(0.01)**
Westernblot						
OxyBlot	1.9(0.2)	*2.9(0.3)*	**2.2(0.2)**	1.7(0.1)	*2.3(0.2)*	**1.9(0.1)**
Caspase 3	0.19(0.01)	*0.35(0.02)*	***0.25(0.02)***	0.09(0.01)	*0.15(0.01)*	**0.11(0.01)**
TNFα	0.07(0.02)	*0.17(0.01)*	***0.14(0.01)***	0.03(0.02)	*0.10(0.01)*	**0.08(0.01)**

Mean(SEM). Lac: lactate. NAA: n-acylaspartate. Glu: glutamate. GSH: reduced glutathione. (*Italic*) P<0.05 vs SHM; (**Bold**) p<0.05 vs VEH

Conclusions: CBD is at least as efficient as neuroprotectant as HT, both therapies showing additive neuroprotective effects.

Supported by FIS PI09/01900, PI12/00192 and PI12/00852, and GWCRI09119.

Map of Zacchera Hotels, Baveno, italy



Baveno ICRS2014

MAIN FLOOR

1 . Entrance
2 . Lobby
3 . Reception
4 . Internet corner
5 . Restaurant Panorama
6 . Restaurant 3 isole
7 . Main Bar
8 . Executive (TV Room)
9 . VIP
10 . President
11 . Royal A
12 . Royal B
13 . Royal C
14 . Royal Garden
15 . Luggage Room

GARDEN FLOOR

16 . Technical Room
17 . Cloakroom
18 . Titano
19 . Saturno
20 . Nettuno
21 . Venere
22 . Marte
23 . Giove
24 . Minerva
25 . Plutone
26 . Urano

GROUND FLOOR

27 . Camino
28 . Birreria
29 . Rustico
30 . Winter Garden
31 . Hall & Bar
32 . Business Office
33 . Rosa
34 . Galassia
35 . Carlo 1
36 . Carlo 2
37 . Carlo 3
38 . Swimming Pool
39 . Relax Area & Jacuzzi
40 . Outside swimming Pool
41 . Children swimming Pool
42 . Soccer

LOWER FLOOR

Planetario A
Planetario B
Planetario C
Planetario D
Planetario E
Planetario F
Planetario G
Planetario H
Planetario I
Planetario L
Planetario M
Planetario N
O . Gallery Hotel Simplon
P . Play room
Q . Fitness room
R . Massage room
S . Solarium
T . Turkish bath
U . Sauna Lady
V . Sauna Gentlemen

ISBN 978-0-9892885-1-4