22ND ANNUAL

SYMPOSIUM

OF THE

INTERNATIONAL CANNABINOID RESEARCH SOCIETY

KONZERTHAUS FREIBURG

FREIBURG IM BREISGAU

GERMANY

JULY 22 - 27, 2012

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SYMPOSIUM OF THE

INTERNATIONAL CANNABINOID RESEARCH SOCIETY

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PROGRAMME AND ABSTRACTS

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Registration: July 22nd, 2012 (16.00 – 19.00)

Welcome Reception: 18.30 – 20.00

Day 1
Monday, July 23rd

7.30	**Breakfast**		
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9.15	Anne-Caroline Schmöle, Daniele Bano, Pierluigi Nicotera, Andreas Zimmer and Judith Alferink	THE ROLE OF THE ENDOCANNABINOID RECEPTOR CB2 IN NEURODEGENERATION	3
9.30	Ryan Graves, Gareth Pryce, Michaela Egertová, Benjamin F. Cravatt, Gavin Giovannoni, Gregory J. Michael, Maurice R. Elphick and David Baker	EFFECTS OF GENETIC DELETION AND PHARMACOLOGICAL INHIBITION OF FATTY ACID AMIDE HYDROLASE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS	4

9.45	Nephi Stella	TARGETING CB1 RECEPTORS AND ABHD6 TO TREAT R6/2 MICE SYMPTOMS	5
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10.15	Jacqueline L. Blankman, Jonathan Z. Long and Benjamin F. Cravatt	GENERATION AND CHARACTERIZATION OF ABHD12–/– MICE, A MODEL OF THE NEURODEGENERATIVE DISEASE PHARC	7
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11.15	Matthew N. Hill, Nicole P. Bowles, Tiffany T. Lee Cecilia J. Hillard and Bruce S. McEwen	ACTIVATION OF HYPOTHALAMIC FATTY ACID AMIDE HYDROLASE BY LEPTIN: REGULATION OF FOOD INTAKE AND EFFECTS OF DIET-INDUCED OBESITY	9
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12.00	William H Hind and Saoirse E O'Sullivan	THE EFFECTS OF ANANDAMIDE ON PERMEABILITY IN A CELL CULTURE MODEL OF THE BLOOD-BRAIN BARRIER	12
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18.00	**PRESIDENTIAL PLENARY SPEAKER** "PLANNING RESEARCH FOR THE NEXT HALF A CENTURY" **RAPHAEL MECHOULAM, PH.D.** *Institute for Drug Research* *Hebrew University of Jerusalem*		

Day 2
Tuesday, July 24th

7.30	**Breakfast**		
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8.30	Mark Bauer, Andrea Chicca, Stefanie Hofer-Reyes, Marco Tamborrini, David Eisen, Oliver Poetz, Gerd Pluschke and Jürg Gertsch	IDENTIFICATION OF A FAMILY OF PEPTIDE ENDOCANNABINOIDS (PEPCANS): EVIDENCE FOR ALLOSTERIC MODULATION OF CB1 RECEPTORS	21
8.45	Maria Grazia Cascio, Daniele Bolognini and Roger G. Pertwee	THE CB1 RECEPTOR ALLOSTERIC MODULATOR, ORG 27569, HAS DIFFERENT EFFECTS ON THE POTENCIES WITH WHICH CP55940, O-2050 AND SR141716A DISPLACE [3H]CP55940 OR [3H]SR141716A FROM MOUSE BRAIN CB1 RECEPTORS	22
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9.15	Michelle Glass, Erin Cawston, Courtney Breen, William Redmond and Mark Connor	ALLOSTERIC MODULATION OF HUMAN CB1 RECEPTORS	24
9.30	Eugen Brailoiu, G. Cristina Brailiou, Elena Deliu, Haleli Sharir, Pingwei Zhao and Mary E. Abood	FUNCTIONAL INTRACELLULAR CANNABINOID RECEPTORS	25

9.45	Lawrence C. Blume, Caroline E. Bass, George D. Dalton, Dana E. Selley and Allyn C. Howlett	CANNABINOID RECEPTOR INTERACTING PROTEIN (CRIP1A) MODULATES CB1 RECEPTOR TRAFFICKING, ACTIVITY AND SIGNALING	26
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10.15	Heather B. Bradshaw, Siham Raboune and Jordyn M. Stuart	21 NOVEL N-ACYL AMIDES, INCLUDING N-DOCOSAHEXEANOYL ETHANOLAMINE, HAVE ACTIVITY AT TRPV1-4 THE PUTATIVE IONOTROPIC CANNABINOID RECEPTORS	28
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10.45	**COFFEE BREAK**		
ORAL SESSION 5. GASTROINTESTINAL REGULATION *CHAIRS*: STEVEN KINSEY AND MARIO VAN DER STELT			
11.00	Steven G. Kinsey, Erica C. Cole and Aron H. Lichtman	REMARKABLE POTENCY OF Δ^9-THC IN BLOCKING GASTRIC HEMORRHAGES CAUSED BY CYCLOOXYGENASE INHIBITION IN MICE	30
11.15	Mohammad Bashashati, Yasmin Nasser, Catherine M. Keenan, Winnie Ho, Fabiana Piscitelli, Giorgio Ortar, Ken Mackie, Martin A. Storr, Vincenzo Di Marzo and Keith A. Sharkey	THE LOCALIZATION AND FUNCTION OF DIACYLGLYCEROL LIPASE (DAGL) IN THE GASTROINTESTINAL TRACT	31
11.30	Luan Koay, Rachael Rigby and Karen Wright	CANNABIDIOL INDUCES NON-CANONICAL AUTOPHAGY	32

11.45	**KANG TSOU MEMORIAL LECTURE** "DO DIFFERENTIATED NEURONS MIGRATE?" ***MICHAEL FROTSCHER, M.D.*** *Institute for Structural Neurobiology* *Center for Molecular Neurobiology (ZMNH)* *Universitätsklinikum Hamburg-Eppendorf (UKE)* *Hamburg*		
12.45-14.00	NIDA TRAINEE LUNCH AND LEARN "CANNABINOID PHARMACEUTICS: HISTORICAL INFLUENCES ON MILLENNIAL RESEARCH" JOHN MCPARTLAND AND ALLYN HOWLETT		
12.45-14.00	**LUNCH**		
ORAL SESSION 6. PAIN *CHAIRS*: KATARZYNA STAROWICZ AND ANDREA HOHMANN			
14.00	Katarzyna Starowicz, Wioletta Makuch, Michal Korostynski, Michal Slezak, Magdalena Zychowska, Stefania Petrosino, Luciano De Petrocellis, Luigia Cristino, Barbara Przewlocka and Vincenzo Di Marzo	FULL INHIBITION OF SPINAL FATTY ACID AMIDE HYDROLASE IN NEUROPATHIC RATS LEADS TO TRPV1-MEDIATED ANALGESIC EFFECTS VIA REMODELING OF THE ENDOCANNABINOID SYSTEM	33
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15.15	Bogna M. Ignatowska-Jankowska, Sudeshna Gosh, Micah J. Niphakis, Scott O'Neal, D. Matthew Walentiny, Jenny L. Wiley, Benjamin F. Cravatt, and Aron H. Lichtman	THE SELECTIVE MONOACYLGLYCEROL LIPASE INHIBITOR KML29 PRODUCES ANTINOCICEPTION IN THE ABSENCE OF CANNABIMIMETIC EFFECTS	38
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16.00-18.00	**POSTER SESSION 2** **COFFEE**		P2
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19.00	István Ujváry, Alessia Ligresti, Rosaria Villano, Marco Allarà and Vincenzo Di Marzo	YANGONIN, A KAVALACTONE FROM *PIPER METHYSTICUM,* IS A NOVEL CB1 RECEPTOR LIGAND	45

Day 3
Wednesday, July 25th

7.30	**Breakfast**		
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8.30	Ryan Vandrey, Maxine L. Stitzer, Miriam Z. Mintzer, Marilyn A. Huestis, Jeannie A. Murray and Dayong Lee	THE DOSE EFFECTS OF DRONABINOL (ORAL THC) IN HEAVY CANNABIS USERS	46
8.45	Margaret Haney, Gillinder Bedi, Ziva D. Cooper, Andrew Glass and Richard W. Foltin	PREDICTORS OF MARIJUANA RELAPSE: ROBUST IMPACT OF CIGARETTE SMOKING	47
9.00	Matthew W. Buczynski, Ilham Y. Polis and Loren H. Parsons	THE VOLITIONAL NATURE OF NICOTINE EXPOSURE DIFFERENTIALLY ALTERS ANANDAMIDE AND OLEOYLETHANOLAMIDE LEVELS IN THE VENTRAL TEGMENTAL AREA	48
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9.45	Jenny L. Wiley, Timothy W. Lefever and Julie A. Marusich	PHARMACOLOGICAL PROFILE OF ABUSED INDOLE-DERIVED CANNABINOIDS CONTAINED IN 'HERBAL INCENSE' IN MICE	51

10.00	Miriam Schneider, Chris M. Friemel and Andreas Zimmer	HEDONIC REWARD PROCESSING IS ATTENUATED IN CB1 RECEPTOR KNOCKOUT MICE	52
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10.30	Zheng-Xiong Xi, Xiao-Qing Peng, Xia Li, Hong-Ju Yang, Jie Li and Eliot L. Gardner	CANNABINOID CB2 RECEPTORS MODULATE COCAINE SELF-ADMINISTRATION IN RATS	54
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14.00	**Excursion to the Black Forest**		

Notes:

Day 4
Thursday, July 26th

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8.20	**NIDA Symposium** Role of Cannabinoid System in Nicotine Addiction Organizers: Vishnudutt Purohit and Rao Rapaka Discussants: Ben Cravatt and Aron Lichtman	
8.30	Sherry Niessen *Scripps Research Institute* *La Jolla, CA USA*	PROTEOMICS OF NICOTINE-DEPENDENT BRAIN
8.55	Daniele Piomelli *University of California* *Irvine, CA USA*	FAAH INHIBITORS AS POTENTIAL LEADS FOR SMOKING CESSATION
9.20	Aron Lichtman *Virginia Commonwealth University* *Richmond, VA USA*	MODULATING NICOTINE REWARD AND DEPENDENCE THROUGH THE ENDOGENOUS CANNABINOID SYSTEM
9.45	Rafael Maldonado *Universitat Pompeu Fabra* *Barcelona, Spain*	TARGETING THE CB1 RECEPTOR TO TREAT NICOTINE ADDICTION
10.10	Commentary and Discussion	
10.35	**Coffee Break**	
11.00	**Young Investigator Awardee Speaker** "Interplay of endocannabinoids and plant derived cannabinoids with inflammation, oxidative stress and cell death: Implications for tissue injury and protection" **Pal Pacher, M.D., Ph.D.** *Laboratory of Physiological Studies* *National Institute on Alcohol Abuse and Alcoholism,* *Bethesda, MD USA*	

12.00	**LUNCH**		
ORAL SESSION 9. ENDOCANNABINOID SYNTHESIS / CATABOLISM *CHAIRS:* DANIEL NOMURA AND NATSUO UEDA			
13.15	Toru Uyama, Natsuki Ikematsu, Manami Inoue, Naoki Shinohara, Xing-Hua Jin, Kazuhito Tsuboi, Takeharu Tonai, Akira Tokumura and Natsuo Ueda	GENERATION OF N-ACYLPHOSPHATIDYLETHANOLAMINE BY MEMBERS OF PHOSPHOLIPASE A/ACYLTRANSFERASE (PLA/AT) FAMILY	55
13.30	Bela Szabo and Mario Lederer	CALCIUM RELEASE FROM THE ENDOPLASMIC RETICULUM CONTRIBUTES TO TRIGGERING ENDOCANNABINOID PRODUCTION IN CEREBELLAR PURKINJE CELLS	56
13.45	Nolan D. Hartley, Daniel J. Hermanson, Lawrence J. Marnett and Sachin Patel	SUBSTRATE SELECTIVE INHIBITION OF COX-2 AS A NOVEL STRATEGY FOR *IN VIVO* ENDOCANNABINOID AUGMENTATION	57
14.00	Melissa V. Turman, Daniel Hermanson, Jeffery J. Prusakiewicz, Philip J. Kingsley, Andrews S. Felts, Kelsey C. Duggan, Sachin Patel and Lawrence J. Marnett	ACETAMINOPHEN AND ITS METABOLITES, 4-AMINOPHENOL AND AM-404, ARE SUBSTRATE-SELECTIVE INHIBITORS OF ENDOCANNABINOID OXYGENATION BY CYCLOOXYGENASE-2	58
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14.30	Attila Oláh, Balázs I. Tóth, Attila G. Szöllősi, Lídia Ambrus, Christos C. Zouboulis and Tamás Bíró	FATTY ACID AMIDE HYDROLASE INHIBITORS EXERT COMPLEX ANTI-ACNE ACTIONS IN HUMAN SEBOCYTES	60

14.45	Julian Romero, Carolyn Tanner, Richard Lennertz, Amanda Smith, Cheryl L. Stucky and Cecilia J. Hillard	LONG-TERM CHANGES IN FATTY ACID AMIDE HYDROLASE KNOCKOUT MICE: INVOLVEMENT OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 RECEPTOR	61
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15.45	William T. Berger, Brian P. Ralph, Jing Sun, Martin Kaczocha, Trent E. Balius, Robert C. Rizzo, Iwao Ojima and Dale G. Deutsch	VIRTUAL SCREENING FOR INHIBITORS OF THE ANANDAMIDE TRANSPORTER (FATTY ACID BINDING PROTEINS)	63
16.00	Carmen Vázquez, Marta Moreno, Lourdes Ruiz-Valdepeñas, Elena Tomás, Samuel Ruiz de Martín, Rosa María Tolón and Julián Romero	FATTY ACID AMIDE HYDROLASE DELETION POTENTIATES *IN VIVO* GLIAL ACTIVITY IN RESPONSE TO ACUTE BRAIN INJURY	64
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16.25	Mauro Maccarrone, Mariangela Pucci, Sara Di Siena, Daniele Di Giacomo, Valentina Pirazzi, Raffaele Geremia and Paola Grimaldi	REGULATION OF THE FAAH GENE BY ESTROGEN ENGAGES HISTONE DEMETHYLASE LSD1	65

16.40	Tiziana Rubino, Pamela Prini, Erica Zamberletti, Simona Speziali and Daniela Parolaro	THE ENDOCANNABINOID SYSTEM PLAYS A ROLE IN NEURONAL REFINEMENT OCCURRING IN THE ADOLESCENT PREFRONTAL CORTEX	66
16.55	Chris M. Friemel, Rainer Spanagel and Miriam Schneider	ACUTE AND CHRONIC CANNABINOID TREATMENT DIFFERENTLY AFFECT ETHANOL INTAKE IN PUBERTAL AND ADULT RATS	67
17.10	Sangeetha Gajendra, Madeleine Oudin, Patrick Doherty and Giovanna Lalli	A CANNABINOID-RALA SIGNALLING PATHWAY CONTROLLING NEURAL PROGENITOR MIGRATION	68
17.25	Sharon Anavi-Goffer, Merav Hajby, Dorian Solomon, Alex Naftaly, Orit Karminsky, Raphael Mechoulam, Iain R. Greig, Ruth A. Ross, Peter McCaffery and Kirsty Shearer	ALTERATIONS IN CANNABINOID SIGNALLING CONTRIBUTE TO ADHD-LIKE SYMPTOMS AT ADULTHOOD	69
17.40-18.40	ICRS BUSINESS MEETING		
19.00–23.00	**ICRS BANQUET** WITH ***LIFETIME ACHIEVEMENT AWARDEE SPEAKER*** **" CANNABINOID RESEARCH AND NIDA: AN UPDATE"** ***RAO RAPAKA, PH.D.*** ***National Institute on Drug Abuse*** ***Bethesda, MD USA***		

DEPARTURE: **FRIDAY, JULY 27TH**

POSTER SESSION 1: TOPICS A - D
DAY 1, MONDAY, JULY 23RD: 15:45 – 17:45

TOPIC A. NEURODEGENERATION

Robert B Laprairie, Melanie E M Kelly and Eileen M Denovan-Wright	TYPE 1 CANNABINOID RECEPTOR (CB1)-MEDIATED INDUCTION OF CB1 EXPRESSION IN CELLULAR MODELS OF HUNTINGTON'S DISEASE	P1-1
Hamad Al-Harbi, David A Kendall and Stephen PH Alexander	EFFECTS OF CANNABIDIOL AND HU210 ON VIABILITY OF SH-SY5Y HUMAN NEUROBLASTOMA CELLS	P1-2
Pazos MR, Mohamed N, Martínez-Pinilla E, Ceprián M, Lafuente H, Santos M, Romero J, Alvarez FJ, Franco R, Hillard C and Martínez-Orgado J.	INVOLVEMENT OF CB2 AND 5HT1A RECEPTORS IN CANNABIDIOL-INDUCED NEUROPROTECTION AFTER HYPOXIA-ISCHEMIA IN NEWBORN PIGS	P1-3
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Pazos MR, Mohammed N, Ceprián M, Santos M and Martínez-Orgado J.	NEUROPROLIFERATIVE EFFECT OF CANNABIDIOL ADMINISTRATION TO NEWBORN RATS AFTER HYPOXIA-ISCHEMIA	P1-5
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Carmen Vázquez, Marta Moreno, Lourdes Ruiz-Valdepeñas, Samuel Ruiz de Martín, Elena Tomás, Rosa María Tolón and Julián Romero	CANNABIDIOL POTENTIATES IN VIVO MICROGLIAL ACTIVITY IN RESPONSE TO ACUTE BRAIN INJURY	P1-7
Balapal S. Basavarajappa, Madhu Shivakumar, Delphine Psychoyos, Shan Xie and Shivakumar Subbanna	AEA/CB1R SIGNALING CONTRIBUTES TO FETAL ALCOHOL SPECTRUM DISORDER	P1-8
Iryna A. Khasabova, Matt Tracy, Natalya Burlakova, Sergey G.Khasabov, Catherine Harding-Rose, Donald A. Simone and Virginia S. Seybold	FACILITATION OF ANANDAMIDE SIGNALING REDUCES NEUROTOXICITY PRODUCED BY CISPLATIN	P1-9
Marie-Louise Zeissler, C. Oliver Hanemann, John P. Zajicek and Camille B. Carroll	THE FAAH INHIBITOR URB597 IS PROTECTIVE AGAINST MPP+ IN A CELL CULTURE MODEL OF PARKINSON'S DISEASE	P1-10

Alex Straiker, Meggie D Beazley, Sherry Shu-Jung Hu, Suresh Viswanathan, Heather Bradshaw and Melanie EM Kelly	EVIDENCE THAT A FUNCTIONAL GPR18-BASED SIGNALING SYSTEM IN THE ANTERIOR MURINE EYE MODULATES INTRAOCULAR PRESSURE	P1-11
John Zajicek	WHAT DO RESULTS FROM THE CANNABINOID USE IN PROGRESSIVE INFLAMMATORY BRAIN DISEASE (CUPID) TRIAL TELL US ABOUT THE POTENTIAL OF CANNABINOIDS FOR NEUROPROTECTION?	P1-12
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Paul A.K.E. Gyimah and W. -S. Vanessa Ho	EFFECTS OF HIGH GLUCOSE ON THE VASCULAR ACTION OF ANANDAMIDE IN YOUNGER AND OLDER RATS	P1-14
Lucie Geurts, Nathalie M. Delzenne and Patrice D. Cani	CHRONIC ENDOCANNABINOID SYSTEM ACTIVATION AND METABOLIC ENDOTOXEMIA DIFFERENTIALLY AFFECT THE ONSET OF GLUCOSE INTOLERANCE AND INFLAMMATION IN HIGH-FAT DIET-INDUCED METABOLIC ALTERATIONS	P1-15
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William H Hind and Saoirse E O'Sullivan	CANNABIDIOL PROTECTS THE BLOOD-BRAIN BARRIER AGAINST INCREASED PERMEABILITY FOLLOWING OXYGEN-GLUCOSE DEPRIVATION	P1-23
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Fabricio A. Moreira, Luciano R. Vilela, Daniel C. Medeiros, Gustavo H. Rezende, Antonio C. Oliveira and Marcio F. Moraes	EFFECTS OF CANNABINOIDS AND ENDOCANNABINOID-HYDROLYSIS INHIBITION ON PENTYLENETETRAZOLE-INDUCED SEIZURES AND ELECTROENCEPHALOGRAPHIC ACTIVITY	P1-31
Martin A. Sticht and Linda A. Parker	THE ROLE OF INTRA-VISCERAL INSULAR CORTEX 2AG IN CONDITIONED NAUSEA-LIKE BEHAVIOR IN RATS	P1-32
Anja A. Goepfrich , Matthias Klugmann, Thomas Enkel and Miriam Schneider	ADULT RATS OVEREXPRESSING THE CB1 RECEPTOR IN THE MPFC DISPLAY ALTERED EMOTIONAL BEHAVIOR AND IMPAIRED EXTINCTION OF FEAR MEMORY	P1-33
Ryan J. McLaughlin, Matthew N. Hill, Tiffany Lee, Jay Schulkin, Cecilia J. Hillard and Bruce S. McEwen	CRH MEDIATES THE EFFECTS OF GLUCOCORTICOIDS ON ENDOCANNABINOID SIGNALING IN THE AMYGDALA	P1-34
Daniela Hauer, Hannah Gola, Patrizia Campolongo, Iris-Tatjana Kolassa and Gustav Schelling	PLASMA LEVELS OF ENDOCANNABINOIDS AND RELATED PRIMARY FATTY ACID AMIDES ARE INCREASED IN PATIENTS WITH POST-TRAUMATIC STRESS DISORDER	P1-35
Tiffany T.-Y. Lee, Matthew N. Hill, Sarah B. Filipski and Bruce S. McEwen	MORPHOLOGICAL AND BEHAVIOURAL EVIDENCE FOR IMPAIRED PREFRONTAL CORTICAL FUNCTION IN FEMALE CB1 RECEPTOR DEFICIENT MICE	P1-36
Vincenzo Micale, Fabricio A. Pamplona, Alexandra Sulcova, Filippo Drago and Carsten T. Wotjak	THE MODULATION OF THE ENDOCANNABINOID SIGNALING MEDIATES THE EXTINCTION OF AVOIDANCE BEHAVIOR BY CONTROLLING SAFETY LEARNING	P1-37
Irena Smaga, Aneta Piwowarczyk, Beata Bystrowska and Małgorzata Filip	CHANGES IN THE LEVEL OF ENDOCANNABINOIDS AND ENDOCANNABINOID-LIKE MOLECULES AFTER ACUTE AND REPEATED ADMINISTRATION OF IMIPRAMINE AND N-ACETYLCYSTEINE IN DIFFERENT RAT BRAIN STRUCTURES	P1-38
Hila Abush and Irit Akirav	CANNABINOIDS AMELIORATE IMPAIRMENTS INDUCED BY CHRONIC STRESS TO SYNAPTIC PLASTICITY AND SHORT-TERM MEMORY	P1-39

Amir Segev and Irit Akirav	CANNABINOID AND GLUCOCORTICOID RECEPTORS IN THE AMYGDALA MODULATE THE STRESS-INDUCED IMPAIRMENT OF LTP IN THE HIPPOCAMPAL-ACCUMBENS PATHWAY	P1-40
Teniel S. Ramikie and Sachin Patel	ENDOCANNABINOID-MEDIATED SYNAPTIC DEPRESSION IN THE CENTRAL AMYGDALA: CHOLINERGIC-CANNABINOID INTERACTIONS	P1-41
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Robert E. Hampson, Cheryl Ann Sexton, John Sesay, Anushka Goonawardena and Sam A. Deadwyler	CANNABINOIDS ALTER REVERSAL OF BEHAVIORAL CONTINGENCY BY SUPPRESSING MEMORY-RELATED ENCODING	P1-43
Yosef Sarne, Miriam Fishbein, Sahar Gov, Ora Keren and Mikhal Gafni	EXTREMELY LOW DOSES OF TETRAHYDROCANNABINOL PROTECT FROM COGNITIVE DEFICITS AND INDUCE LONG-LASTING BIOCHEMICAL CHANGES IN THE MOUSE BRAIN	P1-44
Roberto Colangeli, Ines Santolini, Silvana Gaetani, Ferdinando Nicoletti, Richard Ngomba and Vincenzo Cuomo	ALTERATIONS OF CB1 mRNA EXPRESSION IN STARGAZER MUTANT MICE AND IN PENTYLENETETRAZOLE-INDUCED SPIKE AND WAVE DISCHARGES IN RATS	P1-45
Amir Englund, Paul Morrison, Judith Nottage, Dominic Hague, Fergus Kane, Stefania Bonaccorso, James Stone, Avi Reichenberg, Rudolf Brenneisen, David Holt, Amanda Fielding, Dominic ffytche, Robin Murray and Shitij Kapur	CANNABIDIOL INHIBITS THC-ELICITED PSYCHOSIS AND MEMORY IMPAIRMENTS IN HUMANS	P1-46
Fabricio A. Moreira, Luciano R. Vilela, Daniel C. Medeiros, Gustavo H. Rezende, Antonio C. Oliveira and Marcio F. Moraes	EFFECTS OF CANNABINOIDS AND ENDOCANNABINOID-HYDROLYSIS INHIBITION ON PENTYLENETETRAZOLE-INDUCED SEIZURES AND ELECTROENCEPHALOGRAPHIC ACTIVITY	P1-47
Floor Remmers, Sabine Ruehle, Hector Romo-Parra, Federico Massa, Melanie Wickert, Nadine Kaiser, Giovanni Marsicano, Hans-Christian Pape and Beat Lutz	ENDOCANNABINOID SIGNALLING AND FEAR EXTINCTION IN MOUSE LINES FOR GLUTAMATERGIC AND GABAERGIC-SPECIFIC RESCUE FROM CB1 RECEPTOR DEFICIENCY	P1-48
Vanessa Enk, Martin Häring, Floor Remmers, Sabine Rühle and Beat Lutz	EXPLORATORY BEHAVIOR AND RESPONSE TO THC IN MOUSE LINES FOR GLUTAMATERGIC AND GABAERGIC-SPECIFIC RESCUE FROM CB1 RECEPTOR DEFICIENCY	P1-49

Topic D. Cannabis

Donald P. Land and Kymron B. deCesare	CANNABIS IN (NORTHERN) CALIFORNIA, 2011-2012: STATISTICS AND TRENDS FROM THOUSANDS OF INDIVIDUAL SAMPLES TESTED FOR 15 CANNABINOIDS AND 8 TERPENOIDS	P1-50
Adam Ogden, Andrew Higham and Karen Wright	IMPORTANCE OF REFERENCE GENES IN VALIDATING THE EFFECTS OF CANNABIDIOL ON FIBROTIC PROCESSES	P1-51
Zlatko Mehmedic, Mohamed M. Radwan, Suman Chandra, Mudasir Tantry, Afeef S. Husni, Stephen Cutler, Ikhlas A. Khan and Mahmoud A. Elsohly	VOLATILE OIL FROM HIGH POTENCY *CANNABIS SATIVA* WITH *IN VITRO* BINDING AFFINITY FOR HUMAN CANNABINOID RECEPTORS	P1-52
Patrik Roser, Silke Lissek, Martin Tegenthoff, Volkmar Nicolas, Georg Juckel and Martin Brüne	ALTERATIONS OF THEORY OF MIND NETWORK ACTIVATION IN CHRONIC CANNABIS USERS	P1-53
Kristen Courtney	CANNABIS USE IN PREGNANCY AND BREASTFEEDING	P1-54

POSTER SESSION 2: Topics E - G
Day 2, Tuesday, July 24th: 16:00 – 18:00

Topic E. Receptor Signaling

M. Genedy, N. Hoenig, M. Youseffi and M.C.T. Denyer	EFFECT OF CANNABINOIDS (WIN55,212-2) IN ACCELERATION OF WOUND HEALING IN BONE CELL MONOLAYER	P2-1
M. Genedy, M. Youseffi and M.C.T. Denyer	EFFECT OF CB2 SELECTIVE AGONIST ON WOUNDED BONE CELL MONOLAYERS	P2-2
A. Abdeldayem, M. Yousseffi and M.C.T. Denyer	THE EFFECT OF DIFFERENT CONCENTRATIONS OF CANNABINOID (WIN 55,212-2) ON WOUND HEALING OF CHONDROCYTE MONOLAYER	P2-3

A. Abdeldayem, M. Yousseffi and M.C.T. Denyer	THE EFFECT OF DIFFERENT CONCENTRATIONS OF THE SYNTHETIC CANNABINOIDS WIN 55,212-2, URB602 AND HU-308 ON WOUND HEALING OF CHONDROCYTE MONOLAYER	P2-4
Philip W. Brownjohn and John C. Ashton	PROTEOMIC IDENTIFICATION OF THE RAT CANNABINOID CB2 RECEPTOR USING MASS SPECTROMETRY AND IMMUNOBLOTTING	P2-5
Dina Navia-Paldanius, Niina Aaltonen, Marko Lehtonen, Ulrike Taschler, Franz P. Radner, Robert Zimmermann and Jarmo T. Laitinen	BRAIN REGIONAL DESENSITIZATION OF CANNABINOID CB1 RECEPTOR SIGNALING IN MICE WITH GLOBAL GENETIC KNOCKOUT OF MONOACYLGLYCEROL LIPASE	P2-6
Pekkala S, Wiklund P, Hulmi JJ, Marjomäki V, Alén M, Pöllänen E, Fachada V, Mero AA and Cheng S	CANNABINOID RECEPTOR 1 REGULATES PROTEIN SYNTHESIS AND TRANSLATION IN HUMAN SKELETAL MUSCLE	P2-7
Jahan P. Marcu, Steven N. Popoff and Mary E. Abood	CB1 AND CB2 ANTAGONIST SIGNALING IN OSTEOBLASTS	P2-8
Gemma L. Baillie, Roger G. Pertwee and Ruth A. Ross	SIGNALLING TRAFFICKING AT THE CANNABINOID CB1 RECEPTOR BY THE CB1 ALLOSTERIC MODULATOR, ORG 27569	P2-9
William A. Devane, David L. Stevens, David P. Finn, Ntsang M. Nebane, Zhao-Hui Song, David Peters, Duncan Crawford, J. Michael Walker, Benjamin Applegate, John M. Kennish, Michael P. Cassidy, Dana E. Selley, Anne Zimmer, Andreas Zimmer and William L. Dewey	LEELAMINE, A NOVEL DITERPENE, EXHIBITS CANNABIMIMETIC EFFECTS IN CB1 RECEPTOR KNOCKOUT MICE	P2-10
Richard S. Priestley, Stephen P.H. Alexander and David A. Kendall	EARLY AND LATE PHASE ERK RESPONSES REVEAL A NOVEL FUNCTIONALLY SELECTIVE AGONIST PROFILE AT CANNABINOID TYPE 1 RECEPTORS	P2-11
Chris S. Breivogel and Manan Vaghela	BETA-ARRESTIN1 MEDIATION OF THE EFFECTS OF CANNABINOID LIGANDS	P2-12
Alex Straiker, Laura Pardon, Lisa Walter, Nephi Stella and Suresh Viswanathan	EVIDENCE FOR A FUNCTIONAL ROLE OF CB1 CANNABINOID RECEPTORS IN THE MAMMALIAN CONE PATHWAY	P2-13
Linda Console-Bram, Eugen Brailoiu, Cristina Brailoiu and Mary E. Abood	CALCIUM MOBILIZATION AND MAPK ACTIVITY VIA GPR18	P2-14

Beata Bystrowska, Karolina Wydra, Agata Suder, Irena Smaga and Małgorzata Filip	CHANGES IN CONCENTRATION OF ENDOCANNABINOIDS AND N-ACYL ETHANOLAMIDES IN BRAIN STRUCTURES OF RATS UNDERWENT COCAINE SELF-ADMINISTRATION AND ITS WITHDRAWAL	P2-25
Iain S. McGregor, Alex Wong, Kong M. Li, Jonathon C. Arnold and Wendy Swift	CANNABIS POTENCY IN NEW SOUTH WALES AUSTRALIA: AN ANALYSIS OF POLICE SEIZED STREET SAMPLES	P2-26
Thomas Gamage, Divya Ramesh, Rehab Abdullah, Micah J. Niphakis, Ben Cravatt and Aron Lichtman	DUAL FAAH/MAGL INHIBITION ATTENUATES SOMATIC SIGNS OF SPONTANEOUS MORPHINE WITHDRAWAL IN ICR MICE	P2-27
Matthew F. Lazenka, Eric J. Nestler, Dana E. Selley and Laura J. Sim-Selley	ROLE OF ΔFOSB IN CB1R DESENSITIZATION IN THE BASAL GANGLIA	P2-28
Sofia B. Gustafsson and Stig O. P. Jacobsson	EFFECTS OF CANNABINOIDS ON THE DEVELOPMENT OF CHICK EMBRYOS IN OVO	P2-29
Fabiana Piscitelli, Aron Lichtman, Laura Sim-Selley, Robert Hamm, Tiziana Bisogno, Vincenzo Di Marzo and Raphael Mechoulam	TARGETED LIPIDOMICS PROFILING OF INJURED RAT BRAIN: POSSIBLE IMPLICATIONS FOR NICOTINE DEPENDENCE	P2-30
Emmanuel S. Onaivi, Hiroki Ishiguro, Alvaro Llorente-Berzal, Maria-P Viveros and George R. Uhl	CANNABINOID RECEPTORS: GENE STRUCTURES, SNPS, CNVS, CPG ISLANDS MICRORNA REGULATION AND VARIATION IN NEUROPSYCHIATRIC DISORDERS	P2-31
Bela Szabo, Volker Auwärter, Mario Lederer, Stefan Kneisel and Maren Hermanns-Clausen	ACUTE INTOXICATIONS FOLLOWING CONSUMPTION OF HERBAL PRODUCTS CONTAINING SYNTHETIC CANNABINOIDS	P2-32
TOPIC G. CANCER / IMMUNOLOGY		
Torsten Lowin, Angelika Gräber, Elena Neumann and Rainer H. Straub	ANANDAMIDE ATTENUATES THE INFLAMMATORY PHENOTYPE OF RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS BY ACTIVATING MULTIPLE RECEPTOR PATHWAYS	P2-33
Torsten Lowin, Wentao Zhu, Katja Dettmer, Elena Neumann and Rainer H. Straub	CORTISOL-INDUCED ADHESION OF SYNOVIAL FIBROBLASTS IS MEDIATED BY THE ENDOCANNABINOID SYSTEM	P2-34

Wook Kim, Qizong Lao, Yu-Kyong Shin, Olga D. Carlson, Eun Kyung Lee, Myriam Gorospe, Rohit N. Kulkarni and Josephine M. Egan	CANNABINOIDS INDUCE PANCREATIC BETA-CELL DEATH BY DIRECTLY INHIBITING INSULIN RECEPTOR ACTIVATION	P2-35
Daniel Kerr, Brendan Harhen, David P. Finn and Michelle Roche	PHARMACOLOGICAL INHIBITION OF MONOACYLGLYCEROL LIPASE ATTENUATES LPS-INDUCED INCREASES IN CYTOKINE EXPRESSION IN THE RAT FRONTAL CORTEX AND PLASMA: DIFFERENTIAL MECHANISMS OF ACTION	P2-36
Daniel Roskowski, Douglas McHugh, Siyun Xie and Heather B. Bradshaw	N-ARACHIDONOYL GLYCINE REGULATES THE PHENOTYPIC MORPHOLOGY OF BV-2 MICROGLIA	P2-37
Evan Jameyfield, Siham Raboune, Douglas McHugh and Heather B. Bradshaw	NOVEL ENDOGENOUS N-ACYL AMIDES INDUCE CALCIUM MOBILIZATION IN BV-2 MICROGLIA	P2-38
Douglas McHugh, Jeremy Page and Heather B. Bradshaw	N-ARACHIDONOYL GLYCINE AND Δ^9-THC REGULATE THE CYTOKINE PRODUCTION OF BV-2 MICROGLIA	P2-39
Seok-Woo Park and Myung-Whun Sung	CANNABINOID RECEPTOR-INDEPENDENT ANTI-PROLIFERATIVE EFFECT OF DOCOSAHEXAENOYL ETHANOLAMIDE IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS	P2-40
Benjamin Gennequin, David-Marian Otte, Katrin Zimmermann, Anne Zimmer and Andreas Zimmer	FUNCTIONAL ANALYSIS OF THE HUMAN CB2 RECEPTOR VARIANTS	P2-41
Luciano De Petrocellis, Alessia Ligresti, Aniello Schiano Moriello, Roberta Verde, Pierangelo Orlando, Colin G. Stott and Vincenzo Di Marzo	CANNABINOIDS IN PROSTATE CANCER: AN UPDATE	P2-42
Svenja S. Ternes, Judith Alferink and Andreas Zimmer	REGULATION OF INFLAMMATORY PROCESSES BY THE ENDOCANNABINOID SYSTEM	P2-43
Christeene Haj, Percy F. Sumariwalla, Natalya Kogan, Zhanna Yektin, Marc Feldmann, Raphael Mechoulam and Ruth Gallily	NOVEL CANNABIDIOL DERIVATIVES AND THEIR USE AS ANTI-INFLAMMATORY AGENTS	P2-44
Toguri, J.T., Szczesniak, A.M., Zhou, J., Lehmann, C. and Kelly, M.E.M	CANNABINOID MODULATION OF LEUKOCYTE-ENDOTHELIAL INTERACTIONS IN AN ENDOTOXIN-INDUCED UVEITIS MODEL	P2-45

Anna-Maria Szczesniak and Melanie E.M. Kelly	THE ROLE OF CANNABINOID RECEPTOR 2 IN THE EXPERIMENTAL AUTOIMMUNE UVEORETINITIS	P2-46
Neta Rimmerman, Ana Juknat, Ewa Kozela, Ziv Porat, Danya Ben-Hail, Varda Shoshan-Barmatz and Zvi Vogel	CANNABIDIOL AFFECTS MITOCHONDRIAL TARGETS IN BV-2 MICROGLIAL CELLS	P2-47
Jocelijn Meijerink, Mieke Poland, Michiel Balvers, Zheng Wang, Jvalini Dwarkasing and Renger F. Witkamp	FISHING FOR CANNABINOIDS FROM FISH; IMMUNE-MODULATORY MECHANISMS OF AMIDES DERIVED FROM N-3 FATTY ACIDS	P2-48
Tara Macpherson, John Westwick and Karen Wright	CANNABIDIOL AFFECTS CELLULAR BIOENERGETICS IN COLON CANCER	P2-49
Amy G. Eubanks, Cameron A. Tull, Amber E. Prescott, John R. Sims, Emily R. Coffman, Whitley M. Hoppe, Colmon W. Massey, Nolan J. West, Vikki K. Bennett, Cara L. McKinney, Jessica R. Winston, Larry J. Suva, Robert J. Griffin and Lori L. Hensley	ANTI-TUMOR EFFECTS OF AJULEMIC ACID ON THE EWING'S SARCOMA FAMILY OF TUMORS	P2-50
A.M. Wasik, S. Almestrand, F. Zong, P. Andersson, E. Kimby, B. Christensson and B. Sander	MAPPING OF THE ENDOCANNABINOID SYSTEM IN MANTLE CELL LYMPHOMA	P2-51
Mohd W. Nasser, Zahida Qamri, Konstantin Shilo and Ramesh K. Ganju	CANNABINOID RECEPTOR CB2 CROSSTALK WITH CHEMOKINE RECEPTOR CXCR4 TO MODULATE BREAST CANCER GROWTH AND METASTASIS	P2-52
Somnath Mukhopadhyay, Shalini Jha and Victoria Jones	CB2 RECEPTOR-MEDIATED REGULATION OF I2PP2A (INHIBITOR 2 OF PROTEIN PHOSPHATASE 2A): A NEW TARGET FOR PROSTATE CANCER TREATMENT	P2-53
Benjamin S. Harvey and Scott D. Smid	EFFECTS OF PRO-INFLAMMATORY CYTOKINES AND CANNABINOIDS ON INTESTINAL EPITHELIAL PERMEABILITY	P2-54
June Penman, Alana J. Burns and Andrew J. Irving	L-α-LYSOPHOSPHATIDYLINOSITOL AND AM251 PROMOTE CYTOSKELETAL REORGANISATION IN CANCER CELL LINES THAT EXPRESS GPR55	P2-55

POSTER SESSION 3: TOPICS H - J
DAY 3, WEDNESDAY, JULY 25TH: 10:45 – 12:45

TOPIC H. LIGAND DEVELOPMENT

Daniel J. Hermanson, Nolan D. Hartley, Sachin Patel and Lawrence J. Marnett	DEVELOPMENT OF LM-4131, A SUBSTRATE-SELECTIVE INHIBITOR OF CYCLOOXYGENASE-2	P3-1
Pritesh Kumar, Akhilesh Kumar and Zhao-Hui Song	STRUCTURE-ACTIVITY RELATIONSHIPS OF LIGANDS FOR GPR119 – A GPCR THAT IS RELATED TO CANNABINOID RECEPTORS	P3-2
Jayendra Z. Patel, Teija Parkkari and Tapio Nevalainen	3,4-DISUBSTITUTED THIADIAZOLES AS FAAH AND/OR MGL INHIBITORS	P3-3
Andrea Chicca, Janine Marazzi, Simon Nicolussi and Jürg Gertsch	EVIDENCE FOR A BIDIRECTIONAL ENDOCANNABINOID TRANSPORT ACROSS CELL MEMBRANES AND IDENTIFICATION OF SELECTIVE INHIBITORS	P3-4
Roger Slavik, Daniel Bieri, Konstantin Drandarov, Adrienne Müller, Stefanie Krämer, Roger Schibli, Simon M. Ametamey and Linjing Mu	SYNTHESIS AND EVALUATION OF NOVEL CANNABINOID TYPE 2 RECEPTOR TRACERS FOR PET IMAGING	P3-5
Sari Yrjölä, Tuomo Kalliokoski, Teija Parkkari, Tuomo Laitinen, Antti Poso and Tapio Nevalainen	2,4,6-TRISUBSTITUTED 1,3,5-TRIAZINES AS SELECTIVE CB2 AGONISTS	P3-6
Sara Del Carlo, Giuseppe Saccomanni, Clementina Manera, Giancarlo Pascali, Francesca Castelli, Alessia Ligresti, Vincenzo Di Marzo, Marco Macchia and Piero A. Salvadori	[18F]-LABELED HETEROCYCLIC DERIVATIVES FOR PET IMAGING OF CANNABINOID CB2 RECEPTOR	P3-7
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ICRS2012 - Presidential Plenary Speaker

"Planning research for the next half a century"

Raphael Mechoulam, Ph.D.

Institute for Drug Research
Hebrew University of Jerusalem

In the spring of 1962 – exactly 50 years ago – I started reading on cannabis and planning some limited amount of work on it. A year later the late Yuval Shvo and I reported the structure of cannabidiol - my first paper on cannabinoids. Today – fifty years later – I am still digging the same field.

It's time to plan ahead for the next half a century.

Essentially nothing is known on the chemistry of the human personality, or the individual temperamental differences. Accumulation of such knowledge is essential for a future biochemical basis of psychology.

Assuming that there are chemical differences which are the cause (or one of the causes) of the differences in personalities, we have to look for a large 'catalog' of compounds, which cause CNS effects. The variability of such a cluster of compounds – their levels, their ratios and presumably their effects, not only as individual compounds, but also as a group (a type of entourage effect), should allow an infinite number of individual differences.

Such a cluster of compounds exists in the brain. This cluster consists of fatty acid amides of amino acids, FAAA's (or their derivatives, such as ethanol amides), or fatty acid esters with glycerol and related compounds. A partial list has been published by the Indiana group. A few of the individual components in the cluster have been evaluated for their biological effects. Amongst them are anandamide, 2-AG, NADA, PEA, oleoyl ethanolamide, stearoyl ethanolamide and a few others. The effects of the few components within the cluster that have been evaluated vary considerably; the joint effects of groups of components of the cluster have not been evaluated.

With I. Bab and R. Smoum we looked at oleoyl serine, which is anti-osteoporotic, but is also found in the brain. With E. Shohami, A.Yeshurun and R. Lecker we found that arachidonoyl serine lowers damage caused by closed head injury. Quite recently with A. Lichtman and V. Di Marzo we noted that oleoyl glycine and palmitoyl ethanolamide concentrations are enhanced after damage in a specific brain region.

It is tempting to assume that the huge possible variability of the levels and ratios of substances in such a cluster of compounds may allow an infinite number of individual differences, the raw substance which of course is sculpted by experience. If this intellectual speculation is shown to have some factual basis it may lead to major advances in molecular psychology.

Kang Tsou Memorial Lecture

"Do differentiated neurons migrate?"

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It is generally assumed that postmitotic neurons lose their capacity to migrate as soon as they have arrived at their destination and formed synaptic connections both with incoming afferents and target cells. Hence, altered neuronal positioning associated with epilepsy has generally been deemed to be the result of a migration defect during development. In temporal lobe epilepsy (TLE) and in kainate (KA)-induced experimental epilepsy in mice, the normally compact layer of the granule cells (GCs) in the dentate gyrus is dispersed. Here, we first showed that KA application induced epileptiform activity of the GCs and granule cell dispersion (GCD) both in vivo and in hippocampal slice cultures. As revealed by real-time microscopy, GCD arose from the increased motility of differentiated GCs that translocated their nuclei into apical dendrites. This somal translocation resulted in a reorientation of dendritic processes. Kainate-induced epileptiform activity led to hypermethylation of the reelin promoter region, which in turn resulted in decreased reelin levels as shown by Western blotting, in situ hybridization, and immunocytochemistry. Reelin is known to phosphorylate the actin-depolymerizing protein cofilin, and the decrease in reelin levels was followed by reduced cofilin phosphorylation. Hypophosphorylation of cofilin is associated with enhanced actin-depolymerizing activity of the protein and reorganization of the actin cytoskeleton that is a prerequisite for cell motility. We conclude that an alteration in Reelin-mediated cofilin phosphorylation underlies the increased motility of differentiated granule cells in epilepsy and eventually leads to a dispersion of the normally compact granule cell layer in the dentate gyrus.

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NIDA TRAINEE LUNCH AND LEARN

"CANNABINOID PHARMACEUTICS: HISTORICAL INFLUENCES ON MILLENNIAL RESEARCH"

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Pharmaceutical research on cannabis is truly a millennial pursuit: Ibn Wahshīyah (living in Baghdad) and the founders of Islamic Medicine (from Persia) studied cannabis 1000 years ago. German chemists began extracting cannabis constituents in the 1880s. The modern era of pharmacotherapeutics was advanced by the collaborative work of Roger Adams from the USA and **Walter Siegfried Loewe**, ein deutsch-amerikanischer Pharmakologe. Loewe machte viele Entdeckungen zwischen 1937 und 1950: he pioneered work on drug synergy and invented isobologram analysis. Loewe led the development of humane animal testing and promoted the use of rodents, instead of dogs or rabbits (he devised two components of the Billy Martin tetrad test). He used column chromatography to isolate THC in 1946. This separation of active compounds from the plant led to purification of Δ^9-THC by Raphael Mechoulam in 1964. Loewe emphasized the polypharmaceutical nature of cannabis—that it had more than one active ingredient. He conducted the first pharmacokinetic study of cannabinoids, and discovered the anticonvulsant benefits of cannabinoids. Loewe synthesized cannabinoid compounds, compared their various efficacies, and introduced to pharmacology literature the term "Structure-Activity Relationship." Along the way he ran afoul of Nazi politicians and Harry Anslinger.

In America, cannabis was not recognized for its medicinal potential, but rather, was a commodity that could target populations as part of a political agenda building in the 1930's and enforced by Harry Anslinger. This barrier was difficult to overcome when active compounds were identified in the mid-1960s and extensive pharmacokinetic and physiologic investigations ensued. One study that triggered an interest by major pharmaceutical companies was the demonstration by Wilson and May that an analog of the 11-OH metabolite of Δ^8-THC was 10-times more potent in antinociceptive tests. Big pharma (e.g., Abbott, Lilly, Pfizer) developed novel cannabinoid analgesics (e.g., nabitan, nabilone, nantradol). Unfortunately, the cannabinoid side-effects of sedation and muddled thinking that accompanied the therapeutic pain relief curtailed general use outside of a hospital setting, and by the 1980's these companies had decided not to market their drugs. However, an unmet need to ameliorate the nausea and vomiting that accompanied cancer chemotherapy brought chemically synthesized Δ^9-THC (dronabinol) to the realm of legitimate medicines. Shortly thereafter, with the advent of the AIDS epidemic, dronabinol was approved to treat the wasting accompanying this fatal disease, and nabilone was allowed for similar compassionate purposes.

In conclusion, Loewe's contributions emphasize how the cannabinoid field has led pharmacological research. However, one looks back at a stormy evolution of medicinal applications as research toward legitimate pharmacotherapeutics and political agendas clash.

The views and opinions expressed here are personal views and opinions and do not represent those of GW Pharmaceuticals, the University of Vermont, or Wake Forest School of Medicine.

PLENARY ABSTRACTS

NIDA Symposium

THURSDAY, JULY 26TH

ROLE OF CANNABINOID SYSTEM IN NICOTINE ADDICTION

Tobacco addiction is one of the leading preventable causes of death in the world and nicotine is the main psychoactive component responsible for establishing and maintaining tobacco dependence. Since existing medications are only partially effective in treating tobacco smokers, there is a great need for improved medications for smoking cessation. Increasing evidence suggests that cannabinoid system modulates the addictive properties of nicotine. For example, cannabinoid receptor-1(CB1) antagonist rimonabant has been shown to attenuate several addictive properties of nicotine in animal studies. However, the utility of rimonabant for smoking cessation may be limited because of its known side effects.

Inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for the catabolism of the endogenous cannabinoid anandamide, is another approach used by researchers to examine the role of cannabinoids in nicotine addiction. Administration of the FAAH inhibitor URB597 has been shown to produce species specific effects in preclinical models of nicotine addiction. An initial study showed that URB597-treated wild type mice or FAAH-/- mice display augmented nicotine conditioned place preference (CPP) and increased magnitude of nicotine withdrawal responses. However, subsequent research found that in the rat URB597 prevents development of nicotine-induced CPP, prevents acquisition of nicotine self administration, reduces nicotine-induced reinstatement in both CPP and self administration models of relapse, and reduces nicotine-induced dopamine elevation in the nucleus accumbens. Based on the rat data, FAAH has been proposed to serve as a new target for development of medications for treatment of tobacco dependence. Since FAAH inhibition elicits antidepressant and anxiolytic properties in rodents, targeting this enzyme may be a better strategy to prevent relapse for tobacco smoking when compared to rimonabant, which is known to produce psychiatric side effects such as anxiety, depression, and suicidal ideation. Consistent with this idea, URB597 attenuates the anxiogenic-like effects resulting from nicotine withdrawal in the rat. Distinct neurochemical substrates appear to subserve the opposite effects that FAAH inhibition elicits in mouse and rat models of nicotine addiction.

Another promising approach that is under active investigation is monoacylglycerol lipase (MAG lipase) inhibitors, which elevate brain levels of the endocannabinoid, 2-arachidonic glycerol (2-AG). Since manipulation of endogenous cannabinoids appears to produce less cannabimimetic side effects than systemic administration of exogenous cannabinoids, endocannabinoid metabolizing enzymes or endocannabinoid trafficking molecules may represent promising targets for smoking cessation. In this symposium four speakers will discuss strategies in the development of medications for treatment of tobacco dependence.

Organizers:

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PLENARY ABSTRACTS

Young Investigator Awardee Speaker

"Interplay of endocannabinoids and plant derived cannabinoids with inflammation, oxidative stress and cell death: Implications for tissue injury and protection"

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Oxidative/nitrative stress and inflammation play critical roles in the development of all major diseases affecting humans. These processes may also trigger rapid or chronic activation of the lipid endocannabinoid signaling system (ECS). In turn, ECS may directly or indirectly modulate reactive oxygen species generation, inflammation, and subsequent tissue injury, in addition to its well-recognized metabolic effects. While in certain cases activation of the endocannabinoid system may represent a protective mechanism in limiting inflammation and associated tissue injury in large number of pathological conditions, in other disease states overactivation of the ECS may enhance or even trigger tissue damage via receptor-dependent and independent mechanisms.

This presentation highlights the context dependent role of the endocannabinoids and cannabinoid receptors in modulating inflammation, oxidative/nitrative stress, and tissue injury, in various pathological conditions of the cardiovascular system, diabetic complications, ischemic-reperfusion injury, and nephropathy. The therapeutic potential and novel mechanisms of anti-inflammatory and/or antioxidant effects of certain plant-derived cannabinoids, such as cannabidiol and Δ^9-tetrahydrocannabivarin, which are devoid of psychotropic effects, will also be emphasized.

ENDOCANNABINOID HYDROLYSIS GENERATES BRAIN PROSTAGLANDINS THAT PROMOTE NEUROINFLAMMATION

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Phospholipase A2 (PLA2) enzymes are considered the primary source of arachidonic acid for biosynthesis of pro-inflammatory prostaglandins. We present compelling evidence showing that a distinct pathway exists in brain, where monoacylglycerol lipase (MAGL) hydrolyzes the endocannabinoid 2-arachidonoylglycerol to generate a major arachidonate precursor pool for neuroinflammatory prostaglandins. Both pharmacological and genetic inactivation of MAGL attenuates neuroinflammation and exerts protection against neurodegeneration in a Parkinson's disease mouse model and reduces amyloid plaque deposition in an Alzheimer's disease model. We find that these animals are spared the hemorrhaging caused by COX inhibitors in the gut, where prostaglandins are instead regulated by cytosolic-PLA2. Our findings identify MAGL as a distinct metabolic node that couples endocannabinoid to prostaglandin signaling networks in the nervous system and suggest that inhibition of this enzyme may be a new and potentially safer way to suppress the proinflammatory cascades that underlie neurodegenerative disorders.

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CANNABINOID LIGANDS DIRECTLY MODIFY β-AMYLOID FIBRIL FORMATION AND ARE VARIABLY NEUROPROTECTIVE *IN VITRO*

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Cannabinoid (CB) ligands such as anandamide and cannabidiol afford neuroprotection against β-amyloid exposure. Their mechanism of protection is not clear, particularly with respect to pharmacological selectivity. The possibility that cannabinoid ligands may directly interfere with β-amyloid formation has yet to be investigated, even though some share similar moieties to known polyphenols capable of disrupting β-amyloid aggregation. In this study we determined the capacity for a set of structurally and pharmacology diverse cannabinoid ligands to directly alter amyloid fibril formation and compared this with their neuroprotective properties in vitro. Cannabinoid ligands were assessed for direct interaction with $A\beta_{1-42}$ fibril formation via a fluorometric Thioflavin T (ThT) binding assay. In addition, transmission electron microscopy (TEM) was used to directly visualise the effects of CB ligands on $A\beta_{1-42}$ fibril aggregation and morphology, using the following compounds: anandamide and 2-arachidonoyl glycerol (2-AG) (endogenous CB ligands), ACEA (CB1 receptor-selective agonist), JWH-015 (CB2 receptor-selective agonist), cannabidiol (CBD: phytocannabinoid), Abnormal-cannabidiol (Abn-CBD), O-1602 and O-1918 (putative atypical CB/GPR55 receptor ligands). In addition, human neuroblastoma SH-SY5Y cells were treated with β-amyloid ($A\beta_{1-42}$; 0.1-5 μM) for 24 hours, alone or in the presence of select CB ligands based on results of the ThT and TEM screening. ThT assay revealed maximal $A\beta_{1-42}$ fibril formation at 6 hrs with continued aggregation over a further 8 hrs. Extensive and significant overall inhibition of ThT fluorescence (45-75%) occurred from incubation of $A\beta_{1-42}$ with the following CB ligands over this period (Abn-CBD> > THC = O-1602 > CBD > 2-AG). TEM of fibril imaging however was discordant with the ThT results, with only O-1602 altering $A\beta_{1-42}$ structure to an amorphous state versus control fibrils. O-1918 and 2-AG also altered fibril morphology to an amorphous appearance, without effect on ThT fluorescence. SH-SY5Y cells exhibited reduced cell viability following exposure to $A\beta_{1-42}$, with only ACEA inhibiting $A\beta_{1-42}$-mediated neurotoxicity. Cannabinoid ligand effects on ThT fluorescence alone are not predictive of an anti-amyloid, neuroprotective potential. However, some cannabinoid ligands were found to alter fibril morphology, and the neuroprotective correlates to this effect merit further investigation. Selected cannabinoid agonists targeting CB1 receptors augment neuronal protection to β-amyloid.

THE ROLE OF THE ENDOCANNABINOID RECEPTOR CB2 IN NEURODEGENERATION

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The endocannabinoid system (eCS) encompasses two G-protein-coupled receptors, the cannabinoid receptor 1 (CB1), the cannabinoid receptor 2 (CB2), their ligands and their respective synthesizing and degrading enzymes. Whereas the CB1 is expressed primarily in the brain, the CB2 is exclusively 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. In Alzheimer's disease, microglia are attracted by deposits of accumulated amyloid-ß peptides and exhibit an activated state including enhanced proliferation, increased expression of cell surface markers and production of chemokines and cytokines. In this study, the response of neonatal microglia and bone marrow-derived macrophages from $CB2^{-/-}$ mice to different pro-inflammatory stimuli was investigated. $CB2^{-/-}$ 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. The dimished expression of cell surface markers could not be observed in $CB2^{-/-}$ bone marrow (BM)-derived macrophages. Absence of the CB2 receptor, however, did not result in a difference in amyloid-ß phagocytosis in neonatal microglia, whereas BM-derived macrophages showed enhanced uptake of Aβ. Taken together, we show that $CB2^{-/-}$ microglia but not BM-derived macrophages have a limited capacity to respond to pro- and anti-inflammatory stimuli, whereas the phagocytosis capacity is not influenced. This suggests a functional impact of the CB2 in neuroinflammatory responses associated with neurodegenerative diseases that will be investigated in detail in a mouse model of Alzheimer's disease.

EFFECTS OF GENETIC DELETION AND PHARMACOLOGICAL INHIBITION OF FATTY ACID AMIDE HYDROLASE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Multiple sclerosis (MS) is an immune-mediated demyelinating neurodegenerative disease that typically has a relapsing-remitting pattern of progression superimposed on a gradual worsening of disease symptoms. Experimental autoimmune encephalomyelitis (EAE) is a model of MS where animals develop relapses, demyelination following induction of myelin-restricted autoimmunity and accumulate neurological deficits. Studies using the EAE model have provided evidence that cannabinoids are beneficial in reducing disease symptoms and may impact long term neurodegeneration, but side-effects of exogenous cannabinoid receptor agonists may limit their potential as therapeutic agents for MS. Targeting enzymes involved in degradation of endocannabinoids such as the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH) may be an attractive alternative strategy and here we have employed the *Faah* gene-knockout mouse and a FAAH inhibitor to address this issue.

The effect of *Faah* gene-knockout was investigated in two mouse strains: C57BL/6, which exhibit monophasic chronic EAE, and Biozzi ABH, which exhibit relapsing-remitting EAE. Induction and severity of EAE was comparable to wild-type animals indicating no impact of *Faah* deletion on the primary inflammatory phase of EAE. However, following cessation of inflammation, mice lacking FAAH accumulated significantly less neurological deficit based on clinical scoring of disability and motor control assessed using an accelerating rotarod assay. These data suggest that genetic deletion of FAAH may cause a reduction in neurodegeneration in EAE.

To assess the impact of pharmacological inhibition of FAAH on relapsing remitting EAE, mice were treated with PF-3845. Treatment was initiated following the relapse phase, at 10 $mg.kg^{-1}$ (intraperitoneal) daily or twice daily dose. By comparison with vehicle-treated controls, no significant effect on the clinical score of disability or rotarod assay measurements was observed.

The results indicate that whilst genetic deletion of FAAH provides a beneficial effect during the neurodegenerative phase of EAE, this effect was not necessarily replicated by administration of a FAAH inhibitor. Nevertheless, our data suggest that the life-long elevation of N-acylethanolamines (including anandamide) caused by genetic deletion of FAAH reduces the severity of neurological symptoms in EAE. Further studies are investigating the mechanisms involved as a basis for understanding of the underlying biological processes and potential therapeutic applications.

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TARGETING CB_1 RECEPTORS AND ABHD6 TO TREAT R6/2 MICE SYMPTOMS

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Huntington's disease (HD) is a devastating neurodegenerative disease that affects over 30,000 Americans, with an additional 150,000 at risk. To date, no cure and few palliative treatments are available for these patients. While it is known that the expression of ~3000 genes/proteins are deregulated in HD patients and HD models, specific proteins that are deregulated early in disease progression represent strong candidates for mediating HD pathogenesis and should be investigated as potential therapeutic targets. Down-regulation of CB_1 receptor expression constitutes one of the earliest molecular events occurring in HD and is commonly used as an index of disease initiation; yet few studies have tested the hypothesis that CB_1 down-regulation contributes to HD pathogenesis. Specifically, it is known that daily treatment of R6/2 mice with THC, a CB_1 receptor partial agonist, delays both HD pathogenesis and symptom onset, whereas similar treatment with potent CB_1 receptor agonists, WIN55212, produces no therapeutic benefit in HD mice. Additional evidence for a role of CB1 in HD pathogenesis come from data showing that disease progression is more aggressive in R6/2 mice treated with the CB_1 antagonist SR141617 or bred with $CB_1^{-/-}$ mice.

Based on this evidence, we sought to test if rescuing CB_1 receptor functionality (either genetically or pharmacologically) would affect HD pathogenesis in R6/2 mice, a rapidly progressing HD mouse model. First, we developed a new mouse line that allows for the genetic rescue of CB_1 receptor function in a cell specific manner. Thus, we generated *Rosa26* knock-in mice that contain a flox-stop *Cnr1* gene controlled by the chicken b-actin promoter (referred to as "fs-CB1" mice). In preliminary experiments, we found that Cre-mediated excision of the STOP codon leads to the induction of functional CB_1 receptor protein expression in defined cell populations that reaches expression levels approaching those found in wild-type mice. Our preliminary results also indicate that rescuing CB_1 receptor expression in the medium spiny neurons (MSN) of R6/2 (the major neuronal subpopulation that loose CB_1 receptor expression as a consequence of HD) has no drastic effect of symptoms (rotarod measurements) and has specific/subtle effects on R6/2 pathology (caspase 6 activation in the dorsolateral striatum). Our second approach was to rescue CB_1 receptor signaling by boosting the levels of 2-AG by treating R6/2 mice with the ABHD6 inhibitor WWL-123. Here we found clear therapeutic value in this compound as it reduces the incidence of seizures observed in these mice, with no effect on locomotor impairments (rotarod).

Together, our results suggest that genetically rescuing CB_1 receptors in MSN of R6/2 mice has little impact of HD pathogenesis, whereas inhibition of ABHD6 might represent a therapeutic approach to reduce seizure incidence in HD patients.

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PHYTOCANNABINOID-BASED MEDICINES AS DISEASE-MODIFYING AGENTS IN HUNTINGTON'S DISEASE

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Cannabinoids afford neuroprotection in experimental models of Huntington's disease (HD), although the most effective type of compound depends on the pathological characteristic(s) that mainly operate in each experimental model used. For example, CB_1 receptors become down-regulated, even in presymptomatic phases, in HD and their activation induces positive effects mainly in excitotoxic models (i.e. quinolinate-lesioned mice). CB_2 receptors, however, are up-regulated, mainly in glial elements, so that their pharmacological activation limits glial-derived events that aggravate striatal damage in animal models priming local inflammatory episodes (malonate-lesioned rats) and also excitotoxicity. In addition, antioxidant cannabinoids like cannabidiol (CBD) are effective against oxidative injury of striatal neurons as recapitulated in 3-nitropropionate-lesioned rats. Lastly, combinations of these effects have been found in the transgenic models that best reproduce HD pathogenesis (i.e. R6/2 mice). Therefore, these observations support the idea that the type of cannabinoid compound(s) that may be useful as a disease-modifying therapy in HD patients should be a multi-targeting cannabinoid or a combination of different selective compounds. We have proposed that the cannabis-based medicine Sativex®, which is a combination of botanical extracts enriched in both Δ^9-tetrahydrocannabinol (Δ^9-THC) and CBD, may serve this purpose in HD. Our proposal is based on evidence that a Sativex®-like combination of Δ^9-THC- and CBD-enriched botanical extracts attenuated cytotoxic events and preserved striatal neurons in the above models of HD in which striatal damage depends predominantly on specific cytotoxic mechanisms. Indeed, this Sativex®-like combination of Δ^9-THC- and CBD-enriched botanical extracts rescued the deficiency in endogenous antioxidant defenses and attenuated the up-regulation of calpain that occurs in 3-nitropropionate-lesioned rats (Sagredo et al., J. Neurosci. Res. 89, 1509-1518, 2011). It reduced edema and inflammatory events (astrogliosis and microgliosis) predominantly associated with malonate toxicity in rats (Valdeolivas et al., ACS Chem. Neurosci., in press, 2012). It improved various neurological deficits typical of R6/2 mice in parallel to an increase in the metabolic activity in various CNS structures of these mice (Valdeolivas et al., unpublished results). This potential of the combination of Δ^9-THC- and CBD-enriched botanical extracts in preclinical models has prompted us to study the issue at the clinical level, in a phase II-trial directed at assessing the efficacy of Sativex® as a disease-modifying agent in a population of early symptomatic HD patients (EudraCT 2010-024227-24), whose results will be available soon.

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GENERATION AND CHARACTERIZATION OF ABHD12–/– MICE, A MODEL OF THE NEURODEGENERATIVE DISEASE PHARC

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PHARC (*p*olyneuropathy, *h*earing loss, *a*taxia, *r*etinosis pigmentosa, *c*ataracts) is a recently recognized neurodegenerative disease caused by null mutations in the human *ABHD12* gene [1]. PHARC is a progressive, autosomal-recessive disorder of the peripheral and central nervous systems and the eye characterized by demyelination of sensomotor neurons, cerebellar atrophy and retinal dystrophy. Previously, we identified ABHD12 as a monoacylgycerol hydrolase responsible for a fraction of the 2-arachidonoylglycerol (2-AG) hydrolysis activity of mouse brain membranes [2]. Based on these results, Fiskerstrand et. al. suggested that PHARC may be caused by an "inborn error of endocannabinoid metabolism" [1], however the molecular and cellular mechanisms that drive PHARC disease are unknown.

In order to investigate the functions of ABHD12 in the nervous system and provide a mechanistic explanation of PHARC disease, we have generated and characterized a mouse model bearing a constitutive, targeted disruption of the *Abhd12* gene (ABHD12–/– mice). ABHD12–/– mice are viable, fertile and born at the Mendelian frequency. Young (<6 months old) ABHD12–/– mice are largely indistinguishable from their wild-type and heterozygous littermates, but they develop several PHARC-related phenotypes at advanced age. Biochemical and metabolomic characterization of ABHD12–/– mice has revealed only a very modest defect in bulk brain 2-AG metabolism and ABHD12–/– mice perform as their wild-type littermates in the tetrad tests for cannabimimetic activity. These results suggest that the effects of ABHD12 disruption might be endocannabinoid-independent and we will discuss our recent progress towards the identification of novel ABHD12-regulated metabolites that may become pathogenic in PHARC disease.

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PERIPHERAL CB_1R INVERSE AGONISM REDUCES DIET-INDUCED OBESITY BY REVERSING LEPTIN RESISTANCE

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Obesity-related leptin resistance manifests in loss of leptin's ability to reduce appetite and increase energy expenditure. Obesity is also associated with increased activity of the endocannabinoid system, and CB_1 receptor (CB_1R) inverse agonists reduce body-weight and the associated metabolic complications, although adverse neuropsychiatric effects halted their therapeutic development. Our results indicate that in mice with diet-induced obesity (DIO), the peripherally restricted CB_1R inverse agonist JD-5037 is equieffective with its brain-penetrant parent compound in reducing cumulative food intake, body weight, hepatic steatosis and insulin resistance, even though it does not occupy central CB_1R or induce related behaviors. The hypophagic and weight-reducing effects of JD-5037 are mediated by resensitizing DIO mice to endogenous leptin through rapidly reversing their hyperleptinemia. JD-5037 decreases the elevated plasma leptin levels by decreasing leptin expression and secretion by adipocytes and increasing leptin clearance via the kidney. Thus, inverse agonism at peripheral CB_1R not only improves cardiometabolic risk in obesity, but has antiobesity effects by reversing leptin resistance.

ACTIVATION OF HYPOTHALAMIC FATTY ACID AMIDE HYDROLASE BY LEPTIN: REGULATION OF FOOD INTAKE AND EFFECTS OF DIET-INDUCED OBESITY

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Crosstalk between leptin and endocannabinoid signaling is believed to be critical to the regulation of feeding behavior; however, the mechanism and nature of this interaction has not been well established. In examining this interaction, we determined that leptin exerts positive regulation over metabolism of the endocannabinoid anandamide (AEA), through an ability to activate fatty acid amide hydrolase (FAAH) in the hypothalamus. Leptin deficient mice exhibit increased AEA content and reduced FAAH activity within the hypothalamus, while administration of leptin to wildtype mice increases FAAH activity and reduces AEA content. Functionally, the ability of leptin to induce hypophagia and weight loss following food deprivation is blocked by pre-administration of the FAAH inhibitor URB597 (0.3 mg/kg), indicating that the anorectic actions of leptin require an increase in FAAH activity. Finally, following a nineteen week high fat diet regimen to induce obesity, the ability of leptin to regulate hypothalamic FAAH activity and AEA content is completely lost, suggesting that the uncoupling of leptin to FAAH activity may represent a key factor in leptin resistance in obesity. Collectively, these data provide a novel mechanism linking leptin to endocannabinoid signaling and indicate that the ability of leptin to increase AEA metabolism is a key factor in its anorectic actions, and that the loss of this coupling in obese states may represent a mechanism by which excessive feeding occurs in the face of adequate energy stores.

THE CANNABINOID RECEPTOR INVERSE AGONIST AM251 ALTERS MITOCHONDRIAL PHYSIOLOGY VIA PROTEOLYTIC DEGRADATON OF ERRα

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The endocannabinoid system regulates numerous physiological processes including energy balance and glucose metabolism, and becomes over-activated in obese individuals. While early clinical trials with the cannabinoid 1 receptor (CB1R) inverse agonist rimonabant produced favorable metabolic effects in obese humans, adverse psychiatric side effects were also observed. We have recently reported that the rimonabant analog, AM251, exerts off-target effects in CB1R-null cell lines by inducing the degradation of the orphan nuclear receptor Estrogen Related Receptor alpha (ERRα) protein (Fiori et al., Br. J. Pharmacol. 164 (2011) 1026-40). Since ERRα is a critical regulator of numerous mitochondrial genes, such effects may also translate into altered cellular metabolism. The objectives of this study were to determine the potential effects of AM251-induced ERRα degradation on mitochondrial function and elucidate the molecular mechanisms involved.

Using CB1R-null cell lines, AM251 (5 μM) increased mitochondria biogenesis while negatively impacting mitochondrial membrane potential, which was replicated with ERRα siRNA. Importantly, AM251 and related compounds all induced cell-specific ERRα protein degradation while mRNA expression remained unchanged, suggesting increased proteasomal activity. Indeed, pharmacological inhibition of the proteasome resulted in nuclear accumulation of ERRα in AM251-treated cells. Under these conditions, the integrity of a 220-kDa ERRα-containing oligomeric complex was maintained and its ability to bind to target DNA sequences was preserved in the presence of AM251. AM251 treatment was found to induce protein kinase C-mediated phosphorylation with subsequent ubiquitination of ERRα, two posttranslational modifications associated with efficient proteasomal degradation. Collectively, these data demonstrate a novel mechanism by which a class of CB1R inverse agonists non-specifically alters mitochondrial physiology via destabilization of ERRα.

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CANNABIDIOL IMPROVES IMPAIRED ENDOTHELIAL FUNCTION IN FEMORAL ARTERIES FROM ZUCKER DIABETIC RATS

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Incubation of aortae with cannabidiol (CBD) enhances vasorelaxation in a model of type 2 diabetes (Zucker Diabetic Fatty (ZDF) rats) (Stanley et al., ICRS symposium 2011). The aim of the present study was to investigate whether CBD might also improve endothelium-dependent vasorelaxation in smaller arteries of the ZDF rat model, and to elucidate the underlying mechanisms.

Femoral arterial rings were isolated in a wire myograph from male ZDF rats (340-405g, blood glucose = 25.1 ± 1.1 mM (n=11)), and their lean controls (276-315g, blood glucose = 7.6 ± 0.3 mM (n=8)). The segments were bathed in warmed (37^{o}C) and gassed (95% O_2/5% CO_2) modified Krebs'-Henseleit solution and set to a resting tension of 4.91mN. CBD (10μM), or its vehicle (5μl ethanol), were incubated for 2h before contracting with the α_1-adrenoceptor agonist methoxamine. Following this, cumulative concentration-response curves to the endothelium-dependent vasorelaxant acetylcholine ((ACh) 1nM-100μM) were constructed. The involvement of cyclooxygenase products was investigated by the additional incubation of indomethacin (3μM) or flurbiprofen (10μM). The roles of peroxisome proliferator-activated receptor gamma (PPARγ) and nitric oxide were examined using 1μM GW9662 and 300μM L-NAME respectively.

ACh caused concentration-dependent vasorelaxations in femoral arteries incubated with vehicle in both Lean and ZDF rats, with the maximal relaxation in arteries from ZDF rats being blunted (R_{max}; Lean = 63.8 ± 2.2 % (n=8), ZDF = 40.5 ± 2.1 % (n=6)). Incubation with CBD in arteries from Lean rats had no effect, but CBD significantly enhanced vasorelaxation to ACh in the arteries taken from ZDFs to achieve a maximal relaxation similar to that observed in Lean femoral arteries (R_{max} for ZDF + CBD = 68.5 ± 5.7 % (n=6)). The presence of indomethacin or flurbiprofen abolished the enhancement of Ach responses by CBD in ZDF arteries. Incubation with GW9662 had no effect on vasorelaxation to ACh in arteries from ZDF rats either in the absence or presence of CBD. The presence of L-NAME inhibited maximal relaxations to ACh in arteries from ZDF rats, but arteries that had been incubated with CBD still had an enhanced vasorelaxation compared to control (R_{max}; Lean + L-NAME = 6.7 ± 0.4 % (n=4), ZDF + L-NAME = 38.8 ± 1.2 % (n=6)).

In conclusion, CBD restores endothelium-dependent vasorelaxation to normal levels in ZDF rats. This involves a COX-mediated mechanism, and supports the hypothesis that CBD could benefit type 2 diabetes patients who suffer from endothelial dysfunction.

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THE EFFECTS OF ANANDAMIDE ON PERMEABILITY IN A CELL CULTURE MODEL OF THE BLOOD-BRAIN BARRIER

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The blood-brain barrier (BBB) is a selective barrier formed by the endothelial cells that line cerebral microvessels, and is vital for maintaining a precisely regulated microenvironment for reliable neuronal signalling. Other groups have demonstrated the ability of various cannabinoids (2-AG, CP55940 and WIN55212-2) to attenuate damage caused to the BBB in a variety of pathologies (chronic head injury, HIV infection and multiple sclerosis, respectively). To our knowledge, no studies have investigated the role that AEA plays in regulating BBB permeability, therefore, the aim of the present study is to establish whether AEA modulates BBB permeability.

To model the BBB, co-cultures of human brain microvascular endothelial cells and human astrocytes were grown to confluence on Transwell collagen-coated inserts (0.4 μm pore size, 12 mm diameter – Corning, USA). BBB permeability was measured by transepithelial electrical resistance (TEER) using STX2 electrodes and an EVOM^2 resistance meter (WPI, UK). Resistance readings were taken at various intervals over a 96 hour period, and all readings were compared to their baseline value. AEA and/or antagonist were added at 0 and again at 48 h when the medium was replaced. Statistical analysis was conducted using one-way ANOVA.

Administration of AEA to the luminal (i.e. endothelial) cells at 10 μM, but not 100 nM, 1 μM or vehicle, resulted in a significant, acute increase in BBB resistance compared to baseline (0 h) at 2, 50 and 52 h (i.e. immediately after AEA administration; $n = 9$ from 3 experiments; $P<0.05$-0.001). Receptor involvement was probed, with AM21, GW6471 and GW9662 providing evidence that neither CB_1, PPARα nor PPARγ mediated this response (n range from 4-6 from 2-3 separate experiments). However, the AEA-induced increase in resistance was significantly inhibited by AM630 ($P<0.05$-0.01) and capsazepine ($P<0.05$-0.01), indicating a role for CB_2 and TRPV1 receptor activation (n range from 4-6 from 2 separate experiments). The increase in resistance was also found to be mediated by the metabolites of AEA, as addition of URB597 inhibited the AEA-induced increase in resistance. Interestingly, initial experiments have shown that administration of 1 μM AEA to the abluminal chamber (i.e. the astrocytic side) significantly attenuated the increased resistance of the BBB at 2, 24 and 50 h ($P < 0.05$-0.001) when compared to vehicle ($n = 6$ from 3 experiments). AEA at 10 μM also displayed a significantly lower resistance than vehicle at 2 and 24 h ($P<0.05$-0.01), but not 50 h.

In conclusion, this study shows that luminal administration of AEA results in an acute increase in resistance of the BBB *in vitro*. This is mediated by CB_2, TRPV1 and metabolites of AEA. Ongoing studies are further investigating the effect of abluminal AEA administration, and the effect that AEA has in the presence of oxygen-glucose deprivation (an *in vitro* model of ischaemic stroke) is also be being explored.

MODULATION OF INFLAMMATORY CELL INVASION OF THE CNS BY A CANNABINOID-2 SELECTIVE AGONIST AFTER INJURY

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We have previously demonstrated an attenuation of the inflammatory response by a cannabinoid-2 selective agonist (O-1966) in a variety of CNS injury models including stroke, EAE and spinal cord injury. The goal of the current study was to further investigate mechanisms through which selective CB-2 receptor (CB2R) agonists could contribute to inhibition of inflammatory cell invasion of the CNS following injury. In a spinal cord injury model, treatment with (O-1966) caused a decrease in TLR4 expression, which is associated with activation of nuclear factor-κB. This effect could therefore contribute to the inhibition of the production of a number of proinflammatory cytokines, including TNF-α following injury. Myeloid cells such as dendritic cells, macrophages and monocytes have been postulated to play an important role in inflammatory cell invasion in CNS autoimmune diseases and possibly following CNS injury. We showed recently that selective CB2R agonists inhibit MMP-9 production in activated dendritic cells, macrophages and microglia, and significantly reduce in vitro and in vivo dendritic cell migration. The inhibition of MMP-9 expression was mediated through CB2R, reduction in cAMP, inhibition of MAPK, and finally reduction in AP-1 (c-Fos/c-Jun) DNA binding to the MMP-9 promoter. The inhibition of MMP-9 release and decreased migration of activated dendritic cells could represent an additional important mechanism contributing to the anti-inflammatory effect of CB2R agonists.

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CANNABINOIDS PREVENT THE DEVELOPMENT OF POST-TRAUMA SYMPTOMS IN A RAT MODEL OF PTSD

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Cannabinoids have recently emerged as a possible treatment of stress- and anxiety-related disorders such as post-traumatic stress disorder (PTSD). Here we examined whether cannabinoid receptor activation could prevent the effects of traumatic stress on the development of behavioral and neuroendocrine measures in a rat model of PTSD, the single-prolonged stress (SPS) model.

Rats were injected with the CB1/CB2 receptor agonist WIN55,212-2 (WIN) systemically or into the basolateral amygdala (BLA) at different time points following SPS exposure and were tested one week later for inhibitory avoidance (IA) conditioning and extinction, acoustic startle response (ASR), hypothalamic-pituitary-adrenal (HPA) axis function, and anxiety levels. Exposure to SPS enhanced conditioned avoidance and impaired extinction while enhancing ASR, negative feedback on the HPA axis, and anxiety. WIN (0.5 mg/kg) administered intraperitoneally 2 h or 24 h (but not 48 h) after SPS prevented the trauma-induced alterations in IA conditioning and extinction, ASR potentiation, and HPA axis inhibition. WIN microinjected into the BLA (5 μg/side) prevented SPS-induced alterations in IA and ASR. These effects were blocked by intra-BLA co-administration of the CB1 receptor antagonist AM251 (0.3 ng/side), suggesting the involvement of CB1 receptors.

These findings suggest that: (i) there may be an optimal time window for intervention treatment with cannabinoids after exposure to a highly stressful event, (ii) some of the preventive effects induced by WIN are mediated by an activation of CB1 receptors in the BLA, and (iii) cannabinoids could serve as a pharmacological treatment of stress- and trauma-related disorders.

ANANDAMIDE IN AMYGDALA-MEDIATED FEAR EXTINCTION, THREAT PROCESSING AND STRESS-REACTIVITY: A TRANSLATIONAL STUDY

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Endocannabinoids have been shown to mediate fear- and stress-related behaviors. Here we first investigated the pharmacokinetics of the selective FAAH inhibitor, AM3506, following intraperitoneal, intravenous and oral administration of the drug, by determining endocannabinoid levels in brain and plasma at different time intervals up to 1 day after administration. Next, we tested the effects of intraperitoneal administration of AM3506 on Pavlovian fear extinction in a mouse model of impaired extinction. We found that pre-extinction AM3506 decreased fear during an extinction retrieval test. This effect was reversed by either systemic and intra amygdala administration of the CB1R antagonist, Rimonabant. Anandamide levels in the basolateral amygdala were increased by extinction training and augmented by systemic AM3506 administration. Application of AM3506 to basolateral amygdala slices promoted long-term depression of inhibitory transmission, a form of synaptic plasticity. In healthy human subjects, we identified an association between a common lesser functioning *FAAH* gene variant (C385A) and quicker amygdala threat processing in an fMRI fearful faces task. The same *FAAH* variant was also associated with a higher score on a personality measure of stress-coping. Collectively these convergent findings provide novel evidence of the role of FAAH and anandamide in the modulation of amygdala-related fear behavior.

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MEDICAL CANNABIS/MARIJUANA USE IN POST-TRAUMATIC STRESS DISORDER: A LONGITUDINAL FOLLOW-UP STUDY

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Introduction: Posttraumatic stress disorder (PTSD) is a pervasive and devastating anxiety disorder, associated with other mental and somatic conditions such as depression and chronic pain of various origins. Many PTSD patients frequently use marijuana in order relieve persistent stress and depression and to reduce/alleviate pain. During last years more and more PTSD patients are obtaining a Cannabis/Marijuana as a part of their medical regiment within the frames of pain/psychiatric follow-up clinics. However, we still do not know what kind of the PTSD patients could get the main benefits from such treatment.

Methods: As a part of our routine consulting work, we assessed the mental condition of 167 adult PTSD patients, who applied to the Ministry of Health in order to obtain a license for the Medical Cannabis. The group consisted of patients with "pure" PTSD, PTSD patients with depression and patients suffering from PTSD/chronic pain comorbidity. Clinician-Administered PTSD scale (CAPS) was used for traumatic symptoms assessment and Quality of Life Scale (QOLS) was filled out. The changes in Clinical Global Impression-Improvement scale (CGI-I) were registered. The data on their somatic conditions and pain levels was provided by their treating physicians. Only about 50% were obtained the licenses for Medical Cannabis (study group). We followed up them longitudinally for a period of more than three years.

Results: As we observe in short time (and reported previously in Bonn), in most cases, a significant improvement in Quality of Life and pain scores, with positive changes in CAPS scores was observed. Under combine (Cannabis + conventional medications) treatment, the patients reported a discontinuation or lowering the dosage of pain killers and sedative agents. The vast majority of improved PTSD patients belonged to groups with either pain and/or depression comorbidity. Again, no exacerbations or serious adverse events were reported.

Conclusion: This longitudinal follow-up naturalistic study represents a consequence attempt to assess and to monitor the effectiveness and safety of the Medical Cannabis use in PTSD patients. The results confirms our previous short-term observations, showing good tolerability and other benefits (especially in the quality of life & on CGI-I) of such flexible combine approach, particularly, in the patients with either pain and/or depression comorbidity. Obviously, further large-scale investigations are needed to substantiate our observations and to elaborate the most effective and safe therapeutic approaches to these difficult-to-treat group.

ANXIOGENIC-LIKE EFFECT OF THC IS LOST IN MICE LACKING BETA-ARRESTIN-2: EVIDENCE FOR SEX-DEPENDENT EFFECTS

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Like most G-protein coupled receptors (GPCR), the CB1 cannabinoid receptor is regulated by GRK-mediated phosphorylation and ß-arrestin-dependent internalization. However, recent data from Caron and colleagues regarding D2 receptor signaling indicates that GPCR/ß-arrestin complexes also form signaling nodes in neurons that can couple GPCR activation to several important kinases, including activation of glycogen synthase kinase 3 (GSK3; Cell 132:125-36, 2008).

The cannabis-derived cannabinoid, Δ^9-tetrahydrocannabinol (THC), binds to CB1 receptors with high affinity. However, THC acts as a very weak partial agonist in GDP/GTP exchange assays. On the other hand, we have found that THC is a highly efficacious agonist in assays of activation of GSK3. These biochemical findings lead us to hypothesize that THC promotes ß-arrestin mediated signaling. To test this hypothesis, we examined THC-induced changes in behavior in ß-arrestin-2 knock out mice. Previous studies of Brievogel and colleagues had demonstrated that THC-induced hypothermia and antinociception were enhanced in these mice compared to wild-type (WT), suggesting that ß-arrestin-2 contributes to desensitization (Behav. Pharmacol. 19:298-307, 2008)

We chose to examine the role of ß-arrestin-2 in the effects of THC on mouse behavior in the elevated plus maze (epm). Unlike other CB1 receptor direct and indirect agonists, earlier studies from our laboratory showed that THC produces anxiogenic-type behaviors in the epm, including reduced time spent and entries into the open arms (JPET 318:304-11, 2006). We recapitulated these effects in male WT mice (8-10 weeks of age) in the current study, finding that 3 mg/kg THC produced a significant decrease in open arm entries. However, this effect of THC was lost in ß-arrestin-2 null littermates. There were no effects on THC on total arm entries or distance traveled in either genotype. We examined the effects of THC in female mice of the same age. Remarkably, 3 mg/kg THC produced a significant increase in the time spent in the open arms of the female mice, which was not affected by loss of ß-arrestin-2.

Taken together, these data suggest that the anxiogenic-like effects of THC on the epm are sex-specific (only occurring in male mice) and that this effect of THC requires ß-arrestin-2. These findings are consistent with THC/CB1 receptor-induced formation of ß-arrestin-2 signaling complexes that could influence fear behaviors in a manner opposite to that of CB1 receptor/G protein signaling.

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ENDOCANNABINOID-GLUCOCORTICOID INTERACTION IN THE BASOLATERAL AMYGDALA DURING MEMORY CONSOLIDATION

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Introduction: The basolateral complex of the amygdala (BLA) is a critical component of the neural circuitry mediating emotional arousal and stress hormone effects on the formation of long-term memory for emotionally arousing experiences. Activated endocannabinoid signaling in the brain controls adaptation processes to aversive situations and limits the stress reaction. Circulating glucocorticoid hormones potentiate this effect dose-dependently [1]. Furthermore, it is well known that the glucocorticoid and the endocannabinoid systems interact during memory consolidation [2]. It is, however, unknown if these glucocorticoid effects are mediated by activation of fast acting membrane-associated glucocorticoid receptors (GRs) or via diffusion across the cell membrane and stimulation of cytosolic GRs, resulting in a slow genomic effect.
Here, we show that cannabinoid CB1 receptor blockade attenuates the consolidation of memory induced by a GR agonist when infused directly into the BLA and that memory consolidation by CB1 activation happens downstream from the GR and could therefore not be blocked by a GR antagonist. Additionally, we found that glucocorticoid effects on memory consolidation depend on an activation of membrane-bound GRs.
Methods: Male Sprague-Dawley rats were implanted bilaterally with cannulas in the BLA and trained on an inhibitory avoidance task, using a mild inescapable footshock as an aversive stimulus. Drug administration into the BLA was performed immediately after training to selectively influence the consolidation phase of memory formation. Retention of the traumatic memory was tested 48 h later by recording the time until reentry into the shock compartment of the inhibitory avoidance apparatus.
Results: The GR agonist RU28362 induced a dose-dependent increase in retention latency, which indicates stronger memory consolidation ($p<0.048$). We also found a dose-related enhancement of memory consolidation by corticosterone-BSA ($p=0.008$), a membrane-impermeable glucocorticoid. Both effects were blocked by co-administration of the CB1 receptor antagonist AM251 ($p=0.03$). In a following experiment, the CB1 receptor agonist WIN55,212-2 resulted in memory enhancement and this effect could not be blocked by co-administration of RU38486, a GR antagonist ($p<0.001$).
Conclusions: Memory consolidation induced by a GR agonist in the BLA is blocked by concomitant administration of an CB1 receptor antagonist whereas memory enhancement induced by CB receptor stimulation is not influenced by GR blockade. These findings indicate that the endocannabinoid system plays a crucial regulatory role in mediating memory consolidation induced by activation of membrane-associated GRs, and that the endocannabinid system acts downstream from the glucocorticoid system in the BLA. This is consistent with recent findings in the paraventricular nucleus of hypothalamus [3]. The interaction of membrane-associated GRs with the endocannabinoid system points to new pharmacological approaches to prevent traumatic memory formation in humans during high emotional arousal states.

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CANNABINOIDS MODULATION OF OBJECT RECOGNITION MEMORY IN RATS: INVOLVEMENT OF THE GLUCOCORTICOID SYSTEM

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Although extensive evidence indicates that cannabinoid drugs influence cognitive performance, the findings describing both enhancing and impairing effects independently from the pharmacodynamic properties, have been ambiguous. Here, we investigated the effects of posttraining systemic administration of the cannabinoid receptor agonist WIN55,212-2 on short- and long-term retention of object recognition memory under two conditions that differed in their training-associated emotional arousal. WIN55,212-2 (0.1, 0.3, 1.0 mg/kg) was injected intraperitoneally immediately after a 3-min training trial. In rats that were not previously habituated to the experimental context, WIN55,212-2 impaired 1-hr retention performance in a U-shaped relationship, but enhanced retention when rats were tested 24-hr after training. Conversely, in rats that had received extensive prior habituation to the experimental context, WIN55,212-2 enhanced 1-hr retention without significantly affecting 24-hr performance. Since growing evidence suggests that the endocannabinoid system interacts with the adrenocortical axis, we investigated whether the effects of WIN55,212-2 on object recognition memory could be related to an alteration of plasma corticosterone levels. Interestingly, we found that WIN55,212-2 induced opposite effects on plasma corticosterone levels depending on the training-associated emotional arousal. Particularly WIN55,212-2 enhanced corticosterone levels of rats not habituated to the experimental context while it decreased them in rats previously exposed to the context. We also examined whether the corticosterone synthesis inhibitor Metyrapone (35 mg/kg, i.p.), administered 40 min before the training trial, could prevent WIN55,212-2 effects on short- and long-term performances of rats in the WITHOUT-habituation condition, thus mimicking the performance of rats in the WITH-habituation condition. We found that Metyrapone prevented WIN55,212-2 impairing effect on 1-hr retention memory and WIN55,212-2 enhancing effect on 24-hr retention memory of rats in the WITHOUT-habituation condition. Furthermore, Metyrapone administration was able to prevent the increase of plasma corticosterone levels induced by WIN55,212-2 administration in the WITHOUT-habituated rats. These findings give the first evidence that cannabinoids induce opposing effects on short- and long-term memory performance as well as on plasma corticosterone levels depending on the level of emotional arousal at encoding.

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CANNABINOID RECEPTOR CB2. A NEW DRUG TARGET FOR MULTIPLE MYELOMA INTERVENTION

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Multiple myeloma (MM) remains an incurable plasma cell malignancy with systematic morbidity and a median survival of 3 to 5 years. A diverse spectrum of agents has shown therapeutic potential in myeloma clinic, e.g., thalidomide, arsenic trioxide and bortezomib, but high relapse rates and drug resistance continue to plague these therapies. Thus, novel targets and additional pathways need to be discovered to improve the patient outcomes. Here, we report for the first time a new finding that CB2 but not CB1 was highly expressed in human multiple myeloma (MM) cell lines and primary CD138+ cells. In addition, we have discovered a novel class of CB2 ligands, phenylacetylamide (PAM) analogs, by in silico chemical genomics screening and also biologically validated the compounds by CB2 binding and cellular cAMP bioassays. Our discovered novel CB2 ligands demonstrated potent inhibition of MM cell proliferation (IC_{50}: 0.62~2.5 μM). Such inhibition is believed to be CB2 receptor-dependent as silencing CB2 gene expression markedly attenuated the anti-MM activities. Importantly, PAM can overcome the chemoresistance of MM cells against dexamethasone or melphalan, but exhibit minor cytotoxic effects on human normal mononuclear cells. This unique molecular target warrants further studies to assess *in vivo* response and in combination regimens for MM treatment. (www.CBligand.org/xielab, *Sean Xie, email: xix15@pitt.edu).

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IDENTIFICATION OF A FAMILY OF PEPTIDE ENDOCANNABINOIDS (PEPCANS): EVIDENCE FOR ALLOSTERIC MODULATION OF CB1 RECEPTORS

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The alpha hemoglobin-derived dodecapeptide RVD-hemopressin (RVD-Hpα) has been proposed to be an endogenous agonist for the cannabinoid receptor 1 (CB1 receptor). To study this peptide we have raised monoclonal antibodies (mAbs). Using an immunoaffinity-mass spectrometry approach we have identified a family of N-terminally extended peptides in addition to RVD-Hpα from rodent brain extracts and human and mouse plasma. Immunohistochemical analyses using the mAbs revealed discrete cytoplasmic localizations of these peptides in rodent brain. We designated these peptides Pepcan-12 (RVD-Hpα) to Pepcan-23, referring to peptide length. Using a competitive ELISA for the quantification of Pepcans we found low concentrations of Pepcans in human plasma and significant concentrations in rodent brains. The Pepcans most frequently found were tested for CB receptor binding in different displacement experiments using the cannabinoid radioligands [^{3}HWIN55,212-2], [3H]CP55940, [3H]anandamide, as well as fluorescently labeled Pepcan-12 derivative (FL4). Using FL4 we show that Pepcans-12-17 specifically bind to both CB1 and CB2 receptors with low nanomolar Ki values. Moreover, our data clearly indicate that Pepcans interact with novel CB receptor sites different from the classical endocannabinoid binding site. Functional assays measruing cAMP signaling revealed that the most abundant Pepcan-12 is a potent CB1 receptor allosteric antagonist of the endocannabinoid 2-AG and also modulates the action of other cannabinoids. Overall, the Pepcan family identified here may play a role in the endocannabinoid system by modulating signals from endocannabinoids cell-type or tissue specifically.

THE CB_1 RECEPTOR ALLOSTERIC MODULATOR, ORG 27569, HAS DIFFERENT EFFECTS ON THE POTENCIES WITH WHICH CP55940, O-2050 AND SR141716A DISPLACE [^{3}H]CP55940 OR [^{3}H]SR141716A FROM MOUSE BRAIN CB_1 RECEPTORS

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Previous experiments have shown that Org 27569 binds allosterically to the cannabinoid CB_1 receptor in a manner that increases orthosteric binding of [^{3}H]CP55940 and decreases orthosteric binding of [^{3}H]SR141716A to this receptor [1]. Here we have investigated whether a concentration of Org 27569 (10 μM) that produces such changes in orthosteric binding affects the abilities of CP55940 and SR141716A to displace [^{3}H]CP55940 or [^{3}H]SR141716A from CB_1 receptors. We have also performed experiments with O-2050, which displays similar CB_1 affinity but lower CB_1 efficacy than CP55940 [2].

In our experiments, we tested the ability of CP55940, O-2050 and SR141716A to displace [^{3}H]CP55940 or [^{3}H]SR141716A from specific binding sites present in MF1 mouse whole brain membranes, as described previously [3]. These experiments were performed in the presence and absence of 10 μM Org 27569. EC_{50} values, E_{max} values and 95% confidence limits were calculated using GraphPad Prism 5.0.

Org 27569 significantly decreased the EC_{50} values of CP55940 (29.7-fold) and O-2050 (6.2-fold) for their displacement of [^{3}H]SR141716A from 6.83 nM (3.15 & 14.80) to 0.23 nM (0.08 & 0.71), and from 6.49 nM (3.69 & 11.41) to 1.04 nM (0.39 & 2.76), respectively. The 95% confidence limits of these EC_{50} values are shown in brackets (n=4). Org 27569 did not affect the E_{max} of these agonists or the EC_{50} or E_{max} of either agonist for its displacement of [^{3}H]CP55940. In contrast, Org 27569 increased the EC_{50} of SR141716A for its displacement of [^{3}H]CP55940 but not for its displacement of [^{3}H]SR141716A. In the absence of Org 27569, CP55940 and O-2050 displaced [^{3}H]CP55940 with significantly greater potency than they displaced [^{3}H]SR141716A, whereas SR141716A displaced these two tritiated ligands with equal potency.

Our findings can be explained in terms of the two-state model of receptor activation which proposes that agonists display greater affinity for a precoupled state of the receptor (R*) whereas inverse agonists display greater affinity for an unprecoupled state of the receptor (R). Thus, it is possible that Org 27569 produced its effects on CP55940-, O-2050- and SR141716A-induced displacement of [^{3}H]SR141716A or [^{3}H]CP55940 by increasing the proportion of CB_1 receptors in the R* state. Org 27569 may have enhanced the [^{3}H]SR141716A displacing potency of O-2050 less than that of CP55940 because, being a lower efficacy CB_1 agonist than CP55940, O-2050 binds to the R state of the receptor to a greater extent than CP55940. We are now exploring the possibility that Org 27569 could be used in CB_1 binding experiments to provide a provisional classification of novel compounds as CB_1 agonists, inverse agonists or antagonists, and to predict the efficacy that these compounds display as CB_1 agonists or inverse agonists.

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IDENTIFICATION OF THE ORG27569 BINDING SITE AT THE CB1 RECEPTOR: IMPORTANCE OF K3.28 INTERACTION TO THE ORG27569 EFFECT ON BASAL SIGNALING

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The CB_1 allosteric modulator, ORG27569 has the paradoxical effect of increasing the binding affinity of CP-55940 (a CB_1 orthosteric agonist), while at the same time decreasing its efficacy. In addition, ORG27569 acts as an inverse agonist at the CB_1 receptor by reducing basal activity. In work to be presented, we have used computational methods combined with ligand synthesis, mutation and functional studies to probe the apparent dichotomy between ORG27569's effects on agonist binding and efficacy, as well as its influence on basal signaling. Using a computational model of the CB_1 receptor activated state in complex with CP-55940 (Kapur et al., *Mol. Pharmacol.* **2007**), Glide docking studies were used to identify a binding site for ORG27569 in the THM3/TMH6/TMH7 region that is consistent with previous pharmacological data for ORG27569 (Price et al., *Mol. Pharmacol.* **2005**).

This site explains the ability of ORG27569 to increase the binding affinity of CP-55940, as the combination of two complementary effects: 1) CP-55940 acquires additional interactions when bound to the receptor (in the presence of ORG27569), and 2) ORG27569 sterically blocks the exit of CP-55940 from the receptor more profoundly than it blocks entrance into the receptor (validated by kinetic experiments (Price et al., *Mol. Pharmacol.* **2005**)). The identified ORG27569 binding site also is consistent with a decrease in CP-55940 efficacy, as ORG27569 prevents a key electrostatic interaction between an extracellular loop 3 (EC3) residue K373 and $D2.63^{(176)}$. This interaction is important to orthosteric ligand efficacy, as shown by recent mutation studies (Kapur et al., *J. Pharmacol. Exp. Ther*, **2008**; Marcu et al., *ICRS*, **2011**).Thus, the docking site for ORG27569 at CB_1 is capable of explaining how ORG27569 can increase the binding affinity of CP-55940, yet impair its CB_1 signaling.

Finally, docking studies of ORG27569 (alone) in both the CB_1 inactive and activated states revealed that docking in the inactive state is energetically favored. This energetic preference explains why ORG27569 acts as an inverse agonist. These docking studies identified a key interaction between the piperidine ring nitrogen of ORG27569 and $K3.28^{(192)}$ that only forms when ORG27569 is docked in the inactive state. We have shown previously that interaction with this residue is key to inverse agonism at CB_1 (Hurst et al., *Mol. Pharmacol.* **2002**). To test the importance of this interaction, an ORG27569 analog in which the piperidine ring was replaced with a cyclohexyl ring was synthesized. Functional studies revealed that this analog lacks any effect on basal signaling, but still functions as a negative allosteric modulator. [Support: DA003934 and DA021358 (PHR)]

ALLOSTERIC MODULATION OF HUMAN CB1 RECEPTORS

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Allosteric modulation of G protein-coupled receptors is an emerging therapeutic strategy that may provide improved selectivity and safety, along with maintenance of spatial and temporal regulation associated with native receptor signalling. The binding of an allosteric modulator may cause a conformational change in the receptor protein that is transmitted to the orthosteric site, in essence creating a "new" GPCR with its own set of binding and functional properties. In addition, allosteric modulators may result in additional signalling bias enabling pathway-selective modulation of receptor function.

In addition to activation or inhibition of signalling pathways an important property of orthosteric drug therapy is the resultant desensitization and down regulation of receptors, however, despite an increasing interest in allosteric modulators as potential therapeutics, few studies have investigated the impact of allosteric modulation on these key pathways. For the cannabinoid CB1 receptor several allosteric modulators have been described. Price et al [1] reported a series of novel structurally related small molecules from Organon (Org 27569, Org 27759 and Org 29647) which all enhanced the binding of the agonist CP55,940, but produced insurmountable antagonism in GTPγS assays against both CP55,940 and WIN55,212. PSNCBAM, initially described by Horswill et al [2] likewise enhanced agonist binding while antagonizing GTPγS activation and the ability of the cannabinoid to reduce mIPC frequency in cerebellar neurons [2, 3], but in addition showed more pronounced inhibition of CP55,940 efficacy than of WIN55,212, suggested ligand dependent allosteric modulation.

Given these complex responses, the effect of the allosteric ligands on receptor regulation are not readily predictable. Chronic administration of cannabinoids leads to the rapid development of tolerance. This tolerance is accompanied by changes in CB_1 receptor number and/or function in vivo [4-6]. Previous studies have found that the β-arrestin-2 and GRK3 are capable of desensitizing CB_1 receptor-mediated activation of GIRK channels [7]. Activated CB1 is subsequently internalized [7, 8], and then degraded [9], and resensitization likely requires the delivery of newly synthesized receptors to the cell surface. As the data for each allosteric modulator's efficacy has come from different laboratory groups and predominantly from mouse CB1, we have directly compared these ligands in signalling assays on hCB1, and have then examined the impact of Org 27569 and PSNCBAM on agonist induced desensitization and internalization of the CB1 receptor. Here we report that the allosteric modulators produce complex ligand and signalling bias at human CB1, leading to rapid desensitization but decreased internalisation of the receptor.

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FUNCTIONAL INTRACELLULAR CANNABINOID RECEPTORS

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Endocannabinoids are lipid messengers generated intracellularly, on demand from phospholipid precursors, and are not stored in vesicles. As a result, they may target intracellular or extracellular cannabinoid receptors from the same cells, or in the neighboring cells. We have previously shown that intracellular activation of intracellular CB_1 cannabinoid receptors by anandamide mobilizes Ca^{2+} from endoplasmic reticulum and lysosomes in CB_1-transfected HEK293 cells or NG-108 cells (Brailoiu et al J. Biol. Chem. 28 (2011) 29166-74). Using calcium imaging and microinjection techniques we now examined the functionality of intracellular CB_2 and GPR55 in cells endogenously expressing or transfected with these receptors.

Intracellular injection of 2-arachidonoylglycerol (2-AG) in CB_2-expressing CHO cells produced a robust increase in the cytosolic Ca^{2+} concentration, which was abolished by AM630, a CB_2-selective competitive antagonist. On the other hand, extracellular application of 2-AG produced only a modest increase in cytosolic Ca^{2+}. Inhibition of 2-AG degradation by treatment with JZL184, an irreversible inhibitor for monoacylglycerol lipase, produced a similar increase in Ca^{2+} as the intracellular injection of 2-AG. In endothelial cells, which endogenously express CB_2 receptors, intracellular injection of 2-AG also increased cytosolic Ca^{2+}; the response was prevented by AM630.

In U2OS cells stably transfected with GPR55, intracellular injection of GPR55 agonists lysophosphatidylinositol (LPI) and AM251 produced a faster and more robust increase in cytosolic Ca^{2+} than their extracellular application. LPI- and AM251-induced increase in Ca^{2+} were prevented by pretreatment with the GPR55 antagonist, ML-193 (Heynen-Genel et al, (2011) Probe Reports from the NIH Molecular Libraries Program). Rat cortical neurons, which endogenously express GPR55, responded to intracellular injection of LPI and AM251 by an increase in cytosolic Ca^{2+}; the response was sensitive to ML-193.

We also tested the response of periaqueductal gray (PAG) neurons, which express CB_1 receptors. Intracellular injection of anandamide to PAG neurons increased cytosolic Ca^{2+}; the response was abolished by pretreatment with SR141716A, a CB_1 receptor antagonist.

Taken together, our results support the functionality of intracellular CB_1, CB_2 and GPR55 receptors.

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CANNABINOID RECEPTOR INTERACTING PROTEIN (CRIP1a) MODULATES CB1 RECEPTOR TRAFFICKING, ACTIVITY AND SIGNALING

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The CB_1 receptor (CB_1R) is one of the most abundantly expressed GPCR's in the CNS, where it helps modulate a variety of processes including neuro-protection, appetite, and mood. The CB_1R has been targeted therapeutically for seizures, nausea, dyslipidemia, depression and addiction; however, CB_1 compounds have had limited clinical use due to their side effect profiles. Proteins that interact with GPCR's provide a new avenue for the modulation and fine-tuning of receptor activity. The Cannabinoid Receptor Interacting Protein (CRIP1a) was initially discovered as an accessory protein for the CB_1R. CRIP1a is highly expressed in vertebrates and was first characterized by its ability to reverse the tonic inhibition of voltage-gated Ca^{2+} channels induced by CB_1 (Neihaus JL et al., Mol Pharmacol 2008). Recently, CRIP1a was shown to switch CB_1 neuro-protection from an agonist to antagonist driven mechanism (Stauffer et al., Neuroscience Letters 2011). To date, little is known regarding the cellular mechanisms governing CRIP1a-mediated modulation of CB_1R activity. The objective of this study was to investigate the cellular mechanisms underlying the CRIP1a-CB_1 interaction by manipulating levels of CRIP1a in cells that endogenously express both CB_1R and CRIP1a.

We created clones of the N18TG2 neuroblastoma cell line that stably over-express or knock-down CRIP1a mRNA and protein levels. CB_1 receptor transcript levels were unaltered in CRIP1a over-expressing clones when compared to wild-type cells. CB_1R protein levels in CRIP1a over-expressing clones were unchanged from WT. However, CRIP1a knock-down clones displayed a deficit in total CB_1 protein levels. Over-expression of CRIP1a led to a modest reduction in CB_1 receptor density on the plasma membrane when compared to wild-type cells. Stimulation by the agonist WIN55212-2 led to internalization of CB_1R in WT cells, but not in CRIP1a over-expressing cells. Upon continued exposure to agonist, cell surface CB_1R density was recovered within minutes in both WT and CRIP1a over-expressing clones. Exposure to the CB_1 antagonist rimonabant, led to an increase in cell-surface density of CB_1 receptors in both WT as well as CRIP1a over-expressing cells.

To determine the role that CRIP1a plays in regulating CB_1R-mediated signal transduction, N18TG2 WT and CRIP1a over-expressing or knock-down clones were treated with tetrahydrolipstatin to block 2-arachidonoyl glycerol production, and phosphoERK levels were determined using an in-cell Western assay. CB_1-mediated ERK phosphorylation in WT N18TG2 cells was reduced by rimonabant, consistent with "constitutive activation" of signal transduction. When compared to WT cells, CRIP1a over-expressing clones displayed a reduction in basal phosphoERK levels, whereas CRIP1a knock-down clones showed a significant increase in basal pERK levels. Stimulation of ERK phosphorylation by the CB_1 agonist WIN55212-2 was unaltered in CRIP1a over-expressing clones compared with WT. These findings suggest that the function of CRIP1a may be to modulate internalization-associated CB_1R constitutive signal transduction.

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UNUSUAL TRAFFICKING OF CANNABINOID RECEPTOR 2: AGONIST-INDUCED UPREGULATION OF SURFACE RECEPTORS

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Cannabinoid receptor 2 (CB2) is currently under scrutiny as a potential target in the treatment of various inflammatory conditions and other pathologies. To-date only a few studies have investigated CB2 intracellular trafficking, however as the major determinant of sub-cellular receptor localization these pathways are likely to be central to drug responsivity. We recently published the first thorough characterization of CB2 trafficking and reported that CB2 undergoes agonist-induced internalization and subsequently recycles to the cell surface, while being resistant to degradation even with chronic agonist stimulation [1].

While undertaking these experiments we noted an unexpected trafficking phenotype: although CB2 rapidly internalizes when agonist is applied (monitored by labeling surface receptors with antibody prior to adding agonist), overall surface CB2 expression is maintained and increases above basal levels with prolonged agonist stimulation (measured by labeling surface receptors with antibody at the conclusion of agonist treatment). That is, the receptors originally on the cell surface internalize in response to agonist stimulation but are rapidly replaced by newly-delivered receptors, preventing an overall reduction in cell surface CB2 and ultimately resulting in upregulation. We observe this phenomenon in both stably transfected cell lines and U937 cells endogenously expressing CB2. This is contrary to the internalization phenotype observed for the majority of other G protein-coupled receptors where the continued presence of agonist results in reduced net surface receptor expression through internalization of any receptors newly delivered to the cell surface.

To investigate further, we have developed a human CB2 receptor construct with a thrombin protease-cleavable epitope tag at the extracellular N-terminus. Coordinated use of tag cleavage and antibody labeling allows us to measure delivery of receptors to the surface independently of those present at the start of the experiment, and also directly quantitate intracellular CB2. Using this receptor stably expressed in HEK-293 cells, and our previously reported assays to quantitate receptor trafficking [2], we will report on the rates and mechanisms of agonist-induced surface CB2 upregulation with three CB2 ligands (CP55,940, WIN55,212-2 and anandamide).

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21 NOVEL N-ACYL AMIDES, INCLUDING N-DOCOSAHEXEANOYL ETHANOLAMINE, HAVE ACTIVITY AT TRPV1-4 THE PUTATIVE IONOTROPIC CANNABINOID RECEPTORS

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De Petrocellis and colleagues (2012) have shown that TRPV1-4 are activated by a range of exogenous cannabinoids supporting the hypothesis that the TPRV family of channels are putative ionotropic cannabinoid receptors. Anandamide as well as the structurally similar and highly potent *N*-arachidonoyl- and *N*-oleoyl dopamine (NADA and OLDA) have been shown to activate TRPV1 for over a decade. The regional production of NADA and OLDA (restricted primarily to striatum and hippocampus), however, created doubt that these ligands were activating any spinal or peripheral TRPV1 receptors, which has lead to an ongoing search for additional and/or more ubiquitous TRPV ligands. Therefore, to test the hypothesis that TRPV1-4 would be activated by additional *N*-acyl amides, we screened 81 *N*-acyl amides (the majority of which have now been identified endogenously) for both agonist and antagonist activity.

Calcium mobilization assays were performed on HEK-293 cells over-expressing either TRPV1, TRPV2, TRPV3, or TRPV4 using Fura2AM, a calcium-sensitive dye, monitored by a Molecular Devices FlexStation as previously described (Rimmerman et al, 2008). Agonist screens measured relative florescence changes (an indirect measurement of intracellular calcium) as a result of *N*-acyl amides being added 30 seconds after baseline measurements, whereas, antagonist screens were performed in cells that had been pre-incubated with *N*-acyl amides and then challenged with known exogenous TRPV1-4 activators.

Here, we identified 21 novel *N*-acyl amides as putative ligands for TRPV1-4 channels. Importantly, *N*-docosahexaenoyl ethanolamine (D-EA) is as potent as Anandamide at TRPV1; however, 3 *N*-acyl GABAs, including D-GABA, show a higher potency than either AEA or DEA. In addition, D-serine, D-glycine, and D-aspartic acid activate TRPV1. We also show that D-proline acts as a potent antagonist at TRPV1, therefore, the structure activity relationship for endogenous TRPV1 activity is clustering more around docosahexaenoyl conjugates than arachidonoyl conjugates. *N*-palmitoyl tyrosine (P-Tyr) activates both TRPV2 and TRPV4; however, 5 additional *N*-acyl amides also activate TRPV4. We did not identify an activators of TRPV3; however, we did show that 3 *N*-acyl valines function as TRPV3 antagonists.

These data add to the growing literature that activation of TRPV1-4 follows an opportunistic strategy in which a wide range of structurally similar endogenous and exogenous lipids are capable of regulating calcium mobilization through these channels. In that TRPV1-4 are activated by cannabinoid compounds, an extension of this argument would be that the endogenous lipids identified here that activate these TRPV channels may, likewise, be considered endogenous cannabinoids.

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ASSOCIATION BETWEEN TUMOUR pAKT AND CB_1 RECEPTOR EXPRESSION IN PROSTATE CANCER

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Introduction: We have reported that a high expression of CB_1 receptors in prostate cancer tumour samples obtained at diagnosis is associated with disease severity and with a poor prognosis (Chung *et al., Eur J Cancer* 45 [2009] 174-82). A possible mechanism for this finding has been suggested from studies with transfected astrocytoma cells, where a high CB receptor expression causes the Akt survival pathway to be recruited in response to CB receptor sitmulation (Cudaback *et al., PLoS ONE* 5 [2010] e8702). In the present study, we have investigated the expression of phosphorylated Akt (pAkt) in the same prostate cancer tissue microarray as used for the CB_1 receptor study in order to see whether the two parameters are correlated, and whether they co-influence disease-specific survival.

Method: Formalin-fixed, paraffin-embedded specimens of non-malignant tissue (1-4 cores/patient) and tumour tissue (1-8 cores/patient) obtained at diagnosis from patients who underwent transurethral resection surgery for prostatic enlargement were stained using an antibody directed towards Ser^{473} of pAkt. Epithelial immunoreactive (IR) intensity and distribution were scored by two independent investigators.

Results: A total of 282 cases were scored for tumour pAkt-IR. The pAkt-IR was significantly correlated with tumour CB_1R-IR (Spearman's rho 0.27, $p<0.001$, $n=276$) and with the Gleason score (Spearman's rho 0.39, $p<0.001$, $n=282$). Partial correlation analysis using the Spearman's rho values indicated that the correlation between CB_1R and pAkt-IR was still significant even when controlled for the Gleason score. When CB_1IR was divided into quartiles, and the pAkt-IR split at the median, it was noted that 71% of the cases with the highest CB_1IR and a pAkt-IR score greater or equal to the median had a Gleason score 8-10, as compared with 23 and 40% for CB_1IR in the first three quartiles combined and pAkt < and ≥ the median value, respectively. Approximately 2/3 of the cases were followed by watchful waiting until the appearance of metastases, thereby allowing assessment of the prognostic value of biomarkers. For cases with a Gleason score 6-10, COX proportional-hazards regression analyses indicated that pAkt-IR had modest prognostic value, and in Kaplan-Meier survival plots, the mean 15 year disease-speicific survival was 51 and 36 months for cases with pAkt-IR scores below and ≥ the median value, respectively. pAkt-IR, however, did not provide additive information to that provided by the Gleason score and by the tumour CB_1IR.

Conclusion: pAkt-IR is associated with CB_1IR in prostate cancer. Although association studies do not investigate cause and effect, these data are consistent with the suggestion by Cudaback *et al.* (*ibid.*) that at high levels of CB_1 receptor expression, the Akt survival pathway is recruited.

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REMARKABLE POTENCY OF Δ^9-THC IN BLOCKING GASTRIC HEMORRHAGES CAUSED BY CYCLOOXYGENASE INHIBITION IN MICE

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Cyclooxygenase (COX) inhibitors, which are among the most widely used analgesics in the world, also cause gastrointestinal inflammation that can be life-threatening. Our laboratories recently reported that endocannabinoid catabolic enzyme (i.e., FAAH or MAGL) inhibitors protect against gastropathy induced by the non-selective COX inhibitor diclofenac sodium. In addition, we reported that the plant-derived cannabinoid Δ^9-tetrahydrocannabinol (THC) effectively protected against gastric pathology. The present study further investigates the gastroprotective effects of THC by comparing a range of doses as well as oral (p.o) and intraperitoneal (i.p.) routes of administration. Food deprived, male C57BL/6J mice administered the nonselective cyclooxygenase inhibitor indomethacin (20 mg/kg, p.o.) or diclofenac sodium (100 mg/kg, p.o.) displayed gastric hemorrhages. Whereas the selective COX-1 inhibitor SC-560 (20 mg/kg, p.o.) also elicited gastric hemorrhages, the selective COX-2 inhibitor celecoxib (20 mg/kg, p.o.) did not. Administration of THC via either i.p. (≥0.1 mg/kg) or oral (≥2.5 mg/kg) route significantly attenuated gastric hemorrhages. In a separate group of mice, we compared the behavioral effects of THC following either route of administration in the "tetrad" battery of tests, consisting of assessment of locomotor activity, nociception in the tail withdrawal test, catalepsy in the bar test, and hypothermia. THC significantly increased immobility (≥5 mg/kg), antinociception (≥10 mg/kg), hypothermia (≥5 mg/kg), and catalepsy (≥50 mg/kg), regardless of route of administration. These data indicate that oral administration of the phytocannabinoid THC protects against gastric inflammatory tissue damage induced by COX inhibition at doses that are insufficient to cause cannabimimetic behavioral effects.

THE LOCALIZATION AND FUNCTION OF DIACYLGLYCEROL LIPASE (DAGL) IN THE GASTROINTESTINAL TRACT

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Endocannabinoids (ECs) are involved in the regulation of gastrointestinal (GI) motility. Previous studies suggest that there is ongoing synthesis of ECs, producing an EC tone in the gut. However, the sites of EC biosynthesis in the GI tract are not well defined and the nature of EC tone remains to be fully understood. We have investigated the distribution and function of diacylglycerol lipase (DAGL) in the GI tract of the mouse using immunohistochemistry, PCR and pharmacological blockade of DAGL. CB_1 wild-type (WT) or gene deficient mice ($CB_1^{-/-}$) were used. Immunohistochemistry and real-time PCR were performed for DAGL in the mouse intestine. The effects of the DAGL inhibitors Orlistat and OMDM188 on intestinal contractility induced by electrical field stimulation (EFS; 4Hz) or bethanechol (10μM) were studied *in vitro*. The effects of DAGL inhibitors on whole gut transit (WGT) of an Evan`s Blue marker were studied *in vivo*. Scopolamine or loperamide were used to induce GI hypo-motility and finally the EC levels were measured in the intestinal tissues incubated with scopolamine and/or the DAGL inhibitors.

DAGLα is expressed throughout the length of the gut with higher mRNA expression in the stomach and colon, compared to the small intestine. In the ileum and colon, DAGLα immunoreactivity was localized in the enteric nervous system. In the myenteric plexus, DAGLα immunoreactivity co-localized with the vesicular acetylcholine transporter (VAChT), suggesting it is present in cholinergic nerves. Inhibiting DAGL with Orlistat or OMDM188 did not alter baseline or bethanechol stimulated intestinal contractility *in vitro* (1-5μM of either inhibitor). When cholinergic contractility was pharmacologically reduced by 40-90% with the muscarinic antagonist scopolamine (1-10nM), both Orlistat and OMDM188 significantly reversed this inhibition. When Orlistat or OMDM188 were given *in vivo* (1mg/kg, i.p.) WGT was not altered; however, both inhibitors significantly reversed transit that was slowed with either scopolamine or the opioid agonist loperamide (0.5mg/kg, i.p.) in WT mice. The effect of DAGL inhibition was lost in $CB_1^{-/-}$ mice. Inhibiting DAGL did not change intestinal 2-arachidonoylglycerol (2-AG) levels in normal animals. In animals treated with scopolamine, 2-AG was elevated in the colon; inhibiting DAGL normalized these levels. In conclusion, inhibiting DAGL reverses decreased GI motility and contractility through a 2-AG and CB_1 mediated mechanism.

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CANNABIDIOL INDUCES NON-CANONICAL AUTOPHAGY

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Introduction. Autophagy is a lysosomal degradation pathway that recycles cellular materials, improves cell survival and development, and important for microbial clearance and cytokine production. Impaired autophagy, with the loss of ATG16L, contributes to the pathogenesis of Crohn's Disease (CD), reviewed by Kaser and Blumberg, 2011. Leptin is an important cytokine involved in lipid metabolism and inflammation in the periphery. Increased intestinal leptin in CD is thought to augment this inflammatory setting through increase of pro-inflammatory cytokine production and up-regulation of nuclear factor (NF)-kB (Sitaraman *et al*., 2004). Emerging data from ATG16L-deficient mice show an enhanced expression of leptin in epithelial cells (Cadwell *et al*., 2008). Using fully differentiated CaCo2 intestinal epithelial cells as an *in vitro* enterocytic model, the intersection of leptin and the downstream regulator, suppressor of cytokine signalling (SOCS3), was examined in relation to autophagy. Cannabidiol (CBD), a plant-derived cannabinoid, was used to explore its ability to limit leptin signalling in this context.

Methods. Confluent, mature enterocytic CaCo-2 cells (WT, CB1Rkd and SOCS3kd) were treated with cannabinoids and cytokines in medium complete with amino acids and serum. Protein extracts were subjected to SDS-PAGE electrophoresis, and immunoblotting was performed using Light Chain (LC3B, 1:1000, Cell Signaling, #2775), SOCS3 (1:1000, Cell Signaling, #2923S) and Cannabinoid Receptor (CB)-1 antibodies (1:1000, Caymen Chemical Company, #10006590). Incorporation of dansylacadaverine (MDC, $5x10^{-5}$M, Sigma #30432) into autophagic vacoules was assessed by fluorescent microscopy.

Results/Data Analysis. Cannabidiol does not affect the viability of fully differentiated Caco-2 cells (up to 25mM) under these culture conditions. CBD inhibits autophagy at low doses (0.01-0.1mM) and induces autophagy at high doses (1-10mM). This effect is not mediated by the CB1 receptor. Leptin-induced autophagy is inhibited by the ClassIII PI3K and autophagy inhibitor, 3-methyladenine (3-MA, 5mM), whereas the CBD (1mM) enhancement of leptin-induced autophagy was not blocked by 3-MA. Interestingly, SOCS3 protein levels reciprocally mirror the CBD-induced LC3B-II levels.

Conclusions. Autophagy is an important cellular process during inflammation. Cannabidiol is able to influence inflammatory signalling in gut epithelium by activating non-canonical autophagy. High levels of SOCS3 inhibit autophagic flux, which could influence bacterial clearance.

Futures Directions. The implications of this work indicate that CBD could overcome deficiencies in autophagy in CD through differential utilisation of autophagic pathways. It remains to be established whether SOCS3 plays a direct role in this system and further analysis of SOCS3kd and CB1kd cells will clarify this.

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FULL INHIBITION OF SPINAL FATTY ACID AMIDE HYDROLASE IN NEUROPATHIC RATS LEADS TO TRPV1-MEDIATED ANALGESIC EFFECTS VIA REMODELING OF THE ENDOCANNABINOID SYSTEM

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Despite considerable efforts, no fully satisfactory methods for the management of neuropathic pain have been developed so far. Moreover, the etiological factors that determine the development of neuropathic pain are not fully understood. Cannabinoids, and more importantly endocannabinoids, have the potential to address this unmet need. Recent studies suggest that also endogenous ligands of TRPV1 channels, the endovanilloids, might represent a promising target for pharmacological research aiming at a successful neuropathic pain treatment. TRPV1 and CB_1 co-localization in the spinal cord and dorsal root ganglia opens interesting possibilities for the development of novel therapeutics against neuropathic pain. Administration of FAAH inhibitors, like URB597, to rodents enhances the levels of anandamide (AEA) and/or 2-arachidonoylglycerol (2-AG), as well as of other acylethanolamide FAAH substrates, in various tissues involved in pain perception. Local injection of FAAH inhibitors thus offers the opportunity to investigate the role of FAAH substrates and their molecular targets in pain control. We previously reported that low intrathecal doses of the FAAH inhibitor, URB597, reduce allodynia and hyperalgesia in rats with chronic constriction injury (CCI) of the sciatic nerve via mechanisms that can be mediated by either indirect CB_1 activation or TRPV1 activation/desensitization, depending on the dose of the drug (Starowicz et al., 2012). However, the possibility that higher doses of URB597, leading to full FAAH inhibition, unmask other pathways for AEA metabolism, with likely consequences **on nociception, has not been investigated. Therefore, the present study aimed at elucidating** the mechanism of action of fully effective *analgesic* doses of URB597 in CCI rats by establishing how they modify endocannabinoid and acylethanolamide levels in the spinal cord and determining the contribution of CB_1, TRPV1 and lipoxygenase (LOX) enzymes to their effects.

Among the doses tested, the 200µg/rat dose of URB597 was the only one that elevated the levels of the FAAH non-endocannabinoid and anti-inflammatory substrates, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), and of the endocannabinoid FAAH substrate, 2-AG, and fully inhibited thermal and tactile nociception, although in a manner blocked almost uniquely by TRPV1 antagonism. Surprisingly, this dose of URB597 decreased, rather than increasing, the spinal levels of AEA. Data on altered spinal expression of the mRNAs of LOX enzymes suggested the occurrence of alternative pathways of AEA metabolism. Baicalein, an inhibitor of 12/15-LOX activity, significantly reduced URB597 analgesic effects. Using immunofluorescence techniques, FAAH, 15-LOX and TRPV1 were found to co-localize in the dorsal spinal horn of CCI rats. Finally, 15-hydroxy-AEA, the 15-LOX derivative of AEA, potently and efficaciously activated the rat recombinant TRPV1 channel. We suggest that spinal URB597 at full analgesic efficacy unmasks a secondary route of AEA metabolism via 15-lipoxygenase with possible formation of 15-hydroxy-AEA, which may then cooperate with OEA and PEA at producing TRPV1-mediated analgesic effects in CCI rats.

In conclusion, we suggest that full inhibition of FAAH may determine TRPV1-mediated anti-hyperalgesic and anti-allodynic effects in neuropathic via 15-hydroxy-AEA, OEA and PEA.

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OMDM198, A COMPOUND TARGETING BOTH TRPV1 AND FATTY ACID AMIDE HYDROLASE: A NEW PAIN MANAGEMENT STRATEGY IN OSTEOARTHRITIS?

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Osteoarthritis (OA) is the most common degenerative joint disease, characterized by permanent destruction of articular cartilage and subchondral bone, which leads to pain during joint loading and to chronic physical disability. OA therapy is limited to NSAIDs, which may not always control pain and local inflammation. Thus, there is a strong need to develop new treatments for OA.

The endogenous agonist of cannabinoid receptor 1 (CB1), anandamide (AEA), produces antinociception via this target. It also stimulates transient receptor potential vanilloid channel-1 (TRPV1) channels, which instead plays a key role in the induction of inflammation and the development of chronic pain associated with OA. AEA has a short half-life, due to efficient enzymatic degradation mainly by fatty acid amides hydrolase (FAAH). Previous data, have shown that *N*-arachidonoylserotonin (AA-5-HT), a dual FAAH inhibitor and TRPV1 antagonist, produces more efficacious anti-nociceptive and anti-oedemogenic effects than either selective FAAH or TRPV1 blockers in a model of inflammatory pain (Costa et al., Pharmacol Res 2009). However, this compound is unstable and prone to non-enzymatic oxidation. Therefore, we tested here, in an animal model of OA, another dual, and theoretically more stable, although less potent in vitro, TRPV1-FAAH blocker, OMDM198 (compound **10** in Morera et al., 2009), in comparison to compounds acting exclusively on either of these two targets.

OA was induced in male Wistar rats by intra-articular injection of 3 mg sodium mono-iodo-acetate (MIA) with a recovery period of 14 days. The effect of intraperitoneal (i.p.) OMDM198 on joint pain perception was determined by hindlimb dynamic weight bearing (DWB) and pressure application measurements (PAM) . Immunohistochemistry was used to evaluate the expression of CB_1, TRPV1 and FAAH in the dorsal root ganglia (DRG) and lumbar spinal cord of sham- and MIA-treated animals. By the means of Real Time PCR we evaluated the mRNA expression of AEA-related proteins (CB1 and TRPV1; anabolic enzymes for all three proposed AEA synthetic pathways; AEA catabolic enzymes: FAAH, 12/15-LOX and COX-2) in response to OA. Finally, in order to compare the effectiveness of OMDM198 (0.1, 1, 5 mg/kg, i.p.) against inflammatory pain to that of AA-5-HT, we tested its activity also on carrageenan-induced paw hyperalgesia and oedema in rats under conditions previously used for this latter compound (Costa et al., Pharmacol Res 2009).

OA was accompanied by an increase of the mRNA levels of AEA receptors and metabolic enzymes in both the DRG and spinal cord. The co-expression in spinal neurons of TRPV1 and CB_1 agrees with the concept that some endovanilloids are also endocannabinoids. Additionally the large extent of co-localization of TRPV1 and FAAH in the spinal cord supports the idea that AEA acts as a TRPV1 ligand. OMDM198 showed anti-hperalgesic effects at doses of 1, 2.5 and 5 mg/kg in the MIA model, with maximal activity being observed at the lowest dose. The analgesic effect of 1mg OMDM198 was comparable to that of 20 mg of the selective TRPV1 antagonist, SB366791, and of 5 mg of the selective FAAH inhibitor, URB-597. The effect of OMDM198 was attenuated by the CB_1 receptor antagonist, AM-251, suggesting that this compound acts in part as an "indirect" CB_1 agonist. Finally, OMDM198 was as effective as AA-5-HT at counteracting paw oedema, and slightly more efficacious at reducing hyperalgesia, in rats treated with intraplantar carrageenan, with maximal effects on these two parameters being observed at the 5 mg/kg dose.

These results suggest an innovative strategy for the treatment of OA. Dual targeting of spinal/peripheral FAAH or CB_1, on the one hand, and TRPV1, on the other hand, may yield more successful results also in human OA than those obtained so far with selective FAAH inhibitors.

ORAL TREATMENT WITH THE SELECTIVE FAAH INHIBITOR MM-433593 RELIEVES THERMAL HYPERALGESIA INDUCED BY *E. COLI* LIPOPOLYSACCHARIDE IN C57BLACK6/J MICE VIA CANNABINOID CB1 RECEPTOR

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Systemic treatment of rodents or primates with selective inhibitors of fatty acid amide hydrolase (FAAH) elevates levels of the endocannabinoid anandamide and other fatty acid amides (FAAs) in the central nervous system, peripheral tissues, and systemic circulation. FAAH -/- mutant mice (gene deletion) and wild-type mice or rats treated with FAAH inhibitors show reduced signs of anxiogenic-like, depression-like, pruritic (scratching), and nociceptive (neuropathic, acute, inflammatory) effects and improvements in acquisition of learned responses and sleep. Unlike direct cannabinoid receptor agonists, FAAH inhibitors do not appear to be associated with adverse psychiatric or addictive properties. In the present study, we chemically synthesized and pharmacologically characterized a novel FAAH inhibitor, MM-433593 (structure to be presented), which showed potent inhibition of FAAH [IC50 ~10 nM] in brain membrane preparations from human, monkey, dog, rat, and mouse and in a huFAAH whole cell assay [IC50 ~1 nM]. MM-433593 weakly antagonized CB1 [IC_{50} > 1uM] and showed little or no interaction with CB2, COX1, COX2, FAAH-2, PDE, PLA, other serine hydrolases, hERG, or a broad panel of receptors and channels. Inhibition of FAAH by MM-433593 was not reversible, but, unlike irreversible inhibitors, did not covalently modify the FAAH. Administration of MM-433593 (0.3, 3, or 30 mg/kg) to mice via oral gavage dose-dependently reduced LPS-induced thermal hyperalgesia and concomitantly elevated anandamide levels in plasma and brain tissue in a dose-responsive fashion. These anti-hyperalgesic effects of MM-433593 were blocked by the selective CB1 receptor antagonist rimonabant. MM-433593 is being studied further in other animal models of psychiatric and neurological disorders. In conclusion, we describe the development of a novel FAAH inhibitor that is orally bioavailable and blocks inflammatory pain with high efficacy.

THE FAAH INHIBITORS PF3845 AND URB597 PRODUCE DISTINCT EFFECTS ON ACUTE PAIN-STIMULATED AND PAIN-DEPRESSED BEHAVIOR IN RATS

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Cannabinoid receptor agonists produce reliable antinociception in many preclinical pain assays but inconsistent analgesic efficacy in humans. This disparity suggests that conventional preclinical assays of nociception are not sufficient for prediction of cannabinoid effects related to clinical analgesia. We have previously shown that the low-efficacy cannabinoid receptor agonist Δ9-tetrahydrocannabinol (THC) and the high-efficacy synthetic cannabinoid receptor agonist CP55940 produce antinociception in conventional assays of acute pain-stimulated behavior, but do not produce antinociception in novel assays of acute pain-depressed behavior. Fatty acid amide hydrolase (FAAH) inhibitors increase physiological levels of endocannabinoids and other fatty acid amides and have also been shown to produce antinociception in many conventional preclinical assays of pain-stimulated behavior. In the present study, we compared effects of the highly specific FAAH inhibitor PF3845 and the less specific FAAH inhibitor URB597 on lactic acid-induced abdominal stretching (i.e. acute pain-stimulated behavior) and depression of intracranial self-stimulation (ICSS) of the medial forebrain bundle (i.e., acute pain-depressed behavior) in male Sprague Dawley rats. Drug effects on control ICSS in the absence of the noxious stimulus were also examined. URB597 (1-10 mg/kg IP) dose dependently attenuated acid-stimulated stretching at both 1 and 4 h. Although URB597 significantly decreased control ICSS at 1 h, it failed to block acid-induced depression of ICSS. However, at 4 h a different pattern of effects emerged. At this time point, URB597 no longer affected control ICSS, but significantly attenuated acid-induced depression of ICSS. Thus, after 4 h, URB597 produced antinociception in assays of both acid-stimulated stretching and acid-depressed ICSS. Conversely, PF3845 (1-10 mg/kg IP, 1 and 4 h pretreatment) failed to significantly reduce acid-stimulated stretching or acid-depressed ICSS at doses that produced robust increases in brain and plasma levels of anandamide and other fatty acid amides. These results suggest a dissociation between effects of FAAH inhibitors on acute nociception and brain/plasma levels of anandamide and other fatty acid amides. These results also support further research on mechanisms that mediate acute antinociceptive effects of URB597.

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A NOVEL NAAA INHIBITOR REVERSED UVB-INDUCED INFLAMMATORY RESPONSE BOTH *IN VITRO* AND *IN VIVO*

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Bioactive N-acylethanolamines, including the endocannabinoid anandamide and anti-inflammatory N-palmytoylethanolamine (PEA), are hydrolyzed to fatty acids and ethanolamine in animal tissues by the catalysis of fatty acid amide hydrolase (FAAH). More recently, N-acylethanolamine-hydrolyzing acid amidase (NAAA), another enzyme catalyzing the same reaction has been cloned. NAAA preferentially hydrolyzes PEA over others fatty acid ethanolamides and is localized in lysosomes and highly expressed in macrophages. Previous studies have shown that PEA exerts profound anti-inflammatory and analgesic effects that are due to a large extent to activation of the nuclear receptor peroxisome proliferator-activated receptor-a (PPARa). We recently developed a selective and potent NAAA inhibitor (ARN0077). In the present study we investigated the effect of ARN0077 both in the rat UVB irradiation model of skin hypersensitivity and in UVB-treated human primary keratinocytes.

The exposure of the skin to UV irradiation results in a classical inflammatory reaction characterized by erythema, hyperalgesia and allodynia. Topical administration of ARN0077 produced a significant reversal of UVB-induced thermal hyperalgesia compared to vehicle treated animals. This effect was dose-dependent with the 10% and 30% concentrations producing significant reduction in the thermal hyperalgesia compared to the vehicle treated group at 4 hours post-application. When CB_1 or CB_2 antagonists were injected before ARN0077, they did not block the anti-hyperalgesic effect of the NAAA inhibitor, whereas a PPARα antagonist completely reversed ARN077 activity.

Keratinocytes act as primary sensors of skin injuries. In order to understand the role of NAAA and PEA in UVB-induced inflammation we chose human primary keratinocytes as cell culture model. We therefore tested the effect of NAAA inhibition *in vitro* after exposure of keratinocytes to UVB. Irradiation of the cells caused an increased release of the pro-inflammatory cytokine IL-6 in the medium. Pre-treatment with ARN0077 prevented this effect. Time course measurements of the levels of PEA and OEA indicated that UVB treatment induces an increase of these N-acylethanolamines. Inhibition of NAAA activity by ARN0077 could anticipate and potentiate this effect.

In conclusion, ARN0077 was shown to reverse *in vivo* effects of UVB irradiation through a PPARα dependent pathway. More in depth mechanistic studies revealed that NAAA inhibition in primary keratinocytes could prevent the release of IL-6 from the cells, probably by exacerbating a physiological adaptive augmentation of PEA and OEA levels.

THE SELECTIVE MONOACYLGLYCEROL LIPASE INHIBITOR KML29 PRODUCES ANTINOCICEPTION IN THE ABSENCE OF CANNABIMIMETIC EFFECTS

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Monoacylglycerol lipase (MAGL) is the predominant enzyme responsible for degradation of the endocannabinoid 2-arachidonoyl glycerol (2-AG). While several inhibitors of MAGL have been developed, each possesses varying degrees of non-selectivity. KML29, a novel, highly selective inhibitor of MAGL, allows for examination of selective increase in 2-AG levels without cross-activity with fatty acid amide hydrolase (FAAH), the enzyme responsible for the breakdown of the other major endocannabinoid *N*-arachidonoyl ethanolamine (anandamide; AEA) as well as other fatty acid amides. 2-AG plays an important role in variety of physiological processes, including short-term synaptic activity, and while enhancement in 2-AG signaling has been considered a promising therapeutic target in treatment of pain, neurodegenerative diseases, and inflammatory disorders, its function and effects in vivo remain unclear.

In the present study, we tested whether KML29 reduces mechanical allodynia (i.e., nociceptive responding to a normally non noxious stimulus) in the carrageenan model of inflammatory pain. We also aimed to determine whether this compound produces cannabimimetic effects, as assessed in the "tetrad" assay, consisting of measurements of locomotor activity, body temperature, nociceptive behavior assessed in the tail withdrawal test, and catalepsy assessed in the bar test. Moreover, we used the drug discrimination paradigm to examine whether KML29 mimics subjective effects of Δ9-tetrahydrocannabinol (THC) in C57/BL/6J mice and AEA in FAAH (-/-) mice.

KML29 (40 mg/kg) significantly decreased inflammatory edema and completely reversed allodynia induced by injection of carrageenan. KML29 also elicited antinociception in the tail withdrawal assay, but did not induce catalepsy, changes in locomotor activity, or body temperature. Whereas KML29 (40 mg/kg) failed to substitute for THC in C57/BL/6J mice, it fully and dose-dependently substituted for AEA in FAAH (-/-) mice. Specifically, 40 mg/kg KML29 produced full substitution at the level of 99.5% drug appropriate responding (DAR), 20 mg/kg produced partial AEA substitution (56% DAR), and 5 mg/kg failed to generalize to AEA (3.5% DAR). AEA-like responding following KML29 treatment was fully blocked by pretreatment with the CB1 antagonist, rimonabant (1 mg/kg). Rate of responding was not affected by KML29 administration. The observations that KML29 generalized to AEA in FAAH (-/-) mice, but not to THC in wild type mice, are consistent with a previous study reporting that dual FAAH and MAGL inhibition produces THC-like subjective effects (Long et al., 2009).

These findings indicate that selective enhancement of 2-AG signaling induced by KML29 possesses antinociceptive properties that may be applied to treat inflammatory pain, without THC-like subjective effects or typical cannabimimetic side effects.

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PERIPHERALLY RESTRICTED CB1 AGONISTS FOR TREATMENT OF NEUROPATHIC PAIN

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Cannabinoid agonists reduce the symptoms of hyperalgesia and allodynia associated with persistent pain of inflammatory and neuropathic origin in humans and animals. Furthermore, cannabinoids are effective in alleviating chronic pain symptoms after prolonged repeated treatment, unlike opioids, which have only limited effectiveness. A major impediment to the widespread use of cannabinoid analgesics has been their central nervous system (CNS)-mediated psychotropic side effects. This impediment can potentially be circumvented by the development of peripherally restricted CB1 agonists. The design, synthesis and testing of compounds directed towards this end are reported.

Indole and indene families of cannabinoid agonists were engineered to exhibit high hCB1 affinity, peripheral selectivity, metabolic stability and in vivo efficacy without CNS effects. Introduction of charged (quaternary ammonium), actively exported (carboxylate), and subsequently other moieties evolved indoles and indenes with low nM hCB1 affinity equaling that of CP 55,940 in the best cases. Blood-brain barrier penetration was modeled with the MDCK cell line assay that identified candidates with less than 1% permeation, thus selecting candidates for in vitro metabolic stability studies and subsequent in vivo testing. Modifications were made to increase metabolic stability in in vitro systems.

The candidate CBR agonists were examined for effectiveness in alleviating the painful symptoms of mechanical allodynia in a rat model of neuropathy induced by unilateral sciatic nerve entrapment (SNE). The withdrawal thresholds of the ipsilateral paw were robustly and reversibly increased to 77%, in a selected example, of that of the contralateral paw in the rats treated with test compounds by intraperitoneal injection.

A modified tetrad assay in rats compared the effects of the brain penetrable CB1 agonist HU 210 at doses that alleviate neuropathy symptoms as a positive control to our novel ligands and to vehicle. While HU 210 exhibited strong CNS effects in the tetrad, at systemic doses that relieve neuropathy symptoms, our novel CBR agonists showed a complete lack of side effects in the assays that test for CNS-mediated side effects of catalepsy, hypothermia and motor incoordination.

The potency, peripheral selectivity, in vivo efficacy and absence of side effects of the indole and indene classes of CBR agonists have the potential to afford a viable treatment for neuropathic pain.

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EVIDENCE THAT CANNABINOID CB2 RECEPTORS PLAY A NOVEL ROLE IN THE MODULATION OF SPINAL HYPERSENSITIVITY AND PAIN BEHAVIOUR IN THE RAT MIA MODEL OF OSTEOARTHRITIS

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Osteoarthritis (OA) is a prevalent musculoskeletal disease and a major cause of disability, affecting up to 8.5 million people in the UK alone. The principal characteristic is joint pathology resulting from cartilage degradation and damage to the subchondral bone, with the main presenting symptom being pain. Previous work in our laboratory utilizing the monosodium iodoacetate (MIA) animal model of OA, revealed increased spinal endocannabinoid levels in MIA- vs. saline-treated rats at 14 and 28 days post-injection (of MIA). Furthermore, spinal administration of a CB2 receptor agonist (JWH133) at day 28 post MIA administration significantly reduced evoked spinal (WDR) neuronal responses in MIA-, but not saline-treated rats. Here we further investigated the role of spinal CB2 receptors in MIA-induced OA, by examining the effect of MIA induction, on spinal CB2 receptors, and the ability of a selective CB2 receptor agonist to modulate MIA-induced pain behavior.

Male Sprague Dawley rats (180-200g n= 8 per group) received an intra-articular injection of either MIA (1mg/50µl) or saline into the left knee joint. A second cohort of rats received either MIA or saline and were then given a once daily subcutaneous injections of either the CB2 agonist JWH133 (1 mg/kg from day 1-28 post-MIA) or vehicle (n=8 rats per group). Changes in weight bearing and hindpaw withdrawal (mechanical) thresholds were measured for 28 days post-MIA. On day 28, rats were euthanized via cranial concussion (for fresh tissue collection) or with an overdose of sodium pentobarbital (for PFA perfused fixed tissue collection). Lumbar spinal cords, L3-5 DRGs, and brain were then removed, and either snap frozen (in liquid nitrogen) and stored at -80°c (fresh tissue), or post fixed in 4% PFA and then stored in a sucrose (30%) azide (0.02%) solution at 4°C.

A substantial elevation in ipsilateral versus contralateral spinal CB2 mRNA expression (measured by TAQMAN quantitative RT-PCR) was observed in MIA- but not saline-treated rats (66±15% vs. 10±9%, $p<0.01$). This elevation was accompanied by an increase in the number (measured via immunofluorecence) of CB2-positive microglia cells in the ipsilateral spinal cord of MIA-treated rats (19 ± 3 vs. 13 ± 2 in saline-treated rats, $p<0.05$). Interestingly, we also observed an increase in CB2-expressing Neu-N positive cells in the ipsilateral spinal cord of MIA-treated animals. Daily administration of 1 mg/kg JWH133 significantly attenuated MIA-induced deficits in ipsilateral weightbearing (-44g vs. vehicle, $p<0.001$) and paw withdrawal thresholds (+5.5g vs. vehicle, $p<0.001$). In summary, the data presented here demonstrate, that the CB2 receptor is upregulated in an animal model of OA. Furthermore, our data suggest that CB2 receptor ligands may prove to have a therapeutic potential in treating OA associated pain.

GENOMIC ANALYSIS OF PHYTOCANNABINOID BIOSYNTHESIS IN *CANNABIS SATIVA* L.

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The unique pharmacological activity of *Cannabis sativa* L. (marijuana, hemp; Cannabaceae) is due to the presence of phytocannabinoids, which include Δ^9-tetrahydrocannabinol (THC) and more than 70 related metabolites. We are using a combination of genomics and biochemistry to identify enzymes that produce phytocannabinoids and other specialized metabolites in cannabis. We recently reported the draft genome and transcriptome of the potent medical marijuana strain Purple Kush (http://genome.ccbr.utoronto.ca/) and have produced a gene-expression catalog for glandular trichomes, which are the main site of phytocannabinoid biosynthesis. The first intermediate in the phytocannabinoid pathway is proposed to be olivetolic acid (OA), an alkylresorcinolic acid that forms the polyketide nucleus of the phytocannabinoids. OA is likely synthesized by a type III polyketide synthase (PKS) enzyme but so far PKSs from cannabis have been shown to produce by-products from aberrant cyclization routes instead of OA. We searched the trichome transcriptome for polyketide cyclase-like enzymes that could assist in OA cyclization. We found that a cannabis PKS requires the presence of a polyketide cyclase, olivetolic acid cyclase (OAC), which catalyzes a C2–C7 intramolecular aldol condensation with carboxylate retention to form OA. OAC is a dimeric $\alpha+\beta$ barrel (DABB) protein that is structurally similar to polyketide cyclases from *Streptomyces* species. *OAC* transcript is present at high levels in glandular trichomes, an expression profile that parallels other phytocannabinoid pathway enzymes. Our identification of OAC both clarifies the phytocannabinoid pathway and demonstrates unexpected evolutionary parallels between phytocannabinoid biosynthesis and polyketide pathways in bacteria. The availability of these extensive genomic resources for cannabis will facilitate identification of additional enzymes and transcription factors involved in phytocannabinoid biosynthesis, and may allow for metabolic engineering of phytocannabinoid production in recombinant microorganisms.

CANNABIS USE AS A COMPLEMENTARY ALTERNATIVE MEDICINE IN MULTIPLE SCLEROSIS: EFFECTS ON SOME IMMUNE PARAMETERS

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Cannabinioids have been shown to have anti-inflammatory and immunomodulatory effects in humans. We recently reported differential migratory properties of monocytes in human subjects who either used or did not use *Cannabis*. We wanted to know if patients with Multiple Sclerosis (MS) who are using *Cannabis* for symptom control, have immunomodulatory changes. In this case-control study, several immune parameters as outcome measures were examined for effect of *Cannabis* use on these outcomes. We compared monocyte migration, a panel of cytokines and levels of endocannabinoids in 10 healthy subjects and 11 patients with Multiple Sclerosis, stratifying for current *Cannabis* use. Subjects were recruited according to rules prescribed by the Human Subjects Committee at the University of Washington. Blood was obtained by veni-puncture from the ante-cubital vein under a protocol approved by the Human Subjects Committee at the University of Washington. Donors provided prior written informed consent to the procedure and use of the sample. No *Cannabis* was administered to any subjects in this study.

We observed that monocyte migration toward a cannabinoid "mix" (a set ratio of THC +CBN/CBD) was not different between the cases and controls. When the chemokine CCL2 was the migratory stimulus, there was an inhibition of migration observed in the MS patients who were *Cannabis* users back to basal levels, compared to MS 'non-users', while the serum levels of CCL2 were the same across all cohorts. Panels of TH1 and TH2 cytokines were reduced in both healthy subjects and patients with MS. The cytokine IL17 was significantly reduced in patients who have MS and use *Cannabis*, compared to those who are non-users. Patients with MS had roughly twice the levels of AEA than healthy subjects, while 2AG was increased in patients with MS who were non-users of Cannabis, and reduced in patients with MS who used *Cannabis*.

These results agree with, and extend, previous published results showing immunomodulatory effects of *Cannabis* use in humans. Our data suggests that *Cannabis* use may impair monocyte cell migration, suggesting that *Cannabis* use may reduce the number of monocytes invading the inflamed central nervous system, alter cytokine levels and modulate the endogenous cannabinoid signaling system. These data add to the human data on how *Cannabis* use affects the immune system, and implicate *Cannabis* as a disease modifying therapy for MS. Our data contribute to the body of research on *Cannabis* use in MS, suggesting the need for a further investigation into the potential benefit beyond palliation for patients with MS.

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CANNABIDIOLIC ACID BEHAVES AS A HIGHLY POTENT ENHANCER OF 5-HT$_{1A}$ RECEPTOR ACTIVATION BOTH *IN VITRO* AND *IN VIVO*

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Our previous research has yielded data suggesting that the non-psychoactive phytocannabinoid, cannabidiol (CBD), can enhance the activation of 5-HT$_{1A}$ somatodendritic autoreceptors in rat brainstem membranes and that this action of CBD underlies its ability to attenuate vomiting in house musk shrews and nausea-like behaviour in rats (Rock *et al.*, 2012). Here we present evidence that cannabidiolic acid (CBDA), the immediate precursor of CBD in cannabis, displays markedly greater potency than CBD as an enhancer of 5-HT$_{1A}$ receptor activation both *in vitro* and *in vivo*.

In our *in vitro* experiments, we tested the ability of CBDA to enhance stimulation by the selective 5-HT$_{1A}$ receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), of [^{35}S]GTPγS binding to membranes obtained from rat brainstem, the cerebral region that contains dorsal raphe nuclei. Our *in vivo* experiments were directed at investigating the effects of CBDA on vomiting in house musk shrews (*Suncus murinus*) induced by lithium chloride (LiCl) or cisplatin and on LiCl-induced conditioned gaping (nausea-like behaviour) in rats. The methods used in these *in vitro* and *in vivo* experiments have been described previously (Rock *et al.*, 2012).

8-OH-DPAT stimulated [^{35}S]GTPγS binding to rat brainstem membranes in a concentration-dependent manner, as expected, whereas CBDA (0.1 nM to 1 μM) did not affect [^{35}S]GTPγS binding. At concentrations of 0.1, 1, 10 and 100 nM CBDA did, however, produce statistically significant increases ($P < 0.05$) in the maximal effect (E_{max}) of 8-OH-DPAT for its stimulation of [^{35}S]GTPγS binding. We also found that CBDA (0.5 mg kg^{-1} s.c.) suppressed LiCl- and cisplatin-induced vomiting in shrews, and that at the very low doses of 0.01 and 0.1 mg kg^{-1} s.c., it reduced LiCl-induced conditioned gaping in rats. The latter effect of CBDA was prevented by pretreatment with the selective 5-HT$_{1A}$ receptor antagonist, WAY100635 (0.1 mg kg^{-1} i.p.).

These findings suggest first, that CBDA acts much more potently than CBD (Rock *et al.*, 2012) as an enhancer of 5-HT$_{1A}$ receptor activation and inhibitor of nausea-like behaviour, and second, that, as has been postulated for CBD, the anti-nausea-like effect of CBDA is mediated by 5-HT$_{1A}$ receptors. We conclude that CBDA is a promising candidate drug for the treatment of nausea and vomiting and that it also merits investigation as a potential medicine for the treatment of one or more other disorders that might be ameliorated by enhancing 5-HT$_{1A}$ receptor activation.
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EVIDENCE THAT THE PHYTOCANNABINOID CANNABIGEROL CAN INDUCE ANTINOCICEPTION BY ACTIVATING α2-ADRENORECEPTORS: A COMPUTATIONAL AND A PHARMACOLOGICAL STUDY

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Cannabigerol (CBG) is a poorly characterized phytocannabinoid derived from the *Cannabis sativa* plant and is devoid of any Δ^9-tetrahydrocannabinol (THC)-like psychopharmacological activity *in vivo*. A recent study (Cascio et al., Br. J. Pharmacol. 159:129, 2010) demonstrated in *in vitro* experiments that CBG is a potent α_2-adrenoceptor agonist. This was unexpected since the structure of this plant cannabinoid is very different from known α_2-adrenoceptor ligands, and since no other cannabinoid has been reported so far to display such activity. These observations open the possibility that CBG, like established α_2-adrenoceptor agonists (for example clonidine), could display significant efficacy as an antinociceptive agent when administered *in vivo*. Accordingly, the present study was set up to address this question by investigating a) the manner in which CBG interacts with α_2-adrenoceptors and its affinity for these receptors as indicated by a computational study and b) the antinociceptive properties of CBG in two different murine models of pain, the formalin and the λ-carrageenan tests. Integration of pharmacological and computational data can be useful for achieving a fuller understanding of the molecular mechanism of action of a compound: its binding mode at the active site and its affinity for the receptor. Therefore, in the present study we have modelled the three-dimensional structures of α_{2A}, α_{2B} and α_{2C} isoforms of murine and human adrenergic receptors by comparative modelling and molecular dynamics (MD) simulations. The structures of the models were assessed through the docking of the endogenous ligand norepinephrine, checking its proper placement and its interactions with certain residues in the binding pocket (D113, S200, S201 and S204 for α_{2A}), as described previously (Nyronen et al., Mol. Pharmacol. 1343-1354, 2001). Also, clonidine and CBG docking simulations were performed to obtain preliminary binding affinity data: CBG affinity for the receptor seems to be higher than that of clonidine. The conformations of the receptor collected during 20 ns MD simulations were divided into clusters of structural similarity and the average structure of each cluster was used to perform docking simulations for CBG. This compound seems to bind to the receptor in the same pocket as norepinephrine, but in a different position. In addition, due to the steric effect of the molecule, which is bigger than the natural ligand, a larger number of interactions occur with the receptor, leading to the formation of a highly stable complex. On the basis of these findings confirming an interaction of CBG with α_2-adrenoceptors, CBG antinociceptive efficacy was studied and compared with that evoked by clonidine, the prototypical α_2-adrenergic agonist known to elicit antinociception in experimental models of pain. Preliminary experiments showed that CBG (1, 5, 10 mg/kg, intraperitoneal), when administered in a preventive regimen, was able to reduce in a dose-dependent manner, with a maximum effect elicited by 10 mg/kg, both the first and the second nocifensive phase associated with the intraplantar injection of formalin, and to reduce λ-carrageenan-evoked hypersensitivity. The antinociceptive effects of CBG were comparable with those evoked by clonidine (0.2 mg/kg, intraperitoneal). Antagonism studies directed at investigating the mechanism of action underlying these effects suggested that α_2 receptors contributed to the antinociceptive effects evoked by CBG in both animal models. Collectively, our data convincingly demonstrated that CBG acts as an α_2-adrenoceptor agonist and that it induces antinociceptive effects that are mediated by such receptors.

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YANGONIN, A KAVALACTONE FROM *PIPER METHYSTICUM*, IS A NOVEL CB_1 RECEPTOR LIGAND

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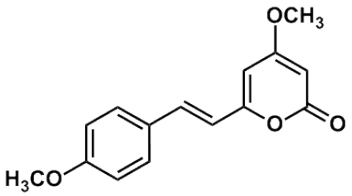
Kava ("intoxicating pepper"; *Piper methysticum* Forster) is a tropical shrub widely cultivated on many islands of the South Pacific. Kava is also the name of the aqueous beverage made from the roots of plant. Traditionally, various kava preparations have been used in local medicine, while the mildly euphoric and narcotic drink has been popular for social and ceremonial purposes. Kava root extracts have recently become available worldwide as over-the-counter antidepressant and anxiolytic medications. The pharmacology and underlying mechanisms of action of the so-called kavalactones, the psychoactive constituents of kava, are only partially understood. Each kavalactone appears to have a distinct pharmacological profile affecting GABA/benzodiazepine receptors, voltage-gated cation channels, monoamine uptake and catabolism, or the arachidonate cascade.

We investigated the binding of five major kavalactones, namely (±)-kavain, 7,8-dihydrokavain, methysticin, 7,8-dihydromethysticin, and yangonin (all from PhytoLab GmbH), to human recombinant CB_1 and CB_2 receptors. The inhibitory effect of these compounds on FAAH and MAGL has also been examined. Of the compounds tested, only yangonin had notable binding affinity to the hCB_1 receptor (K_i = 0.72 mM, [^{3}H]CP-55,940). None of the test compounds displaced the radioligand at the hCB_2 receptor (K_i >10 mM). Furthermore, the kavalactones showed negligible inhibitory effect on the two endocannabinoid degrading enzymes (IC_{50} > 10mM).



Yangonin

The observed affinity of yangonin to the hCB_1 receptor, though moderate, suggests that the endocannabinoid system might be involved in the complex human psychopharmacology of the traditional kava drink and the anxiolytic preparations obtained from the kava plant. Further studies with natural and synthetic compounds belonging to this new cannabinoid chemotype are thus warranted.

THE DOSE EFFECTS OF DRONABINOL (ORAL THC) IN HEAVY CANNABIS USERS

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Prior studies have separately examined the effects of dronabinol (oral THC) on cannabis withdrawal, cognitive performance, and the acute effects of smoked cannabis. A study examining each of these clinically relevant domains would benefit the continued evaluation of dronabinol as a potential medication in the treatment of cannabis use disorders. Thirteen daily cannabis users completed a within-subject crossover study in which they received 0 mg, 30 mg, 60 mg and 120 mg dronabinol per day for 5 consecutive days. Vital signs and subjective ratings of cannabis withdrawal, craving and sleep were obtained daily. On the 5th day of medication maintenance, participants completed a comprehensive cognitive performance battery and then smoked 5 controlled puffs of cannabis. Each dronabinol maintenance period occurred in a counterbalanced order and was separated by 9 days of ad-libitum cannabis use.

Dronabinol dose-dependently attenuated cannabis withdrawal and resulted in few adverse side effects or consequences on cognitive performance. Surprisingly, dronabinol did not alter the subjective effects of smoked cannabis, but cannabis-induced increases in heart rate were attenuated by the 60 mg and 120 mg doses. Withdrawal suppression may be therapeutically beneficial to individuals trying to stop cannabis use, and these data corroborate prior reports that withdrawal can be suppressed safely with dronabinol. While dronabinol did not attenuate the subjective effects of smoked cannabis, it is possible that this effect could be achieved at higher doses while maintaining the benefits of withdrawal suppression, a pattern that would better support therapeutic utility.

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PREDICTORS OF MARIJUANA RELAPSE:
ROBUST IMPACT OF CIGARETTE SMOKING

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Few marijuana smokers in treatment achieve sustained abstinence, yet factors contributing to these high marijuana relapse rates are unknown. In Study 1, data from five inpatient laboratory studies assessing marijuana intoxication, withdrawal and relapse were combined to assess factors predicting the likelihood and severity of marijuana relapse. Daily, nontreatment-seeking marijuana smokers (n=51; 10 $\pm$ 5 marijuana cigarettes/day) were enrolled. The objective of Study 2 was to follow-up on the findings of Study 1 to isolate the effects of cigarette smoking on marijuana relapse. Thus, marijuana intoxication, withdrawal and relapse were assessed in daily marijuana and cigarette smokers (n=15) under two within-subject, counter-balanced conditions: while smoking cigarettes as usual (SAU) and after 1-2 weeks without cigarettes (Quit).

The results from Study 1 showed that 49% of participants relapsed the first day active marijuana became available. Those who were also cigarette smokers (75%), who were not abstaining from cigarettes, were far more likely to relapse to marijuana than non-cigarette smokers (OR=19, $p<0.01$). Individuals experiencing more positive subjective effects (i.e. feeling "high") after marijuana administration and those with more negative affect and sleep disruption during marijuana withdrawal were more likely to have severe relapse episodes ($p< 0.05$). In Study 2, most participants (87%) relapsed to marijuana whether in the SAU or Quit phase. Cigarette smoking as usual did not significantly increase relapse, nor did it affect peak marijuana intoxication or most symptoms of marijuana withdrawal relative to tobacco cessation. Overall, daily marijuana smokers who also smoke cigarettes have high rates of marijuana relapse, and cigarette smoking versus recent abstinence does not directly influence this association. These data indicate that current cigarette smoking is a clinically important marker for increased risk of marijuana relapse.

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THE VOLITIONAL NATURE OF NICOTINE EXPOSURE DIFFERENTIALLY ALTERS ANANDAMIDE AND OLEOYLETHANOLAMIDE LEVELS IN THE VENTRAL TEGMENTAL AREA

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Cannabinoid CB_1 receptors play an important role in nicotine reward and CB_1 function is disrupted by chronic nicotine exposure, suggesting nicotine-induced alterations in endocannabinoid (eCB) signaling. However, the effects of nicotine on brain eCB levels have not been rigorously evaluated. Volitional drug intake produces physiological and behavioral effects distinct from forced drug administration, though the mechanisms underlying these differential drug effects are not known. This study compared the effects of volitional nicotine self-administration (SA) and forced nicotine exposure (YA) on levels of eCBs and related neuroactive lipids in the ventral tegmental area (VTA) and other brain regions. Brain lipid levels were indexed both by extractions of bulk brain tissues and by *in vivo* microdialysis in the VTA using liquid chromatography mass spectrometry (LC-MS).

The effects of nicotine SA vs. YA were first assessed on brain bulk tissue levels of eCBs, ethanolamides, and n-arachidonoyl conjugates of neurotransmitters (NA-NTs) in the ventral tegmental area (VTA) and six additional regions of interest. Measures of total tissue content identified greater effects of forced (YA) vs. self-administered (SA) nicotine on AEA and did not detect nicotine-induced changes in 2-AG content in any region. Interestingly, regionally-distinct alterations in brain tissue NA-NT content occurred following chronic nicotine exposure, including alterations in NA-GLU, NA-GABA, and NA-TAU (but not NA-DA).

Subsequently, the effects of nicotine exposure on eCB levels were assessed using *in vivo* microdialysis, an approach that indexes signaling-relevant changes in a manner that is temporally aligned with ongoing drug exposure. Repeated nicotine exposure significantly reduced baseline VTA dialysate oleoylethanolamide (OEA) levels relative to nicotine-naïve controls, and this was more pronounced following nicotine SA vs. YA. Nicotine SA increased interstitial levels of 2-AG, AEA and OEA in the VTA, while forced nicotine YA increased only 2-AG without altering AEA or OEA. Nicotine differentially modulates brain levels of 2-AG, AEA and OEA and the magnitude of these modulations is influenced by the volitional nature of the drug exposure. OEA inhibits VTA dopamine activity through non-CB_1 mechanisms, thus deficient baseline OEA levels along with increased AEA and 2-AG levels during nicotine SA may contribute to enhanced excitability and activity of the mesolimbic dopamine system. Collectively these findings implicate disrupted eCB and ethanolamide signaling in the motivational effects of nicotine.

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Δ^9-THC "REINTOXICATION" EFFECTS IN RATS AND HUMANS

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An important question in forensic toxicology is the extent to which plasma or urinary levels of cannabinoids are reliable indicators of recent cannabis use. One complicating factor is that the main psychoactive component of cannabis, Δ9-THC (THC), accumulates in fat tissue from where it can be released slowly back into the circulation. This makes it uncertain whether detection of THC, or its metabolite THC-COOH, reflects proximal or distal cannabis use. Our group has reported that manipulations that increase fat utilization (such as food deprivation or injections of the lipolytic hormone ACTH) can increase blood THC and THC-COOH levels in rats that have been repeatedly pre-exposed to THC (Gunsekaran et al. (2009) *Brit J Pharmacol*). We named this phenomenon "reintoxication". More recently we have extended our demonstrations of "reintoxication" as follows:

(1) Single dose effects in rats. Rats received a single injection of THC (5 mg/kg) and then experienced either 20 h food deprivation or normal food access. The food-deprived rats had significantly higher plasma THC-COOH levels than controls. This indicates that "reintoxication" effects can be observed after a single moderate dose of THC.

(2) **Exercise-induced effects.** Rats were pre-trained over several days to run on a treadmill. They then received a single injection of THC (5 mg/kg) and 24 h later were run on a treadmill for 30 min. Rats that ran on the treadmill had higher levels of plasma THC-COOH than non-exercising controls showing for the first time that exercise can also cause "reintoxication" effects.

(3) Functional reintoxication. After 5 daily injections of THC (5 mg/kg) and 4 days of washout, rats that were 24 h food deprived showed lower locomotor activity than non-fasted THC exposed controls. This hypoactivity was associated with higher plasma levels of THC-COOH and higher brain THC levels. This demonstrates that fat-derived THC can have significant functional behavioural effects in rodents.

(4) Reintoxication effects in humans. An ongoing study is examining whether regular cannabis users subjected to overnight fasting and a 30 min session on an exercise bike demonstrate elevated plasma levels of THC and THC-COOH and whether this is associated with cognitive impairment. Results will be reported at the ICRS meeting.

In summary, these observations give further proof of the concept of "reintoxication". This phenomenon may be relevant to the interpretation of the results of workplace and roadside drug tests.

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ANALYSIS OF SMOKE COMPOSITION FROM PRODUCTS CONTAINING SYNTHETIC CANNABINOIDS

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Designer drugs, such as synthetic cannabinoids have become increasingly prevalent, as have their interrelated health and societal consequences. Currently very little is known about the pharmacological and toxicological profiles of these products. Synthetic cannabinoids are typically processed into herbal formulations and smoked, yet very few studies have been published on components of smoke produced by burning the cannabinoids in this form. These formulations are typically prepared by people with little experience and so impurities with unexpected effects may be present in the smoked product. By determining these components we can assess what the user is actually being exposed to and the potential pharmacological and toxicological consequences.

A solution of AM-2201 was spiked into a rolled cigarette of "Marshmallow Leaf" (*Althaea Officinalis*) plant material using a Hamilton syringe to evenly distribute it along the length of the cigarette. Commercial "Spice" products "Yeah Right Head Funk" and "Mr Nice Guy" were also rolled into cigarettes. The cigarettes were smoked on a Borgwaldt KC RM20D smoking machine and the mainstream smoke was trapped using two glass impingers connected in series. The first impinger was used dry and cooled using liquid nitrogen. The second impinger contained methanol and was cooled in a dry ice-acetone bath. The trapped material from the first impinger was recovered using different solvents (polar and non-polar). Unspiked cigarettes were smoked to provide a matrix blank. The samples were analyzed by LC-MS using a Waters Synapt G2 HDMS QTOF mass spectrometer, interfaced with a Water Acquity UPLC. Data analysis was performed using manual mass defect filtering to identify the parent compound and related degradation and/or pyrolysis products. The solution of AM-2201 was analyzed as a control.

In smoke from cigarettes spiked with AM-2201, besides the AM-2201 compound, other related compounds generated during smoking such as JWH-022 were also identified by LC-MS. The "Head Funk" sample, which is known to contain AM-2201, showed the same related compounds as the sample spiked with AM-2201. The smoke sample from "Mr Nice Guy", which was previously found to contain JWH-018 and JWH-019, showed these compounds, but no other related cannabinoid degradants were noted. A more thorough assessment of this data is under way using the Synapt software to determine whether other pharmacologically or toxicologically important components can be elucidated from the data collected. Analysis by GC-MS is also in progress.

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PHARMACOLOGICAL PROFILE OF ABUSED INDOLE-DERIVED CANNABINOIDS CONTAINED IN 'HERBAL INCENSE' IN MICE

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Smoking of synthetic cannabinoid-enhanced "herbal incense" is an emerging substance abuse problem, with one study reporting that 8% of a sample of college students had used synthetic cannabinoids at least once [Hu et al., 2011, *Subst Abuse Treat Prev Policy*, **6**: 16]. The indole-derived cannabinoids identified in these products were originally developed (primarily by ICRS members, John Huffman and Alex Makriyannis) as research tools, and are structurally distinct from cannabinoids in the cannabis plant. Anecdotal reports from humans suggest that smoking of products containing synthetic cannabinoids result in a marijuana-like high as well as other physiological effects, some of which differ from those of marijuana (e.g., elevated blood pressure, vomiting) [Vardakou et al., 2010, *Toxicol Lett*, **197:** 157-162].

Although abused by humans, most published research on this class of compounds has been performed in vitro. Hence, the degree to which their in vivo effects overlap with those of Δ^9-tetrahydrocannabinol (THC) is largely unexplored. The purpose of this study was to assess the effects of a sample of indole-derived cannabinoids with a range of CB_1 receptor binding affinities in a functional observational battery (FOB) in mice and to compare their effects to those of THC. The FOB consisted of observations of adult male albino mice in their home cage and in an open field, their response to handling, and manipulative behavioral measures. Results revealed considerable overlap between THC and the active synthetic indole-derived cannabinoids in measures of CNS activity, with all cannabinoids decreasing activity, alertness and rearing. In addition, shared effects between THC and the active indole-derived cannabinoids within the muscle tone/equilibrium domain were apparent on some, but not all, measures. At higher doses of all cannabinoids, mice exhibited abnormal gait, with ataxia and flattened body posture. Greater impairment of muscle tone (more hypertonia) was noted for the indole-derived cannabinoids than for THC. In contrast, neither THC nor the indole-derived cannabinoids affected the autonomic reflexes of lacrimation or salivation, and neither produced stimulant-like behavior (e.g., circling, stereotypy). Effects that were observed uniquely for some of the indole-derived cannabinoids included measures in the domains of CNS excitability (increased difficulty in handling), sensorimotor activity (abnormal approach and startle responses), and autonomic effects (palpebral closure, gaping, and piloerection). Together with previous research, the results of this study suggest only partial overlap in the in vivo pharmacological profiles of THC and indole-derived cannabinoids. Further, the fact that the presence of unique effects of synthetic cannabinoids was not correlated with their CB_1 receptor affinity suggests they may have been mediated via a noncannabinoid mechanism.

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HEDONIC REWARD PROCESSING IS ATTENUATED IN CB1 RECEPTOR KNOCKOUT MICE

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The endocannabinoid (ECB) system has emerged recently as a key neurochemical mediator for reward processing. It is well known that cannabinoid signaling affects learning processes and can induce reinforcing and rewarding effects in humans and animals alike. However, the involvement of the ECB system in hedonic aspects of reward-related behavior is not completely understood so far. With the present study we investigated the modulatory role of the ECB system on hedonic perception, measured by the pleasure attenuated startle (PAS) paradigm in CB1 receptor knockout (CB1-KO) mice. During PAS, the aversive acoustic startle reflex (ASR) is reduced in the presence of a conditioned odor stimulus which has been previously paired with a food reward (sweetened condensed milk). This odor-induced reduction in ASR is not related to attentional alterations or a more general arousal by odor presentation, the conditioned olfactory cue rather elicits a pleasant emotional state during which the ASR is inhibited. Reward intake as well as PAS were significantly attenuated in CB1-KO mice compared to wild-type littermates. We observed no differences between wild-type controls and CB1-KO animals for startle-reactivity, startle habituation and the ability for olfactoric discrimination. These data therefore indicate that the ECB system, beside its important role for motivation and reward learning, appears to be also highly important for the mediation of hedonic aspects of reward processing.

COMBINED EFFECTS OF A MONOACYLGLYCEROL LIPASE AND FATTY ACID AMIDE HYDROLASE INHIBITOR IN MICE DISCRIMINATING Δ^9-TETRAHYDROCANNABINOL

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In pre-clinical studies, inhibitors of the degradative enzymes of endogenous cannabinoids produce antinociception. However, the extent to which enzyme inhibitors share subjective effects with cannabis has not been established. This study examined the combined effects of a monoacylglycerol lipase inhibitor (JZL-184) and a fatty acid amide hydrolase inhibitor (PF-3845) in a pre-clinical assay predictive of the subjective effects of cannabis. Male C57BL/6J mice (n=8) were trained to discriminate 5.6 mg/kg of Δ^9-tetrahydrocannabinol (Δ^9-THC) i.p. from vehicle under a fixed ratio 10 schedule of food presentation. Δ^9-THC dose-dependently increased Δ^9-THC responses; the ED_{50} value (95% confidence limits) of Δ^9-THC to produce discriminative stimulus effects was 3.3 (2.9-3.8) mg/kg. When administered separately, JZL-184 (4 and 40 mg/kg i.p. or doses that partially and fully inhibited monoacylglycerol lipase, respectively), and PF-3845 (10 mg/kg i.p. or a dose that fully inhibited fatty acid amide hydrolase), produced predominantly vehicle responses. When PF-3845 (10 mg/kg) was combined with 4 and 40 mg/kg of JZL-184, mean ± S.E.M. Δ^9-THC responding was 12 ± 4% and 56 ± 16%, respectively. In contrast, PF-3845 (10 mg/kg) and a relatively small dose of JZL-184 (4 mg/kg) produced an enhanced anti-allodynic effect in the chronic constrictive injury of the sciatic nerve, a mouse model of neuropathic pain. Taken together, these results indicate that combined inhibition of monoacylglycerol lipase and fatty acid amide hydrolase produces analgesia without cannabis-like subjective effects and should be considered as an alternative to Δ^9-THC and other CB_1 receptor agonists for pain management.

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CANNABINOID CB2 RECEPTORS MODULATE COCAINE SELF-ADMINISTRATION IN RATS

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We recently reported that brain cannabinoid CB2 receptors (CB2Rs) modulate cocaine self-administration *in mice* (Xi et al, *Nature Neurosci* 14:1160-1166, 2011). However, it is unknown if brain CB2Rs similarly modulate cocaine self-administration *in rats*. We now report that, in rats, systemic administration of the selective CB2R agonist JWH133 (0, 10, 20mg/kg, i.p., 30 min prior to testing) fails to alter cocaine self-administration under fixed-ratio reinforcement, but dose-dependently increases progressive-ratio (PR) break-point for cocaine self-administration. This effect is blocked by pretreatment with the selective CB2R antagonist AM630 (10 mg/kg, i.p.), suggesting CB2R mediation. To determine if this effect is mediated by brain or peripheral CB2Rs, JWH133 was locally administered into the nose, by which it may directly enter the brain. Intranasal JWH133 microinjections (0, 50, 100 mg/5 ml/nostril) also dose-dependently increased break-point for cocaine. At very high doses (200 mg/5 ml/nostril), intranasal JWH133 significantly decreased PR break-point. JWH133 microinjections into the lateral ventricles (20, 40 mg/4 ml/side), but not nucleus accumbens (NAc) (5, 10 mg/1 ml/side), dose-dependently decreased break-point, suggesting mediation outside the NAc. If break-point is taken to reflect motivation to work for drug, low-to-moderate JWH133 doses may partly attenuate cocaine reward causing a compensatory increase in drug-taking, while high doses may produce near total inhibition of cocaine reward causing a decrease in motivation to take cocaine. An alternative explanation may lie in the notion that PR break-point can reflect *either* a change in reward priming-threshold *or* satiety-threshold (Norman and Tsibulsky, *Brain Res* 1116:143-152, 2006), and that priming and satiety thresholds can move inde-pendently of each other (Wasserman et al, *Pharmacol Biochem Behav* 17:783-787, 1982). Low-to-moderate dose JWH133 may raise the *satiety* threshold – increasing break-point. High dose JWH133 may raise the *priming* threshold – decreasing break-point. Supporting this supposition are our findings using *in vivo* brain microdialysis – in which we find that systemic (10, 20 mg/kg, i.p.) or intra-NAc (1, 10, 100 mM) administration of JWH133 fails to alter basal or cocaine-enhanced extracellular NAc dopamine (DA), suggesting a non-DA mechanism underlying JWH133's action on cocaine taking. Further, systemic JWH133 significantly and dose-dependently increased, while intra-NAc JWH133 perfusion dose-dependently decreased, extracellular glutamate – suggesting that the increased NAc glutamate produced by systemic JWH133 is a final net effect of direct inhibition and indirect augmentation of glutamatergic input to the NAc. This altered glutamatergic signaling may underlie alteration in motivational salience of cocaine-associated environmental cues (Suto et al, *Psychopharmacology* 211:267-275, 2010) after brain CB2R activation – leading to altered PR break-points. In sum, brain CB2R activation alters cocaine reward and/or cocaine-associated cue-induced incentive salience in rats, possibly by complex brain mechanisms involving glutamatergic neural substrates.

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GENERATION OF *N*-ACYLPHOSPHATIDYLETHANOLAMINE BY MEMBERS OF PHOSPHOLIPASE A/ACYLTRANSFERASE (PLA/AT) FAMILY

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Bioactive *N*-acylethanolamines (NAEs), including anandamide, are enzymatically biosynthesized from their precursors, *N*-acylphosphatidylethanolamines (NAPEs). NAPE is formed in animal tissues by *N*-acylation of phosphatidylethanolamine by *N*-acyltransferase. Although Ca^{2+}-dependent *N*-acyltransferase is not molecularly characterized, we recently revealed that all five members of the HRASLS tumor suppressor family, which we renamed as the phospholipase A/acyltransferase (PLA/AT) family, possess *N*-acyltransferase activities together with $PLA_{1/2}$ and lysophospholipid *O*-acyltransferase activities. However, it remained unclear whether these proteins can form NAPE in living cells. In the present studies we first show that COS-7 cells transiently expressing recombinant PLA/AT-1, -2, -4 or -5 generate significant amounts of [^{14}C]NAPE and [^{14}C]NAE when metabolically labeled with [^{14}C]ethanolamine. Second, as analyzed by liquid chromatography-tandem mass spectrometry, the stable expression of PLA/AT-2 in HEK293 cells remarkably increased endogenous levels of various species of NAPEs and NAEs, including anandamide. Third, when NAPE-hydrolyzing phospholipase D was additionally expressed in the PLA/AT-2-expressing cells, the accumulating NAPE was efficiently converted to NAE. Finally, purified recombinant PLA/AT-2 selectively used *sn*-1 acyl group of the donor phospholipid to form NAPE and was not stimulated by Ca^{2+}. These results suggest that the PLA/AT family proteins may be involved in Ca^{2+}-independent generation of bioactive NAEs *in vivo*.

CALCIUM RELEASE FROM THE ENDOPLASMIC RETICULUM CONTRIBUTES TO TRIGGERING ENDOCANNABINOID PRODUCTION IN CEREBELLAR PURKINJE CELLS

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Introduction. Presynaptic CB_1 cannabinoid receptors can be activated by endogenous cannabinoids (endocannabinoids) synthesized by postsynaptic neurons. This phenomenon is termed endocannabinoid-mediated retrograde synaptic signaling (for review see Kano et al., Physiol Rev 89: 309–380, 2009). The production of endocannabinoids in postsynaptic neurons can be triggered by an increase in intracellular Ca^{2+} concentration and by activation of $G\alpha_{q/11}$ protein-coupled receptors. The aim of the present study was to clarify the role of Ca^{2+} released from the endoplasmic reticulum in endocannabinoid production triggered by activation of the $G\alpha_{q/11}$ protein-coupled metabotropic glutamate receptor type 1 (mGluR1 receptor).

Methods. The experiments were performed on slices prepared from the mouse cerebellum. Glutamatergic excitatory synaptic currents (eEPSCs) were elicited by electrical stimulation in the molecular layer of the cerebellar cortex and were recorded in Purkinje cells with patch-clamp techniques. In order to observe Ca^{2+} concentration changes in dendrites and dendritic spines, Purkinje cells were loaded via the patch pipette with the Ca^{2+}-sensitive fluorescent dye Oregon green 488 BAPTA2.

Results. Superfusion of the mGluR1 agonist DHPG (5×10^{-5} M) elicited an inward current (probably mediated by the TRPC3 channel), increased the Ca^{2+} concentration in dendrites and dendritic spines and suppressed the eEPSCs. Expectedly, the effects of DHPG were prevented by the mGluR1 antagonist CPCCOEt (10^{-4} M). The CB_1 antagonist rimonabant (10^{-6} M) prevented the suppression of eEPSCs, pointing to the involvement of endocannabinoids and CB_1 receptors. The DHPG-induced suppression of eEPSCs was strongly attenuated after depletion of the endoplasmic reticulum Ca^{2+} stores by the SERCA pump inhibitors thapsigargin (5×10^{-5} M) and cyclopiazonic acid (3×10^{-5} M). mGluR1 receptors in the postsynaptic Purkinje cells were also activated by endogenous glutamate released by burst stimulation (10 pulses / 100 Hz) of the presynaptic parallel fibres. The burst stimulation elicited fast eEPSCs (sensitive to the AMPA/kainate receptor antagonist DNQX), slow eEPSCs (sensitive to CPCCOEt) and Ca^{2+} increases in denditic compartiments (sensitive to DNQX and CPCCOEt). The burst stimulation suppressed eEPSCs, and abolishment of this suppression by rimonabant verified the role of endocannabinods and CB_1 receptors. Ca^{2+} store depletion by thapsigargin strongly attenuated the burst-induced suppression of eEPSCs.

Conclusions. DHPG and endogenous glutamate increased the intracellular Ca^{2+} concentration and suppressed glutamatergic synaptic transmission. This suppression was attenuated in experiments in which intracellular Ca^{2+} stores were depleted by SERCA pump inhibitors. Thus, Ca^{2+} release from the endoplasmic reticulum is important for endocannabinoid production, when this production is initiated by activated mGluR1 receptors.

SUBSTRATE SELECTIVE INHIBITION OF COX-2 AS A NOVEL STRATEGY FOR *IN VIVO* ENDOCANNABINOID AUGMENTATION

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Development of endocannabinoid degradation inhibitors has significantly advanced the therapeutic potential of the eCB system for a variety of pathological conditions including mood and anxiety disorders. COX-2 oxygenates both AEA and 2-AG, however, the utility of COX-2 inhibitors is limited by the substantial role COX-2 plays in prostaglandin (PG) synthesis. We have recently developed substrate-selective inhibitors of COX-2 (SSI-COX-2) that selectively inhibit the oxygenation of 2-AG and AEA, but not arachidonic acid (AA), by COX-2. The aim of the current study was to test the hypothesis that SSI-COX-2 increase brain eCB levels via a COX-2 dependent mechanism, without affecting PG formation.

The novel SSI-COX-2, LM-4131 (a morpholino amide derivative of indomethacin; described in accompanying abstract Hermanson et al.) increased brain AEA levels without effecting 2-AG or AA levels 2 hours after i.p administration. LM-4131 had minimal effect on brain PG levels. Indomethacin also increased brain AEA levels, but profoundly decreased PG levels. The ability of LM-4131 to increase brain AEA levels was absent in COX-2 knock-out mice, but present in WT littermates.

LM-4131 dose dependently increased exploratory behavior in the open field, as well as center time exploration, which was most pronounced during minutes 40-60 of the one-hour test. These effects were similar to those seen with the FAAH inhibitor PF-3845 and indomethacin. Importantly, these behavioral effects of LM-4131 were absent in COX-2 KO mice. LM-4131 also increased the number of light compartment entries in the light-dark box test. Ongoing studies are aimed at determining the role of cannabinoid receptors in mediating the behavioral effects of LM-4131. These data indicate that SSI-COX-2 is a viable strategy for *in vivo* augmentation of eCB signaling, and that substrate selective inhibitor LM-4131 has anxiolytic-like actions in animal models.

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ACETAMINOPHEN AND ITS METABOLITES, 4-AMINOPHENOL AND AM-404, ARE SUBSTRATE-SELECTIVE INHIBITORS OF ENDOCANNABINOID OXYGENATION BY CYCLOOXYGENASE-2

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Cyclooxygenease-2 (COX-2) catalyzes the oxygenation of arachidonic acid (AA) and the endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA), to prostaglandin endoperoxide derivatives. Non-steroidal anti-inflammatory drugs such as ibuprofen, naproxen, and lumiracoxib, which are weak, competitive inhibitors of AA oxygenation, are potent non-competitive inhibitors of 2-AG and AEA oxygenation. These compounds are as much as 1,000-fold more potent at inhibiting endocannabinoid oxygenation than AA oxygenation. We have termed this phenomenon substrate-selective inhibition (SSI-COX-2) and propose that it contributes to the pharmacological effects of certain NSAIDs by preventing oxidative endocannabinoid metabolism at sites of COX-2 induction.

The analgesic agent, acetaminophen (APAP), and its *in vivo* metabolites, 4-aminophenol and *N*-(4-hydroxyphenyl)arachidonoylamide (AM404), exhibit SSI-COX-2 with IC_{50}'s of 233 μM, 71 μM and 97 nM for inhibition of 2-AG oxygenation, respectively. No inhibition of AA oxygenation is observed up to 1 mM for APAP or 4-aminophenol. AM404 exhibits 700-fold selectivity for inhibition of 2-AG and AEA oxygenation compared to AA oxygenation. APAP and AM404 selectively inhibit 2-AG oxygenation by lipopolysaccharide-activated RAW264.7 cells with IC_{50}'s of 97 μM and 46 nM, respectively. Structure-activity analysis of a series of AM404 analogs reveals that the presence of a hydroxyl group at the *para*-position of the phenylamide ring is essential for SSI-COX-2. Dissociation of AM404 from COX-2 is slow in the absence of substrate but is rapid in the presence of AA. Inhibition of endocannabinoid oxygenation is reversed by addition of nM concentrations of AA. The observations are consistent with a model for SSI-COX-2 in which APAP, 4-aminophenol or AM404 bind to the allosteric subunit of COX-2 to inhibit endocannabinoid oxygenation in the catalytic subunit. Substrate-selective inhibition of endocannabinoid oxygenation may contribute to the pharmacological actions of APAP and its metabolites.

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FLAT IS NOT AN INTRACELLULAR ANANDAMIDE TRANSPORTER

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The endocannabinoid anandamide (AEA) regulates numerous physiological processes including nociception, inflammation, and reproduction. AEA is inactivated through cellular uptake and subsequent intracellular hydrolysis by fatty acid amide hydrolase (FAAH). Prior to reaching FAAH, AEA must diffuse across the aqueous cytosol. The intracellular transport of AEA was proposed to be mediated by fatty acid binding proteins, heat shock protein 70, and most recently FAAH-like anandamide transporter (FLAT) (Kaczocha et al., 2009; Oddi et al., 2009; Fu et al., 2011). FLAT, a catalytically silent, truncated variant of FAAH (~56 kDa) was found to be co-expressed with FAAH and to enhance the uptake of AEA when overexpressed in cells. Additionally, an inhibitor of FLAT reduced nociception in rodent models, indicating that this protein may represent a target for the development of novel therapeutics.

In contrast to the findings of Fu et al (2012), previous reports examining FAAH expression by western blotting detected a single band at ~63 kDa corresponding to FAAH. This apparent discrepancy prompted us to investigate FLAT expression and function. In contrast to the findings of Fu et al (2012) and in agreement with previous work, western blotting revealed a single band corresponding to FAAH and failed to detect the presence of FLAT in brain, neuroblastoma, and astrocytoma cells. In our hands, FAAH activity was not detected in astrocytoma cells, contrasting the reported presence of FAAH and FLAT in these cells as observed by Fu et al (2012). To provide a mechanistic rationale for FLAT's ability to drive AEA uptake into cells, the catalytic activity of FLAT was explored. When overexpressed in HeLa cells, FLAT was found to be catalytically active. Its enzymatic activity was lower than that of FAAH and was inhibited by the FAAH inhibitors URB597, PF3845, and MAFP. Compared to FAAH, overexpression of FLAT in HeLa cells modestly potentiated AEA uptake and its transport enhancing effects were reduced in the presence of URB597. Immunofluorescence revealed that FLAT localized to internal membranes and was not found at sites proximal to the plasma membrane, indicating that it is unlikely to serve as a freely diffusible protein. Taken together, our present study casts into doubt the existence and function of FLAT as an intracellular AEA transport protein.

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FATTY ACID AMIDE HYDROLASE INHIBITORS EXERT COMPLEX ANTI-ACNE ACTIONS IN HUMAN SEBOCYTES

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We have previously shown that prototypic endocannabinoids (anandamide [AEA] and 2-arachidonoylglycerol), also produced in the human sebaceous glands (hSGs), play essential role in the maintenance of the basal lipid (sebum) production of sebocytes. Moreover, we have shown that "exogenous" application of these endocannabinoids resulted in dramatically elevated sebaceous lipid synthesis (Dobrosi et al., FASEB J. 22 (2008) 3685-3695.). These data implicated that the modulation of the "sebaceous endocannabinoid tone" may play an essential role in the regulation of the sebum production. Therefore, in the current study, we aimed at investigating the expression and potential role of the endocannabinoid degrading enzyme fatty acid amide hydrolase (FAAH) in the hSGs.

Using a well characterized human immortalized sebocyte cell line (SZ95 sebocytes), we have first shown that FAAH is expressed in the cells (RT-qPCR, Western blot). In order to investigate its biological significance, we have applied two FAAH-inhibitors (URB597 and JP104). In contrast of our expectations (i.e. elevated sebum synthesis by the increase of the endocannabinoid tone) we found that non-cytotoxic doses (determined by MTT-assay and combined fluorescent $DilC_1(5)$-SYTOX Green staining) of these agents did not alter the basal lipid production of the cells (Oil Red O and fluorescent Nile Red staining). Of further importance, we have also shown that, quite unexpectedly, high concentrations (1-10 μM) of the inhibitors were able to significantly suppress the lipogenic effect of AEA. Moreover, both substances inhibited proliferation (CyQUANT assay) and decreased expression of molecules (IL-18, TNF-α, IGF-I receptor) involved in the pathogenesis of acne vulgaris, the most common human skin disease.

Since substances exerting similar, complex "anti-acne" effects are known to increase intracellular $[Ca^{2+}]_{IC}$ of the sebocytes, in order to reveal potential signaling mechanisms underlying the above findings, we have investigated the effects of URB597 and JP104 on the Ca^{2+}-homeostasis of SZ95 sebocytes. Importantly, both inhibitors evoked transient increase in $[Ca^{2+}]_{IC}$ (Fluo-4 AM; FlexStation). Furthermore, these effects were successfully prevented by either the decrease of the extracellular $[Ca^{2+}]_{EC}$ or the non-selective transient receptor potential (TRP) channel inhibitor ruthenium red suggesting the involvement of TRP channel(s) in these actions. Our ongoing RNAi studies intend to reveal the target molecule.

Collectively, these results strongly argue for that FAAH inhibitors – most probably via the activation of TRP channel(s) – target multiple steps of the pathogenesis of acne vulgaris and hence should be exploited in future clinical studies as promising, novel anti-acne agents.

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LONG-TERM CHANGES IN FATTY ACID AMIDE HYDROLASE KNOCKOUT MICE: INVOLVEMENT OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 RECEPTOR

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Fatty acid amide hydrolase (FAAH) is a critical enzyme in the regulation of endocannabinoid/endovanilloid signaling through effects on the contents of N-arachidonylethanolamine (AEA) and *N*-arachidonoyldopamine (NADA). Inhibition of FAAH activity has been suggested as a therapeutic approach for the treatment of several human disorders, including pain and anxiety. However, very little is known regarding the impact of long-term inhibition of FAAH on endovanilloid signaling. We found age-related differences in body temperature between wild type (WT) and FAAHko mice. FAAHko mice are significantly hypothermic at 2mo but significantly hyperthermic at 9mo of age compared to age-matched WT mice. Transient receptor potential vanilloid 1 receptor (TRPV1) antagonists produce a significant hyperthermia, indicating that endogenous activation of this receptor reduces body temperature. Since the FAAH substrates AEA and NADA are agonists of TRPV1, we explored the hypothesis that age-dependent TRPV1 down-regulation or desensitization occurs in the 9 mo FAAHkos, resulting in an increase in body temperature. To test this hypothesis, dorsal root ganglion (DRG) cells were isolated from 2-3 and 9 mo old mice of both genotypes and TRPV1 function was determined using Fura-2 calcium imaging. DRG cells from 9mo, FAAHko mice revealed a significant increase in the percentage of neurons responsive to the TRPV1 agonist, capsaicin, compared to WT (52% versus 33%, $p<0.001$). However, the magnitude of response to capsaicin in was significantly decreased in FAAHko DRGs compared to WT (262±22% versus 386±42% increase in FURA 340:380 ratio; $p<0.05$). There were no significant differences in the percentage of responsive neurons or the response per neuron between DRGs from 3mo WT and FAAHko mice (42% versus 45%, $p=0.65$; and 334±32% versus 253±24%, $p=0.11$). These in vitro studies are consistent with down-regulation of TRPV1 receptors in response to long-term FAAH loss and suggest further that FAAH loss could trigger an increase in TRPV1 expression by other DRGs as a compensatory mechanism. In the first, 9mo, but not 2mo, FAAHko mice were significantly more likely eat food with high capsaicin content (habanero chili peppers) than WT mice. These data suggest that the long-term increase in TRPV1 agonists that results from genetic deletion of FAAH induces desensitization of TRPV1 channels, leading to hyperthermia and loss of sensitivity to capsaicin.

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REGULATION OF MONOACYLGLYCEROL LIPASE FUNCTION BY PHOSPHORYLATION

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Monoacylglycerol lipase (MAGL) is a serine hydrolase that hydrolyzes the endocannabinoid, 2-arachidonoylglycerol (2-AG), into arachidonic acid and glycerol. Activity based protein profiling (ABPP) has shown that MAGL contributes approximately 85% of 2-AG hydrolysis in mouse brain [Blankman *et al.,* 2007, Chemistry and Biology 14(12): 1347]. Blockade of MAGL using JZL184, a selective MAGL-inhibitor, prolongs the duration of cannabinoid receptor type 1 (CB1R) signaling [Pan *et al.*, 2009*,* JPET 331(2): 591-7]. However, very little is known about the mechanisms by which MAGL is regulated.

Post-translational modifications are one of the major mechanisms of regulation of enzyme function. *In silico* analysis of the primary amino acid sequence of MAGL proteins of various species (i.e. mouse, rat and human) reveals several potential phosphorylation sites. Native neuronal MAGL migrates as multiple electrophoretic species on two-dimensional gel electrophoresis, suggesting post-translational modifications. These findings lead us to hypothesize that MAGL is phosphorylated at multiple residues, and that phosphorylation is one of the mechanisms of MAGL regulation.

Vectors encoding N-terminally flag and myc tagged human MAGL (hMAGL) were used to heterologously express MAGL in human embryonic kidney cells (HEKs). Thus expressed hMAGL was immunoprecipitated using an anti-Flag antibody and hMAGL was subjected to mass spectrometric analyses. Three phosphorylated residues were identified by this analysis: threonine 10, threonine 228 and serine 244. We used a site-directed mutagenesis approach to study the effects of each of these residues on MAGL function. MAGL activity was determined using radiolabelled 2-mono-oleyolglycerol as the substrate. Centrifugal fractionation approach was used to determine the relative distribution of MAGL in various sub-cellular compartments. Our results indicate that phosphorylation at serine 244 is important for both enzymatic activity and association with the plasma membrane of cells. These findings support the hypothesis that MAGL is phosphorylated at multiple residues and proves that phosphorylation is one of the mechanisms by which MAGL function is regulated.

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VIRTUAL SCREENING FOR INHIBITORS OF THE ANANDAMIDE TRANSPORTER (FATTY ACID BINDING PROTEINS)

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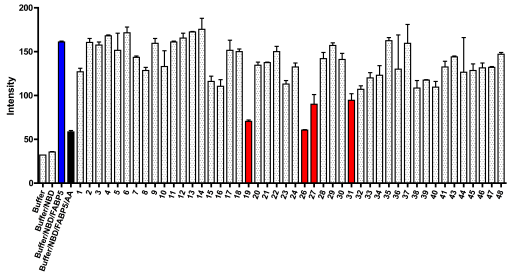
Lipids, owing to their water insolubility, require a variety of fatty acid binding proteins (FABPs) as chaperones or transporters to carry them throughout cells. Recently, it was shown that anandamide also uses FABPs as intracellular transporters (1). The FABPs are part of the pathway for anandamide inactivation by the fatty acid amide hydrolase (FAAH), an enzyme localized inside the cell on the endoplasmic reticulum. FABPs are drug targets, similar to FAAH, that decrease hydrolysis of anandamide and its uptake into cells, raising the levels of extracellular anandamide. Few specific FABP inhibitors have been described. There are those that were originally designed to inhibit a putative anandamide transmembrane transporter and have subsequently been shown to bind to FABPs (2). A few have specifically been designed for FABP4, such as BMS309403 (3), that has protective effects in metabolic syndrome and atherosclerosis. BMS309403 also binds some FABPs that carry anandamide.

In order to find new specific inhibitors, we carried out an *in silico* screening of approximately a million compounds employing a new the footprint similarity scoring function (*4*) to identify ligands whose binding profiles are similar to oleic acid, a natural FABP substrate. Human FABP5 was purified to homogeneity and a fluorescent displacement-binding assay was employed using NBD-stearate as a fluorophore. The inhibitory activities were measured by monitoring decrease in fluorescence using a multi-plate reader spectrofluorimeter. The methods employed for footprint scoring eliminated approximately 1,057,000 compounds with the identification of 48 compounds selected for binding assay. Approximately half of the compounds identified by virtual screening produced inhibition. Four of these compounds were found to be as good or better inhibitors of FABP5 than BMS309403.



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FATTY ACID AMIDE HYDROLASE DELETION POTENTIATES *IN VIVO* GLIAL ACTIVITY IN RESPONSE TO ACUTE BRAIN INJURY

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Glial cells are involved in the inflammatory responses of the brain against different kinds of insults. Astrocytes and microglia are the main types of cells that result activated after an alteration of the brain parenchyma homeostasis. We have recently reported that the enhancement of the endocannabinoid tone through the genetic deletion of the fatty acid amide hydrolase (FAAH) induces profound phenotypic changes in glial cells in vitro[1]. In the present experiments we have used an in vivo model of acute insult to the brain in order to explore the effects of FAAH gene deletion on glial responses. To that end, we generated a new transgenic mouse ($Cx3cr1^{eGFP/+}/FAAH^{-/-}$) that exhibits green fluorescence in microglial cells while lacking the enzyme FAAH and compared them with their corresponding controls ($Cx3cr1^{eGFP/+}/FAAH^{+/+}$). To study glial activity in vivo, we used an intravital multiphoton microscopy system[2,3]. By opening a small cranial window (3mm diameter), we could observe cellular responses against acute laser injuries (15microns diameter) induced in the brain parenchyma. Our data indicate that the absence of FAAH exacerbated the microglial response by increasing their ability to direct cell processes towards the sites of injury. Furthermore, this effect was mediated by cannabinoid CB_1 receptors as was prevented by the preincubation with SR141716. In addition, astrocytic hemichannels exhibited a differential pattern of activation in mice lacking FAAH, which could partially account for the observed effect in microglial cells. These results confirm the relevant impact that FAAH gene deletion has on glial function.

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REGULATION OF THE FAAH GENE BY ESTROGEN ENGAGES HISTONE DEMETHYLASE LSD1

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Estrogen (E_2) regulates spermatogenesis, yet its direct target genes have not yet been identified. Here, we cloned the proximal 5' flanking region of the mouse fatty acid amide hydrolase (*faah*) gene upstream of the luciferase reporter gene, and demonstrated its promoter activity and E_2 inducibility in primary mouse Sertoli cells. Specific mutations in the E_2 response elements (ERE) of the *faah* gene showed that two proximal ERE sequences (ERE2/3) are essential for E_2-induced transcription, and chromatin immunoprecipitation experiments showed that E_2 induced estrogen receptor β binding at ERE2/3 sites in the *faah* promoter *in vivo*. Moreover, the histone demethylase LSD1 was found to be associated with ERE2/3 sites and to play a role in mediating E_2 induction of FAAH expression. E_2 induced epigenetic modifications at the *faah* proximal promoter compatible with transcriptional activation, by remarkably decreasing methylation of both DNA at CpG site and histone H3 at lysine 9. Finally, FAAH silencing abolished E_2 protection against apoptosis induced by the FAAH substrate anandamide.

Conclusions. Taken together, our results identify FAAH as the first direct target of E_2.

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THE ENDOCANNABINOID SYSTEM PLAYS A ROLE IN NEURONAL REFINEMENT OCCURRING IN THE ADOLESCENT PREFRONTAL CORTEX

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Adult female rats exposed to THC during adolescence exhibit a complex behavioral phenotype characterized by impairments in spatial working memory and recognition memory, decreased social behavior, anhedonia, passive coping strategy. This behavioral picture was also paralleled by different cellular alterations, among all decreased synaptic functionality in the prefrontal cortex. It is worth noting that this complex phenotype did not develop when THC was administered at adulthood, thus suggesting that the compound may affect maturational refinement peculiar of the adolescent brain. One of the most marked changes in postnatal brain development is reorganization of synapses, which include synaptic stabilization and elimination (pruning). The eliminated synapses in this process of "synaptic pruning" are mainly glutamatergic. Therefore to test whether the endocannabinoid system is involved in the neuronal refinement peculiar of adolescence, we monitored PSD95, a major structural protein of the postsynaptic density of the glutamate synapse, extensively used to mark synaptic sites, as well as the gluA1 and gluA2 subunits of the AMPA receptor from mid (PND46) to late adolescence (PND60) and into adulthood (PND75). These markers show a specific course during this temporal window that is altered by AM251 treatment, thus suggesting that the endocannabinoid tone controls, at least in part, their course during adolescence. Moreover, adolescent exposure to THC was able to alter the fluctuations in PSD95 levels observed from PND46 to adulthood as well as in gluA1 and gluA2. Golgi staining was also used to better clarify the pruning event both in control and THC-exposed animals.

These results clearly suggest that the endocannabinoid system plays a role in the neuronal refinement present in the adolescent prefrontal cortex and exposure to THC during this still vulnerable developmental window may disrupt it. Specifically the endocannabinoid tone seems to be involved in glutamate synapse development in adolescence, a process impaired by adolescent exposure to THC. This is relevant since dysfunction of glutamatergic transmission is considered the core feature and fundamental pathology of several mental disorders.

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IN MEMORIAM OF PROF. ESTER FRIDE

ACUTE AND CHRONIC CANNABINOID TREATMENT DIFFERENTLY AFFECT ETHANOL INTAKE IN PUBERTAL AND ADULT RATS

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Adolescence is a highly critical period for the initiation of drug use and abuse with alcohol, nicotin and cannabis being the most commonly used substances. Teenagers often engage in polydrug use which was found to increase the risk of later drug abuse and adverse health consequences. The rates of simultaneous polydrug use are especially high among cannabis users however, the short and lasting consequences of the simultaneous use of cannabis and alcohol during adolescence on later alcohol reward are poorly studied. The present study assessed the acute, chronic and lasting effects of pubertal and adult cannabinoid treatment on repeated ethanol (10 vol%) intake and on alcohol reward in male Wistar rats. Therefore, a pubertal (pd40) and an adult group of rats were either treated with the synthetic cannabinoid agonist WIN 55,212 (WIN) or vehicle. Acute administration of WIN was found to exclusively stimulate initial alcohol intake in pubertal rats, whereas no effects were observed in adult rats. A subsequent chronic WIN treatment with daily injections stimulated alcohol intake in pubertal and adult rats, but was more pronounced for the pubertal treatment. After an abstinence phase, the lasting effects of pubertal and adult WIN treatment were evaluated in adult rats with limited-access intake and progressive ratio testing for alcohol. Whereas no lasting WIN effect could be found for adult-treated rats, pubertal cannabinoid treatment significantly increased consumption and the reinforcing effects of ethanol in adulthood. These data clearly demonstrate a higher vulnerability for the acute and chronic effects of cannabinoids during puberty on alcohol intake. Additionally, pubertal but not adult cannabinoid treatment affected the rewarding properties of ethanol persistently.

A CANNABINOID-RALA SIGNALLING PATHWAY CONTROLLING NEURAL PROGENITOR MIGRATION

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In the postnatal mammalian brain, stem cell-derived neural progenitors migrate from the subventricular zone towards the olfactory bulb following a very well defined path, the rostral migratory stream (RMS). Importantly, these cells have the capacity to migrate away from their native route to areas of pathological damage in the adult brain. While our understanding of neural progenitor migration has increased over the years, the exact molecular mechanisms remain to be fully elucidated. Understanding the migratory properties of these cells is essential to fully exploit their potential in neuroregenerative strategies.

The endocannabinoid system has been previously shown to play an important role in the regulation of neural stem cell proliferation. We have recently shown that an endogenous cannabinoid tone is also involved in controlling the polarised migration of RMS progenitors both *in vitro* and *in vivo*. Indeed, agonists of the G protein-coupled cannabinoid receptors CB1 and CB2 markedly increase neural progenitor migration, while CB receptor antagonists significantly impair it.

In an effort to analyse the CB-dependent signalling pathways regulating neural progenitor motility, we found that stimulation of CB1/CB2 receptors leads to a significant activation of RalA, a Ras-like GTPase previously shown to be involved in the control of neuronal morphology and polarity. RalA appears to be abundantly expressed in a vesicular pattern in migrating neural progenitors. Using time-lapse imaging of RMS explants, we show that siRNA-mediated knockdown of RalA abolishes cannabinoid-stimulated motility and strongly impairs nucleokinesis, a crucial step for efficient migration. Moreover, expression of dominant negative RalA using in vivo postnatal electroporation significantly disrupts the typical polarised neural progenitor morphology.

Current work is aimed at dissecting the molecular components of this cannabinoid-RalA signalling pathway, including other small GTPases and motility regulators. We are also further examining the effect of CB agonists/antagonists and RalA knockdown on neural progenitor migration using *in vivo* electroporation in the postnatal mouse forebrain together with confocal time-lapse imaging of fluorescently labelled progenitors in cultured brain slices.

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ALTERATIONS IN CANNABINOID SIGNALLING CONTRIBUTE TO ADHD-LIKE SYMPTOMS AT ADULTHOOD

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Following birth the environment can influence the postnatal phase of cerebral development. Such factors act within specific critical periods but their effects can be life-long. Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common developmental disorders, appearing first in childhood with symptoms sometimes continuing through adolescence and into adulthood. We have previously presented a new animal model of ADHD in which postnatal administration of the CB_1 antagonist SR141716A led to the development of ADHD–like behaviour in adult mice. Cannabis is frequently consumed by patients with ADHD, however the effect of Δ^9-THC in this model has not been investigated. In this study postnatal administration of SR141716A (5 mg/kg) significantly impaired the attention behaviour of eight week old male mice in the pre-pulse inhibition test, mimicking inattention behaviour of adult ADHD. Ten-twelve week old mice were tested in the open-field test. SR141716A significantly increased ambulation behaviour, mimicking hyperactivity behaviour which is associated with ADHD. In male mice, co-treatment with Δ^9-THC (5 mg/kg) significantly reversed the effects of SR141716A-induced hyperactive behaviour and significantly improved the attention to startle. Interestingly, similar results were obtained when retinoic acid, a lipid derived from vitamin A, was administrated exogenously. Administration of retinoic acid (5 mg/kg) resulted in a significant reduction in postnatal body weight similarly to that induced by SR141716A. Retinoic acid also induced alterations in the level of CB_1 receptor in the brain stem and cerebellum, cerebral areas which gate auditory and locomotor activity. These results suggest that both the endocannabinoid system and retinoid signalling system are intertwined, leading to an ADHD-like disorder in adults.

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TYPE 1 CANNABINOID RECEPTOR (CB_1)-MEDIATED INDUCTION OF CB_1 EXPRESSION IN CELLULAR MODELS OF HUNTINGTON'S DISEASE

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In the central nervous system, the type 1 cannabinoid receptor (CB_1) is expressed at the highest levels in the striatum, compared to other tissues[1]. Activation of CB_1 inhibits Ca^{2+}-dependent neurotransmitter release and increases expression of pro-survival genes[1]. Treatment of cultured hepatocytes, T cells, and colorectal cancer cells with cannabinoids, such as 2-arachidonyl glycerol, anandamide, or Δ^9-tetrahydrocannabinol, increases CB_1 mRNA levels[2,3,4]. Huntington's disease (HD) is an inherited, progressive, neurodegenerative disorder characterized by cognitive, behavioural, and motor control deficits[1]. HD is caused by expression of one copy of the *huntingtin* gene containing an expanded CAG repeat, which codes for mutant huntingtin protein (mHtt)[1]. mHtt causes cell-specific transcriptional dysregulation, ATP deficit, and eventual cell death[1]. CB_1 mRNA levels are lower in human HD patients relative to healthy controls, and in the striatum of adult HD mice relative to age-matched wild-type controls, prior to other pathogenic changes[1]. We hypothesized that cannabinoid treatment could increase CB_1 levels and improve cellular viability in a cell culture model of HD.

We found that CB_1 mRNA and protein levels were increased in a cell culture model of HD following treatment with arachidonyl-2'-chloroethylamide (ACEA), methanandamide, and anandamide. This increase was cannabinoid dose-dependent (0.01 – 5.00 μM) and mediated by activated CB_1 receptors signalling through Akt to NF-κB. CB_1 levels were increased in wild-type and mHtt-expressing cells. The magnitude of cannabinoid-dependent CB_1 increase was less in mHtt-expressing cells compared to wild-type cells. Treatment of HD cells with ACEA increased PGC1α and BDNF-2 mRNA levels, improved cellular viability, and increased cellular ATP production. Cannabinoid treatment may represent a viable means of improving cellular health in HD. We are continuing to explore the molecular mechanism mediating cannabinoid-dependent CB_1 induction, and understanding how mHtt affects this induction.

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EFFECTS OF CANNABIDIOL AND HU210 ON VIABILITY OF SH-SY5Y HUMAN NEUROBLASTOMA CELLS

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SH-SY5Y cells represent a useful model to investigate neurotoxicity *in vitro*. We have examined the sensitivity of these cells in undifferentiated and retinoic acid-differentiated forms to assess the effects of cannabidiol (CBD) and HU210 on cell viability. Cell viability was monitored using the MTT assay. In the presence of 5 % serum, numbers of undifferentiated cells plated in 24-well plates were not altered following 24 hours in the presence of CBD or HU210 at concentrations up to 10 μM. However, following 48 hours exposure, there were significant reductions in cell viability in the presence of 10 μM CBD (66 ± 6 % control, $P<0.001$), but not 1 μM CBD (92 ± 1). In the absence of serum, cell adherence to the wells was reduced. At the same time, there was an increased sensitivity to CBD (24 hrs, 1 μM 102 ± 1; 10 μM, 57 ± 3, $P<0.001$) and HU210 (24 hrs, 1 μM 105 ± 3; 10 μM 108 ± 3), particularly following 48 hrs exposure (CBD 1 μM 96 ± 1; 10 μM 56 ± 2 %, $P<0.001$; HU210 1 μM 94 ± 2; 10 μM 80 ± 4 %, $P<0.001$).

Following differentiation in the presence of 10 μM retinoic acid for four days, the effects of a further 48 hrs exposure to CBD and HU210 were assessed. In the presence of 5 % serum, 10 μM CBD inhibited cell viability (84 ± 2 %, $P<0.001$), while 300 nM AM251 or 10 μM LY320135 were without significant effect (112 ± 2 and 107 ± 1 %, respectively). Combining CBD and AM251, however, elicited a modest further reduction in cell viability (76 ± 3, $P<0.05$), while co-incubation of CBD and LY320135 led to no change in viability compared to CBD alone (85 ± 3 %). In differentiated cells in the presence of 5% serum, HU210 failed to alter cell viability in the absence or presence of AM251 or LY320135. In the absence of serum, the cannabinoids evoked a greater inhibition of differentiated cell viability. Thus, 10 μM CBD and 10 μM HU210 inhibited viability (82 ± 4, $P<0.001$; 62 ± 1, $P<0.001$), while AM251 and LY320135 were without effect (101 ± 1 and 108 ± 1 %, respectively). Combining CBD with AM251 evoked a further inhibition (60 ± 2, $P<0.001$), while LY320135 was ineffective (84 ± 3 %). In contrast, combining HU210 with AM251 or LY320135 was without further effect (63 ± 1 and 70 ± 1 %, respectively).

We conclude that serum protects SH-SY5Y cells from CBD-evoked damage, while the mechanism of damage appears to be independent of CB_1 cannabinoid receptors.

INVOLVEMENT OF CB2 AND 5HT1A RECEPTORS IN CANNABIDIOL-INDUCED NEUROPROTECTION AFTER HYPOXIA-ISCHEMIA IN NEWBORN PIGS

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Background: 5HT1A receptors are involved in cannabidiol (CBD) neuroprotection in animal models of adult stroke. Although CBD is thought to not bind CB1 or CB2 receptors, we demonstrated in previous in vitro studies on newborn mice forebrain slices exposed to oxygen-glucose deprivation that CB2 antagonists abolished CBD-induced neuroprotection.
Aim: to study the involvement of CB2 and 5HT1A receptors on CBD neuroprotection in immature brain in vivo.
Methods: sedated and ventilated newborn pigs (1-2 day-old) underwent HI damage (hypoxia -FiO2 10%- + bilateral carotid artery compression for 30 min). Thirty min later HI piglets received i.v. vehicle (HV, n=8) or CBD 1 mg/kg (HC, n=10), alone or with the antagonist for CB2 AM630 1 mg/kg(HCA, n=5) or for 5HT1A WAY100638 0.1 mg/kg (HCW, n=5) . Hemodynamic parameters and aEEG were monitorized up to six hours post-HI. The, brains were obtained to perform the following studies: histological (Nissl staininig and GFAP imunohistochemistry); biochemical (cytokine microarrays); proton magnetic spectroscopy (H-MRS) (NAA/Cho [neuronal death], Lac/Cr [metabolic impairment], Glu/NAA [excitotoxicity] and GSH/Cr [oxidative stress] ratios); Western blot (Oxyblot, protein oxidation); and isotope-dilution, liquid chromatography–mass spectrometry (determination of AEA, 2-AG and PEA concentration in brain). Finally, ioluminescence Resonance Energy Transfer (BRET) was carried out on HEK-293T cells to detect CB2-5HT1A heteromers. Similarly studied animals without HI insult served as controls (SH, n=5)
Results: HI led to severe brain damage as shown by histology (26±3 vs. 5±1% dead neurons for HV and SH, $p<0.05$), brain activity (final aEEG amplitude: 18±3% vs 85±7% baseline) and H-MRS ratios (NAA/Cho: 4.7±0.5 vs. 7.1±0.6, for HV and SH, $p<0.05$; Lac/Cr: 5.7±0.9 vs. 2.7±0.3, for HV and SH, $p<0.05$). CBD administration reduced neuron death (9±3% dead neurons, $p<0.05$ vs. HV), increased astrocyte survival (33.0±3.2, 31.6±2.2 and 39.4±2.4 GFAP+ cells/field, $p<0.05$), improved brain activity recovery (aEEG: 65.5±9% baseline, $p<0.05$ vs HV) and normalized H-MRS ratios (NAA/Cho: 7.8±0.3, Lac/Cr: 3.4±0.3, both $p<0.05$ vs. HV). CBD effect related with the reduction of glutamate release (Glu/NAA: 0.5±0.02, 0.62±0.03 y 0.5±0.03 for SH, HV y HC, $p<0.05$), oxidative stress (GSH/Cr: 0.17±0.005, 0.11±0.01 and 0.17±0.01 for SH, HV and HC, $p<0.05$) and inflammation (IL-1: 116.7±7, 138±9 and 121±4 pg/mL; Il-6: 23±2, 28±3 and 22±2 pg/mL, for SH, HV y HC, $p<0.05$). CBD protective effects were abolished by coadministration of AM630 or WAY100630. CB2 involvement was not related to a CBD-induced increase in brain endocannabinoid concentration (AEA: 2.6±0.6, 3.9±0.7 and 2.8±0.4 pg/g; 2-AG: 1.6±0.4, 6.5±4.3 and 1.6±0.3 pg/mg; PEA: 48.7±9.8, 586.5±227.8 and 73.7±18.2 pg/g, for SHM, HV and HC, respect., all $p<0.05$). BRET studies demonstrate the existence of CB2-5HT1A heterodimers.
Conclusions: administration of CBD after a HI insult in newborn pigs reduces brain damage as assessed by histological, biochemical and metabolic studies, by acting on the more important mechanisms inducing brain damage as are excitotoxicity, oxidative stress and inflammation. CB2 and 5HT1A receptors are involved in CBD neuroprotection. CB2 involvement is likely due to the formation of CB2-5HT1A heterodimers.

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THE CB2 AGONIST HU308 REDUCES BRAIN HYPOXIC-ISCHEMIC DAMAGE IN NEWBORN RATS

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Background: Several data emerged lately pointing to a role for CB2 receptors in hypoxic-ischemic (HI) brain damage, thus suggesting that CB2 agonists may protect brain from acute ischemic brain damage in adult animals.
Aim: to demonstrate that a CB2 agonist is protective for the immature brain after a HI insult.

Methods: unilateral HI brain damage was induced in newborn Wister rats (7-10 day-old: P7-P10) by exposure to hypoxia (10% FiO2) for 110 min after left carotid artery electrocoagulation under anaesthesia. Ten minutes after the end of HI pups were treated s.c. with vehicle (HV, n=8) or with the CB2 agonist HU308 1 mg/kg single dose (HU, n=10). Other pups remained as controls (SHM, n=6). Seven days after HI rats were sacrificed, transcardially perfused with paraformaldehide (PFH) 4% and their brains removed. Then, a histological study by Nissl staining was carried out in brain frontoparietal cortex, striatum and CA1 area of hippocampus of the ipsilateral hemisphere, scoring the tissue damage from 0 (normal) to 5 (massive destruction). In addition, GFAP and IBA-1 inmmunohistochemistry was carried out to identify astrocytes and microglial cells, respectively. Astroglial or microglial response was quantified by calculating the areal percentage of GFAP- or IBA-1-immunoreactive processes and cell bodies by using the ImageJ 1.43s software (NIH, Bethesda, USA).

Results: HU reduced HI-induced neuronal death (neuropathological score: 0.5±0.2, 2.6±0.3 and 1.2±0.2 points for SHM, HV and HU, respectively, $p<0.05$). This was associated with the reduction of astrogliosis (GFAP+: 1410±771, 3338±825 and 1803±578 pixels^2/field, $p<0.05$) and a trend to reduce microglial response (IBA-1+: 1447±114, 1700±306 and 1552±236 pixels^2/field, $p=0.07$). The beneficial effect of HU was more apparent in cortex. No side effects were observed in pups treated with HU.
Conclusions: treatment with the CB2 agonist HU308 reduced brain damage in the short term without apparent side effects.

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NEUROPROLIFERATIVE EFFECT OF CANNABIDIOL ADMINISTRATION TO NEWBORN RATS AFTER HYPOXIA-ISCHEMIA

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Background: Administration of CBD to hypoxic-ischemic (HI) newborn rat leads to a full functional recovery but to just a mild reduction of the volume of brain damage. This suggests that CBD might support a functional compensation by the surviving brain tissue, which could theoretically be accomplished by enhancing neuroproliferation.

Aim: to demonstrate that CBD enhances neuroproliferation in immature brain after a HI insult.

Methods: unilateral HI brain damage was induced in newborn Wister rats (7-10 day-old: P7-P10) by exposure to hypoxia (10% FiO2) for 120 min after left carotid artery electrocoagulation under anaesthesia. Ten minutes after the end of HI pups were treated s.c. with vehicle (HV, n=9) or with the CBD 1 mg/kg single dose (HC, n=9). Other pups remained as controls (SHM, n= 11). Seven days after HI rats were studied by H^+-Magnetic Resonance Spectroscopy (HMRS) to quantify the NAA/Cho ratio, which is proportional to the neuronal density of the tissue. Then, rats were sacrificed, transcardially perfused with paraformaldehide (PFH) 4% and their brains removed and cut off into sagital slices for immunohistochemical study on the subventricular zone (SVZ), hippocampus and cortex. Nestin was used to identify stem cells and KI67 to detect proliferating cells. GFAP and IBA-1 were used as astrocyte and microglial specific markers, respectively.

Results: In the HMRS study, NAA/Cho was reduced in HV rats both in ipsilateral (7.1±0.3 vs 5.1±0.3 for SHM and HV, $p<0.05$) and contralateral hemispheres (7.4±0.2 vs 6.5±0.2 for SHM and HV, $p<0.05$). CBD prevented NAA/Cho decrease in ipsilateral hemispheres (6.7±0.3, $p<0.05$ vs HV) and increase NAA/Cho in contralateral hemispheres (8.9±0.3, $p<0.05$ vs HV and vs. SHM). CBD increased the density of nestine+ cells but not that of GFAP+ or IBA1+ cells in cortex, as compared with HV. KI67 staining revealed that CBD increased the density of proliferating cells in SVZ (20±2 vs 28±1 KI67+ cells per field for HV and HC, respectively, $p<0.05$).
Conclusions: CBD has a neuroproliferative effect in immature brain after HI. This effect might account for CBD neuroprotective effects.

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THE GPR55 LIGAND L-α-LYSOPHOSPHATIDYLINOSITOL (LPI) EXERTS NEUROPROTECTIVE EFFECTS AFTER EXCITOTOXIC LESION *IN VITRO*

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Cannabinoids have a great capacity to limit inflammation and neuronal death by acting upon the G-protein coupled receptors (GPR) cannabinoid $(CB)_1$ and CB_2. However, the neuroprotective potential of cannabinoids varies significantly among the different members of the cannabinoid family. Pharmacological experiments and the availability of CB_1/CB_2-deficient mice unraveled the existence of so-called non-CB_1/non-CB_2 receptor subtypes, such as abnormal-cannabidiol-sensitive receptor located on microglia mediating the neuroprotective effects of the endogenous cannabinoid 2-AG. Currently, GPR55 has been discussed as a putative novel non-CB_1/non-CB_2 receptor subtype with l-α-lysophosphatidylinositol (LPI) being the best characterized agonist so far. Quantitative PCR revealed that *Gpr55* is present in primary microglia and the brain, but the exact regional and cellular distribution and the physiological/pathological effects downstream of GPR55 activation in the CNS still remain open. Here, we showed that LPI (1nM-10mM) protected dentate gyrus granule cells *in vitro* after excitotoxic lesion as induced by NMDA application for 4 hours in organotypic hippocampal slice cultures (OHSCs). In parallel, LPI also reduced the number of microglia in the dentate gyrus (0.1mM-10mM). By use of the bisphosphonate clodronate, microglia was removed from OHSCs before excitotoxic damage. In microglia depleted OHSCs, LPI lost its neuroprotective ability, indicating a strong involvement of microglia in GPR55-mediated neuroprotection. In conclusion, the present study unmasked a yet unknown role for GPR55 in neuroprotection which seems to be driven by modulation of microglia function.

CANNABIDIOL POTENTIATES IN VIVO MICROGLIAL ACTIVITY IN RESPONSE TO ACUTE BRAIN INJURY

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Cannabidiol (CBD) is a natural cannabinoid that lacks psychoactivity and that has been shown to exhibit antioxidant and neuroprotective properties. Some of these effects of CBD may be partially mediated by microglial cells. These cells are involved in the inflammatory responses of the brain against different kinds of insults. In the present experiments we have used an in vivo model of acute insult to the brain in order to explore the effects of CBD on microglial responses. To that end, we used $Cx3cr1^{eGFP/+}$ mice, that exhibit green fluorescence in microglial cells. To study glial activity in vivo, we used a intravital multiphoton microscopy system[1,2]. By opening a small cranial window (3mm diameter), we could observe cellular responses against acute laser injuries (15microns diameter) induced in the brain parenchyma. Our data indicate that CBD exacerbated microglial response by increasing their ability to direct cell processes towards the sites of injury. Furthermore, this effect was partially mediated by cannabinoid CB_2 receptors as was prevented by the preincubation with SR144528. These results deserve further clarification, but are sugestive of a direct role of CBD on microglial function in vivo.
[1]Ruiz-Valdepeñas et al, J Neuroinflamm 2011, [2]Davalos et al, Nat Neurosci 2005.

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AEA/CB1R SIGNALING CONTRIBUTES TO FETAL ALCOHOL SPECTRUM DISORDER

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The transient exposure of immature rodents to ethanol during postnatal day 7 (P7), which is comparable to the third trimester human pregnancy, induces neuropathophysiological changes. However, the molecular mechanisms underlying these changes are still poorly understood. Although the endocannabinoid system has been shown to be an important modulator of ethanol sensitivity in adult mice, its potential role in modulating neuropathophysiological function in mice exposed to ethanol during early brain development has not yet been examined. In this study, we investigated the potential role of endocannabinoids (ECs) and the cannabinoid receptor type 1 (CB1R) in neonatal neurodegeneration and adult synaptic deficits in mice exposed to ethanol at P7. We found that enhanced anandamide (AEA) synthesis and CB1R protein expression are regulated by the transcriptional activation of the genes encoding, respectively, *N*-arachidonoyl phosphatidylethanolamine-phospholipase D (NAPE-PLD) and CB1Rs in the hippocampus and cortex, two brain areas that are important for memory formation and storage, respectively. We also found that ethanol reorganizes the enzymes that synthesize and degrade 2-arachidonylglycerol (2-AG) through the transcriptional activation of the genes that encode them; however, this change results in no significant increase in 2-AG levels. In addition, ethanol caused a marked reduction in ERK1/2 and AKT phosphorylation and neuropathology in neonatal mice. The pharmacological blockade of CB1Rs prior to P7 ethanol treatment rescued pERK1/2 but not PI3K/AKT deficits and prevented neonatal neuropathology. CB1R knockout (KO) mice were resistant to ethanol-induced ERK1/2- but not AKT-phosphorylation-mediated neonatal neuropathology. The protective effects of CB1R blockade resulted in normal adult synaptic plasticity in mice exposed to ethanol at P7. These findings reveal a novel function for developmental EC-CB1R-regulated pERK1/2 signaling pathways following ethanol exposure and represent molecular factors that may directly modulate the long-lasting synaptic deficits associated with FASD.

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FACILITATION OF ANANDAMIDE SIGNALING REDUCES NEUROTOXICITY PRODUCED BY CISPLATIN

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Since dorsal root ganglion (DRG) neurons and sensory nerves (SN) are not protected by a blood-brain barrier, peripheral neurotoxicity is a common dose-limiting complication of cisplatin chemotherapy treatment. The neuroprotective effect of URB597, an inhibitor of anandamide (AEA) hydrolysis was investigated in a murine model of cisplatin-induced peripheral neurotoxicity (1 mg/kg, i.p. for 7 days). Cisplatin produced several indices of neurotoxicity including up-regulation of activating transcription factor (ATF)-3 in DRG neurons and a decrease in conduction velocity of Aα/Aβ fibers in the tibial nerve. These changes were accompanied by a decrease in tubulin-immunoreactivity in the tibial nerve. Co-injections of URB597 (0.3 mg/kg daily, i.p.) with cisplatin blocked the increase of ATF-3 expression in DRG neurons as well as normalized the conduction velocity of Aα/Aβ fibers and the tubulin immunoreactivity in the tibial verve.

Since direct mitochondrial DNA (mtDNA) damage in DRG neurons contributes to cisplatin-induced neurotoxicity (Podratz et al., 2011), the effect of cisplatin (4 µg/ml for 24 h) was studied on cultured DRG neurons. Mitochondria were labeled with the MitoTracker deep red (25 nM). Treatment with cisplatin reduced the density of mitochondria in DRG neurons and co-treatment with URB597 (100 nM) attenuated these changes.

Collectively, these results suggest that pharmacological facilitation of AEA signaling is a promising strategy for attenuating cisplatin-associated peripheral neurotoxicity.

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THE FAAH INHIBITOR URB597 IS PROTECTIVE AGAINST MPP^+ IN A CELL CULTURE MODEL OF PARKINSON'S DISEASE

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Background: Levels of the endocannabinoid anandamide (AEA) as well as the cannabinoid receptor 1 (CB1) are elevated in Parkinson's disease (PD) patients. We have recently demonstrated that the cannabinoid Δ^9-tetrahydrocannabinol is neuroprotective via activation of the peroxisome proliferator-activated receptor gamma (PPARγ) receptor which AEA can also activate. Levels of AEA can be raised endogenously by inhibiting its hydrolysis through fatty acid amide hydrolase (FAAH). We therefore investigated whether FAAH inhibition is protective in our cell culture model of PD.

Methods: SH-SY5Y neuroblastoma cells were differentiated with retinoic acid. The PD relevant neurotoxin MPP^+ was co-administered with the FAAH and PPARγ inhibitors (URB597 and T0070907) for 48 hours after which cell death was determined using the LDH assay and production of reactive oxygen species (ROS) measured. Protein levels were determined by Western blotting.

Results: URB597 was protective against MPP^+ toxicity which was blocked by T0070907 but not the CB1 antagonist AM251. However, administration of URB597 alone or with MPP^+ did not significantly increase PPARγ expression. Increased expression of the receptor is thought to be an indicator of its activation. Furthermore, the MPP^+ induced increase in ROS production was reduced by URB597. This effect was not reversed by T0070907. We then examined the expression levels of proteins involved in oxidative stress defence and found no significant difference in superoxide dismutase 1 and catalase expression. However, MPP^+ decreased levels of the PPARγ co-factor 1 α (PGC1α), an inducer of mitochondrial biogenesis. This was restored by URB597 treatment and remained unaltered by T0070907 co-application. Furthermore, the active form of NRF2 was decreased by MPP^+ treatment and fully restored by URB597 which remained unchanged by the addition of T0070907. In contrast the increase in total NRF2 levels induced by MPP^+ treatment was not changed by addition of URB597 or T0070907.

Conclusions: Although our results are thus far inconclusive regarding the involvement of the PPARγ receptor, we have shown that URB597 may be neuroprotective by influencing the expressions of proteins involved in oxidative stress responses.

EVIDENCE THAT A FUNCTIONAL GPR18-BASED SIGNALING SYSTEM IN THE ANTERIOR MURINE EYE MODULATES INTRAOCULAR PRESSURE

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GPR18 is a recently deorphanized lipid receptor that is activated by the chief psychoactive ingredient of marijuana, THC, as well as behaviorally-inactive cannabinoids (e.g. abnormal cannabidiol (Abn-CBD)). This G protein-coupled receptor is related to CB_1/CB_2 and GPR55 receptors. Strong evidence now supports N-arachidonoyl glycine (NAGly) as the endogenous ligand of GPR18. The presence and/or function of any GPR18-based ocular signaling system remains essentially unstudied. The objectives of this research are: 1) to determine the disposition of GPR18 receptors and ligands in anterior murine eye, 2) examine the effect on intraocular pressure (IOP) by GPR18 and GPR55 in a murine model, including knockout mice for CB_1 ($CB1^{-/-}$), CB2 ($CB2^{-/-}$) or GPR55 ($GPR55^{-/-}$).

IOP was measured by rebound tonometry in C57Bl/J6 (C57), $CB_1^{-/-}$, and $GPR55^{-/-}$ anesthetized mice (isoflurane 2-3%) following a 5 µl topical application of 2% Abn-CBD, 1% 0-1602, 1% NAGly or vehicle (Tocrisolve®). O-1918, an antagonist at both GPR55 and GPR18, was injected i.p at 2 mg/kg. GPR18 protein localization was assessed with immunohistochemistry in frozen sections of mouse eye using an antibody developed against GPR18 protein. Endo-cannabinoids were measured using LC-MS.

GPR18 protein is expressed most prominently in the ciliary epithelium and the corneal epithelium though it is also expressed elsewhere at lower levels. Interestingly, GPR18 staining is detected in the trabecular meshwork. The GPR18 ligand NAGly is also detected in mouse eye at levels comparable to the brain. Abn-CBD (2%) significantly reduced IOP in C57, $CB_1^{-/-}$, $CB2^{-/-}$ and $GPR55^{-/-}$ mice, with decreases of 1.17±0.15, 1.37±0.16, 0.68±0.08 and 0.85±0.15 mmHg, respectively. NAGly (1%) produced similar decreases in IOP of 0.72±0.18, 1.41±0.08, 1.22±0.23 and 0.86±0.18, respectively, when tested in all four groups of mice. The GPR55 receptor agonist, 0-1602 (1%) failed to decrease IOP in WT mice ($p > 0.05$). Abn-CBD and NAGly did not significantly reduce IOP when co-administered with 0-1918, in C57, $CB_1^{-/-}$, or $GPR55^{-/-}$ mice ($p > 0.05$ per respective group).

We present evidence for a functional GPR18-based signaling system in the murine anterior eye, including receptors and ligands. GPR18 agonists, Abn-CBD and NAGly reduce IOP independently of CB_1, CB_2, or GPR55. These findings suggest that GPR18 may serve as a desirable target for the development of novel ocular hypotensive medications.

WHAT DO RESULTS FROM THE CANNABINOID USE IN PROGRESSIVE INFLAMMATORY BRAIN DISEASE (CUPID) TRIAL TELL US ABOUT THE POTENTIAL OF CANNABINOIDS FOR NEUROPROTECTION?

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Cannabinoids have been shown to reduce MS-related symptoms, as well as potentially having a longer-term beneficial effect. This potential disease modifying effect is supported by in vitro evidence for neuroprotection. This led to the development of the CUPID trial, a 30 centre, 493 patient parallel group randomised, placebo controlled trial over a 3-year treatment period. Patients with primary (PPMS) or secondary progressive MS (SPMS), deemed to be deteriorating by their treating neurologists, with an Expanded Disability Status Score (EDSS) of between 4.0 and 6.5, were allocated to either oral D^9 THC, or placebo in a 2:1 ratio over the period May 2006- July 2008. An initial dose titration phase was used to optimise dosage whilst minimising side effects. Patients were followed up at 6 monthly intervals for 3 years before coming off medication. Primary outcome measures were reduction in time to EDSS progression maintained for at least 6 months (physician measure), and overall mean change from baseline to the end of the study in the MSIS-20 version 2 (patient-orientated measure). A variety of secondary outcome measures included the MSFC and MRI outcomes. The study was powered to allow for 15% loss to follow-up, with a reduction in progression from 70% to 55% over 3 years.

Overall, 40% of recruited patients had PPMS, 60% SPMS, but EDSS progression was less than predicted at 44% over 3 years. Results showed no significant treatment effect on the primary outcome, with a hazard ratio for patients on active treatment, compared to placebo, having adjusted for the covariates age, centre, gender, disease type, weight and baseline EDSS score, of 0.9183 (95% CI: 0.6847 to 1.2317). However, there were significant treatment effects in people with lower disability scores (EDSS<6.0) with strong evidence that treatment affects time to EDSS progression (P = 0.00014). The hazard ratio for patients on active treatment with lower EDSS scores, compared to placebo, having adjusted for covariates, was 0.21 (95% CI: 0.094 to 0.47). There were no significant treatment effects on MRI parameters, or in most of the secondary outcome measures. Rasch analysis also provided some evidence for treatment effects at lower levels of disability, with effect sizes of between 0.3 and 0.67.

The results present a complex picture of overall negative results, with apparent disease modifying effects in less disabled patients – a finding not obtained in any previous study of progressive MS. These findings will be invaluable in informing future trial design and probably reflect a combination of population effects (less disability progression than expected in most outcomes), possible differential effects across the disease spectrum, and issues with our ability to pick up change in many of the currently used outcome measures.

ANANDAMIDE HAS DISTINCT EFFECTS ON HUMAN MYELOID AND PLASMACYTOID DENDRITIC CELLS IN MULTIPLE SCLEROSIS

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The immunopathogenesis of multiple sclerosis (MS) has always been thought to be driven by chronically activated and autoreactive Th-1 and Th-17 cells. Recently, also dendritic cells (DC) have been thought to significantly contribute to antigenic spread and to maturation of adaptive immunity, and have been linked with disease progression and exacerbation. Yet, the role of DC in MS pathogenesis remains poorly understood.
We compared the level of cytokines production from myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) in healthy subjects and MS patients. We also evaluated the effect of the main endocannabinoid, anandamide (AEA), in these DC subsets and correlated cytokine levels with defects in the endocannabinoid system.
In MS patients mDC produce higher levels of interleukin-12 and interleukin-6, whereas pDC account for lower levels of interferon-α compared to healthy subjects. Moreover, we found that in MS patients only pDC lack responsiveness to cytokine inhibition induced by AEA. Consistently, this specific cell subset expresses higher levels of the anandamide hydrolase FAAH (fatty acid amide hydrolase).

Conclusions. Our data disclose a distinct immunomodulation by AEA of mDC and pDC in MS patients, which may reflect an alteration of the expression of FAAH, thus forming the basis for the rational design of new endocannabinoid-based immunotherapeutics targeting a specific cell subset.

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EFFECTS OF HIGH GLUCOSE ON THE VASCULAR ACTION OF ANANDAMIDE IN YOUNGER AND OLDER RATS

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Endocannabinoids, acting via central and peripheral cannabinoid receptors, are involved in the control of food intake and energy balance. One of the metabolic parameters, high glucose has been shown to modulate the actions and tissue content of anandamide (an endocannabinoid) in pancreatic beta-cells, adipoctyes and serum (1-2). Whilst anandamide is also a potent vasorelaxant and has been implicated in blood pressure regulation (3), the influence of glucose on the vascular effects of anandamide remains undetermined. In this study, the main mesenteric artery was isolated from 2 groups of male Wistar rats (10-14 weeks & 27-31 weeks) and maintained at 37^{o}C in oxygenated Krebs-Henseleit solution (with 10mM glucose) for isomertric tension recording. Vessels were precontracted with 10μM methoxamine (an α_1-adrenoceptor agonist), followed by cumulative additions of anandamide. Data are expressed as mean±s.e.m (n>4rats) and analysed by Student's *t*-tests or 2-way analysis of variance. $P<0.05$ was considered statistically significant.

In younger rats, incubation of endothelium-intact vessels with high glucose (30mM for 1h) significantly reduced the potency of anandamide (control: $pEC_{40\%}$=7.2±0.3 R_{max}=86±3% vs high glucose: $pEC_{40\%}$=6.2±0.3 R_{max}=71±7%). The effect of high glucose was not due to increases in osmolarity as Krebs-Henseleit solution with 10mM glucose plus 20mM mannitol had no effect (data not shown). Removal of the endothelium reduced anandamide responses and also the inhibitory effect of high glucose (control: R_{max}=34±10% vs high glucose: R_{max}=24±10%). In contrast, high glucose had no effect on relaxation to the muscarinic agonist carbachol which was entirely endothelium-dependent (control: pEC_{50}=7.1±0.1 R_{max}=98±4% vs high glucose: pEC_{50}=7.2±0.1 R_{max}=95±4%). Advancing age (by about 20 weeks) greatly reduced responses to anandamide and, to a lesser extent, carbachol. The resultant relaxation to anandamide was not significantly affected by high glucose in endothelium-intact (control: R_{max}=35±8% vs high glucose: R_{max}=32±10%) or endothelium-denuded vessels (control: R_{max}=21±10% vs high glucose: R_{max}=22±8%). High glucose also had no effect on carbachol relaxations in older rats (control: pEC_{50}=6.5±0.2 R_{max}=91±9% vs high glucose: pEC_{50}=6.6±0.2 R_{max}=99±1%). We conclude that high glucose selectively reduces endothelium-dependent relaxation to anandamide, which might contribute to vascular changes seen in hyperglycaemia. Anandamide responses are greatly compromised by ageing, but high glucose has no further effect in older animals.

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CHRONIC ENDOCANNABINOID SYSTEM ACTIVATION AND METABOLIC ENDOTOXEMIA DIFFERENTIALLY AFFECT THE ONSET OF GLUCOSE INTOLERANCE AND INFLAMMATION IN HIGH-FAT DIET-INDUCED METABOLIC ALTERATIONS

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Obesity and associated metabolic disorders are characterised by low-grade inflammation, metabolic endotoxemia and altered endocannabinoid (eCB)-system tone. eCB and lipopolysaccharides (LPS) are associated with several metabolic features observed in individuals with obesity, but the metabolic interactions between these two parameters are still under investigation. The aim of this study was to decipher the specific role of eCB-system activation or metabolic endotoxemia in the onset of metabolic disorders associated with obesity. To this purpose, a set of 9-week-old male C57BL/6J mice were treated with an eCB agonist (HU210, 50µg/kg/d) or LPS (300µg/kg/d) via subcutaneous mini-pumps for 6 weeks. After 3 weeks of treatment, half of the mice were challenged with a high-fat (HF) diet for the following 3-week period.

Chronic eCB stimulation induces glucose intolerance, metabolic endotoxemia, and increases macrophages infiltration in the muscles, a phenomenon associated with an altered lipid metabolism in this tissue. Chronic LPS treatment increases body weight and fat mass with minor effect on the other metabolic parameters. Challenging mice with a HF-diet after eCB or LPS pre-treatment exacerbates glucose intolerance, inflammation and alters insulin secretion without affecting fat mass development. Concomitant treatment with a HF-diet and an eCB agonist altered muscles lipid oxidation markers.

In conclusion, eCB stimulation under basal conditions induces glucose intolerance, inflammation (i.e., metabolic endotoxemia, muscles macrophages infiltration), and alters lipid metabolism in muscle. These effects are exacerbated by the concomitant ingestion of a HF-diet. This study highlights the different effects of eCB stimulation and LPS on metabolic features associated with obesity under basal and pathological conditions. These results support new roles played by LPS and eCB in both the onset and the physiopathology of obesity and type 2 diabetes.

FUNCTIONAL MEASUREMENT OF FLAT-SENSITIVE ANANDAMIDE UPTAKE INTO THREE RAT CELL LINES

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Introduction: FAAH-like anandamide transporter (FLAT) is a catalytically silent variant of FAAH that acts as a shuttle in order to facilitate the transport of anandamide (AEA) into cells (1). mRNA for FLAT was detected both centrally, in peripheral organs such as the liver and intestine, and in Neuro-2A neuroblastoma and CFCF-STTG1 astrocytoma cells (1), but it is not known if FLAT is expressed functionally in peripheral cell lines. Here we investigate the expression of FLAT in rat cell lines by determination of the sensitivity of the uptake of AEA to inhibition by the FLAT inhibitor ARN272 (1).

Method: AEA uptake was evaluated as described previously (2). Unless otherwise stated, cells were preincubated with the compounds for 10 min and then incubated with [^{3}H]AEA (labelled in the arachidonoyl part of the molecule) for 4 min at 37 °C.

Results: The cell lines used were C6 glioma, R3327 AT1 prostate cancer and RBL2H3 basophilic leukaemia cells. In C6 and R3327 AT1 cells, ARN272 concentration-dependently inhibited AEA uptake, but not to the extent seen following preincubation with the FAAH inhibitor URB597. The effects of ARN272 and URB597 upon AEA uptake were not additive. The inhibition of uptake was also seen in C6 cells when [^{3}H]AEA labelled in the ethanolamine part of the molecule was used. In RBL2H3 cells, on the other hand, the uptake was greatly reduced by URB597, but was not affected by ARN272 over the concentration range used (0.3-30 μM). AEA uptake was also measured in R3327 AT1 cells using different incubation times (1, 4, 7 and 10 min). The mean rates of uptake (i.e. the slope replot of the data) were 83, 78, 53, 26 and 9 fmol per min and well for R3327 AT1 cells in buffer, vehicle, 10 μM ARN272, 1 μM URB597 and for wells alone, respectively. The intercept replots (which reflect the initial rapid association of AEA with the cells), were not affected by either ARN272 or URB597, consistent with the intracellular trafficking role of FLAT.

Conclusion: FLAT, as visualised by measurement of ARN272-sensitive AEA uptake, is functionally expressed in C6 glioma and R3327 AT1 prostate cancer cells, but not in RBL2H3 basophilic leukaemia cells. The greater effect of URB597 than of ARN272, and the lack of additivity of the two compounds, would suggest that in the cells investigated, FLAT-independent pathways are also involved in the transport of AEA to FAAH.

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ROLE AND MECHANISM OF CANNABINOID CB2 RECEPTOR ACTIVATION IN REGULATING THE EFFEROCYTOSIS FUNCTION OF MACROPHAGE

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Background Recent proof indicates that accumulation of too much apoptotic cells and defective abilities of clearing apoptotic cells (efferocytosis) in macrophage within the atherosclerotic plaque are an important reason of the formation and progression of unstable plaque. Previous studies have confirmed that the cannabinoid CB2 receptor agonists have strong anti-inflammatory and antioxidant properties, and also inhibit the progression of atherosclerosis. In this study, it is therefore investigated that the regulatory role in the efferocytosis function of macrophage and the possible-involved mechanisms with selective activation of cannabinoid CB2 receptor.

Methods The UV irradiation induced RAW264.7 murine macrophage apoptosis. Flow cytometry method and laser scanning confocal microscope are used to detect the phagocytic index of apoptotic cells in normal and oxLDL-loaded RAW264.7 macrophages and primary C57BL murine peritoneal lavage macrophages by the incubations with different concentrations of the selective cannabinoid CB2 receptor agonists JWH-133 and HU-308 (0.1μM-10μM). And also, the mechanisms are explored by analyzing the expression of the pro-phagocytosis receptor MerTK, Tyro3 and Axl with western blotting, and detecting the level of tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS) with the enzyme-linked immunosorbent assay (Elisa) or flow cytometry.

Results (1) Cannabinoid CB2 receptor agonists JWH-133 and HU-308 have the tendency to increase the phagocytic index of apoptotic cells in normal RAW264.7 macrophage and primary murine peritoneal lavage macrophages in a concentration-dependent manner. (2) JWH-133 and HU-308 have a significant role in promoting clearance of the apoptotic cells in oxLDL-loaded RAW264.7 macrophages and primary murine peritoneal lavage macrophages. (3) JWH-133 and HU-308 increase the receptor expression of Tyro3 family receptors MerTK, Tyro3 and Axl in normal and oxLDL-lorded RAW264.7 macrophages. (4) JWH-133 and HU-308 also significantly reduce the levels of TNF-α and ROS induced by oxLDL in RAW264.7 macrophages.

Conclusion Activation of cannabinoid CB2 receptors can increase the abilities of normal and oxLDL-lorded macrophages to clear apoptotic cells, the mechanisms of which might be linked with up-regulating of expression of the pro-phagocytosis receptors MerTK, Tyro3, Axl and inhibiting the oxidative/inflammatory reaction.

DESIGN, SYNTHESIS AND PHARMACOLOGICAL ACTIVITY OF O-7460, A NOVEL FLUOROPHOSPHONATE INHIBITOR OF THE BIOSYNTHESIS OF 2-ARACHIDONOYL-GLYCEROL

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The development of potent and selective inhibitors of the biosynthesis of the endocannabinoid 2-AG via diacylglycerol lipases (DAGL) a and b is just starting to be considered as a novel and promising source of pharmaceuticals for the treatment of disorders that might benefit from a reduction of endocannabinoid tone, such as hyperphagia in obese subjects. Here we describe the synthesis and pharmacological characterization *in vitro* and *in vivo* of four new fluorophosphonate compounds: 1-((fluoro(methyl)phosphoryl)oxy)-3-(penthyloxy)propan-2-yl oleate (O-7458); 1-ethoxy-3-((fluoro(methyl)phosphoryl)oxy)propan-2-yl oleate (O-7459); 1-((fluoro(methyl)phosphoryl)oxy)-3-isopropoxypropan-2-yl oleate (O-7460); and (S)-1-metoxy-5-oxopentan-2-yl oleate (O-7344).

Of the four compounds, only O-7460, exhibited both high potency (IC_{50}=690 nM) against the human recombinant DAGLa, and selectivity (IC_{50}>10 mM) towards both COS cell monoacylglycerol lipase (MAGL) and rat brain fatty acid amide hydrolase (FAAH). Activity-based protein profiling confirmed that O-7460 inhibits mouse brain MAGL only at concentrations $\geq$ 10 mM, and showed that this compound has only one major "off-target", i.e. the serine hydrolase KIAA1363. O-7460 did not exhibit measurable affinity for human recombinant CB_1 or CB_2 cannabinoid receptors (K_i>10 mM). In intact mouse neuroblastoma N18TG2 cells stimulated with ionomycin, O-7460 (10 mM) reduced de novo biosynthesized 2-AG levels. When administered i.p. to mice, O-7460 dose-dependently inhibited the intake of high fat food over a 14 h observation period and, subsequently, slightly but significantly reduced body weight, as one might expect from an inhibitor of endocannabinoid biosynthesis.

O-7460 might be considered a useful pharmacological tool to investigate the role played by 2-AG both *in vitro* and *in vivo* under physiological as well as pathological conditions, and to provide proof-of-concept status to the development of new anti-obesity drugs from DAGLa inhibitors.

ABSTRACT WITHDRAWN

ACUTE VASCULAR EFFECTS OF ENDOCANNABINOIDS IN THORACIC AORTAE FROM ZUCKER DIABETIC RATS

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Increased plasma levels of endocannabinoids have been found in type 2 diabetes. The cardiovascular complications of this disease, including endothelial dysfunction have been well documented. This study aimed to investigate whether vascular responses to the endocannabinoids anandamide or 2-arachidonoyl glycerol (2-AG) were also altered in a model of type 2 diabetes (Zucker Diabetic Fatty (ZDF) rats).

Thoracic aortic rings from male ZDF rats, and their Lean controls, were isolated and mounted on fixed pins in a myograph, and then contracted with methoxamine (α_1-adrenoceptor agonist). Cumulative concentration-response curves to anandamide (10nM-100μM) or 2-AG (10nM-10μM) were constructed. The roles of the endothelium, cyclooxygenase, nitric oxide, or endocannabinoid metabolism were investigated.

Vasorelaxation to anandamide was impaired in ZDF rat aortae compared to the Lean rats (% relaxation at 1μM anandamide; Lean = 34.2 ± 9.4% (n=6), ZDF 9.6 ± 4.9% (n=5) (P<0.001)). Removal of the endothelium, or inhibition of nitric oxide synthase with L-NAME, reduced vasorelaxation in aortae from Lean rats, and even abolished the response at concentrations of anandamide less than 1μM making the responses comparable to those observed in aortae from ZDF rats. Endothelial denudation or L-NAME pre-treatment of aortae from ZDF rats did not further reduce vasorelaxation to anandamide. The blunted vasorelaxation of aortae from ZDF rats was restored by both inhibition of FAAH (with 1μM URB597) and cyclooxygenases (% relaxation at 1μM anandamide; Lean controls = 34. 2 ± 9.4 % (n=6), ZDF + indomethacin = 34.3 ± 6.2 % (n=5)).

In ZDF aortae, responses were absent up to 3μM 2-AG, and at high concentrations a contractile response was uncovered (% contraction at 10μM 2-AG = 17.98 ± 7.63 % (n=5)). The presence of indomethacin uncovered vasorelaxation in ZDF aortae that was comparable to control relaxations in aortae from Lean rats (% relaxation at 1μM 2-AG, Lean control = 15.97 ± 8.63 % (n=6), ZDF + indomethacin = 13.57 ± 7.81 % (n=7)). In arteries from both Lean and ZDF rats, the presence of JZL184 also uncovered a vasorelaxation to 2-AG.

There are impaired endothelium-dependent vasorelaxant responses to anandamide in aortae from ZDF rats, which appear due to dysfunction of endothelial nitric oxide pathways, enhanced metabolism and the production of vasoconstrictor prostanoids. Impaired vasorelaxation to 2-AG occurs in this model of diabetes, and may also be due to over-production of vasoconstrictor prostanoids.

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INVOLVEMENT OF THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS IN THE PRESSOR EFFECT OF THE CANNABINOID RECEPTOR AGONIST CP55940

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Intravenous injection (*i.v.*) of the endogenous cannabinoid receptor agonist anandamide and its stable analogue methanandamide (methAEA) to urethane anaesthetized rats induces triphasic changes in cardiovascular parameters. The mechanism(s) of the pressor effect (phase II) still remains to be established. We have demonstrated previously that central thromboxane A_2, NMDA (N-methyl-D-aspartate) receptors and β_2-adrenoceptors play a role in the pressor effect of anandamide given both *i.v.* and intracerebroventricularly (Malinowska et al., Naunyn-Schmiedeberg's Arch. Pharmacol. 381 (2010) 349-360). The paraventricular nucleus of the hypothalamus (PVN) is a major integrative site for autonomic function including cardiovascular and respiratory functions. **The aim** of the present study was to determine the potential involvement of the PVN in the pressor effect of the cannabinoid receptor agonist CP55940.

Experiments were performed in male Wistar rats anaesthetized with urethane. Stainless cannulae were implanted in the PVN. Basal diastolic blood pressure (DBP) was about 60 mmHg. Microinjection of MethAEA (0.01 μmol/rat) and CP55940 (0.1 μmol/rat) into the PVN decreased DBP by about 24% each. In the presence of the CB_1 receptor antagonist AM251 (3 μmol/kg, *i.v.*) the same doses of methAEA and CP55940 increased DBP by about 28% each. The pressor effect of CP55940 (0.1 μmol/rat) in the presence of AM251 was reduced by MK-801 and ICI118551 (1 μmol/kg, *i.v.* each), antagonists of NMDA and β_2-adrenergic receptors, by 60 and 50%, respectively, but not affected by ruthenium red (3 μmol/kg, *i.v.*), an antagonist of TRPV1 receptors. In the presence of the $GABA_A$ receptor antagonist bicuculline (5 μmol/kg, *i.v.*), CP55940 (0.1 μmol/rat) induced comparable increases in DBP (by about 30%) both in the absence and in the presence of AM251. The increase in DBP induced by NMDA given into the PVN (1 mmol/rat; by 99%) was reduced by the simultaneous injection of AM251 and ruthenium red (3 μmol/kg, *i.v.* each) by 70%. In conclusion, our results suggest that, in addition to CB_1 receptors, also β_2-adrenergic, NMDA and $GABA_A$ receptors in the paraventricular nucleus of the hypothalamus are involved in the blood pressure effects of the cannabinoid receptor agonist CP55940.

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THE PROSTAMIDE ANALOGUE BIMATOPROST INHIBITS ADIPOGENESIS

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Bimatoprost (LUMIGAN®) is a synthetic analogue of the anandamide/COX-2-derived prostamide, prostaglandin $F_{2\alpha}$ ethanolamide ($PGF_{2\alpha}EA$), and is utilized as a glaucoma therapy. Heterodimerization between the prsotaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) FP receptor and its splice variants is required for prostamide/Bimatoprost sensitivity. Various prostaglandins are known to have distinct and opposing effects on adipocyte differentiation. While the molecular mechanisms are not completely understood, $PGF_{2\alpha}$ is known to inhibit adipogenesis. We have therefore investigated the possibility that Bimatoprost is able to regulate adipogenesis. In fact, Bimatoprost efficiently inhibited adipogenesis in both murine 3T3-L1 and primary human subcutaneous preadipocytes based on analysis of mature adipocyte genetic markers and assessment of triglyceride accumulation. Interestingly, this inhibition was observed whether the cells were exposed to Bimatoprost during the first two days or the entire duration of the differentiation protocol. Similarly, exogenous $PGF_{2\alpha}EA$ is able to inhibit adipogenesis and its endogenous levels are drastically decreased in differentiating adipocytes concomitant with *Cox2* expression. This decrease in *Cox2* expression is reversed by both Bimatoprost and $PGF_{2\alpha}EA$, which in fact greatly stimulate *Cox2* levels. Incubation of 3T3-L1 preadipocytes with anandamide results in significant increases in $PGF_{2\alpha}EA$ levels, and after 2 days of differentiation this increase is significantly reduced. Both human pre- and mature adipocytes were found to express the wild-type FP receptor as well as several splice variants and the expression of the wild-type and alt.4 versions, through which Bimatoprost signals, are decreased during adipogensis. Pharmacological inhibition of the splice variant receptor mitigated the ability of Bimatoprost to inhibit adipogensis, as did inhibition of Erk1/2 phosphoyrlation. Futhermore, Bimatoprost, $PGF_{2\alpha}EA$ and $PGF_{2\alpha}$ appear to be able to induce a posttranslational modification of *Pparg*, as observed with western blotting, which may result in its decreased transcriptional activity. These studies point to $PGF_{2\alpha}EA$, signalling through FP receptor splice variants as a novel endocannabinoid-derived mechanism for the regulation of adipogenesis, and to Bimatoprost as a potent pharmacological tool for studying this process.

CANNABIDIOL PROTECTS THE BLOOD-BRAIN BARRIER AGAINST INCREASED PERMEABILITY FOLLOWING OXYGEN-GLUCOSE DEPRIVATION

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During stroke, the blood brain barrier (BBB) is subjected to a series of events which increase its permeability and can result in brain oedema. Cannabidiol (CBD) is beneficial in animal models of stroke, and other groups have demonstrated the ability of cannabinoids to attenuate the damage caused to the BBB in various pathologies. The aim of the present study was to establish whether CBD can modulate BBB permeability following oxygen-glucose deprivation.

To model the BBB, co-cultures of human brain microvascular endothelial cells and human astrocytes were grown to confluence on Transwell collagen-coated inserts (0.4 μm pore size, 12 mm diameter – Corning, USA). BBB permeability was measured by transepithelial electrical resistance (TEER) using STX2 electrodes and an EVOM^2 resistance meter (WPI, UK). Inserts were subjected to 4 h OGD by incubating them in GasPak EZ Anaerobe pouches (BD, UK) with RPMI (no glucose) medium. Reperfusion was established by returning the cells to normoxia and their specialised medium. CBD (100 nM, 1 μM, 10 μM), receptor antagonists, or vehicle were added to the luminal chamber either before or after OGD. Statistical analysis was conducted using one-way ANOVA.

Pre-treatment with 10 μM CBD attenuated the initial increase in permeability caused by 4 h OGD (P<0.05), and 100 nM, 1 or 10 μM CBD increased barrier resistance at 6, 8, 10, 12 and 14 h during reperfusion in a concentration dependent manner (P<0.05-0.001; n range 8-11 inserts from 4-6 separate experiments). Administration of CBD post-OGD increased barrier resistance at 8, 10 and 24 h during the reperfusion period in a concentration dependent manner (P<0.05-0.01*;* n range 4-13 from 5 separate experiments). Receptor involvement was probed, with AM251, AM630 and capsazepine, providing evidence that neither CB_1, CB_2 nor TRPV1 mediated this response (n range from 4-6 from 2-3 separate experiments). However, the CBD-associated increase in TEER was significantly inhibited by GW9662 at 4 and 8 hours into the reperfusion period (P<0.001 and P<0.05 respectively), indicating a role for PPARγ activation (n =6 from 3 experiments).

In conclusion, our data demonstrates that CBD attenuates the initial OGD-induced increase in permeability of the BBB when given pre-OGD. CBD given post-OGD increased barrier resistance during the reperfusion period, an effect which was mediated through PPARγ

2-AG RESPONSES ARE BLUNTED IN PATIENTS WITH CARDIOVASCULAR DISEASE AND CARDIOVASCULAR DISEASE RISK FACTORS

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We have previously shown that 2-AG causes vasorelaxation of human mesenteric arteries that is dependent on cyclooxygenase metabolism (Stanley & O'Sullivan, ICRS 2011). Previous studies have shown that 2-AG levels are often elevated in cardiovascular disease. The aim of the present study was to investigate whether the vascular responses to 2-AG are altered in patients diagnosed with cardiovascular disease or cardiovascular disease risk factors.

With ethical approval and written informed consent, human mesenteric arteries were taken from patients (37 male, 15 female) undergoing colorectal surgery. Arteries were dissected free of fat and connective tissue, mounted on a Mulvany-Halpern wire myograph and bathed in oxygenated physiological salt solution at 37°C under a set pressure of 90% of 100 mmHg. U46619 and endothelin-1 were used to increase tension by a minimum of 5 mN. Once a stable contraction had been achieved, cumulative concentration-response curves to 2-AG were constructed. Comparisons were made between patients with a given diagnosis and those without (2-way ANOVA) or correlated using Pearson correlation coefficient.

2-AG induced concentration-dependent vasorelaxation of human mesenteric arteries (R_{max} 74 ± 3 %, pEC$_{50}$ 5.5 ± 0.1, n = 52). The maximal vasorelaxant response to 2-AG was negatively correlated with age our patient sample (r^2 0.09, P <0.05 (n = 52)). 2-AG responses were significantly reduced in patients diagnosed with hypertension (P<0.05), cardiovascular disease (ischaemic heart disease and myocardial infarction) (P<0.001) and type-2 diabetes (P<0.01). Vasorelaxation to 2-AG was significantly reduced in the arteries of patients with risk factors associated with cardiovascular disease such as hyperlipidaemia (P<0.001) and a BMI above 25kg/m^2 (P<0.01), and were also blunted in those patients taking cyclooxygenase-inhibiting medication (P<0.001). There were no differences in 2-AG responses between patients with cancer and functional bowel disease, the type of operation performed, or between patients who smoked and those that did not smoke.

We have shown for the first time that 2-AG responses decline with age, and that the vasorelaxant responses to 2-AG are universally blunted in patients with cardiovascular disease and cardiovascular disease risk factors such as hyperlipidaemia and obesity.

CANNABIDIOL INDUCED VASORELAXATION OF HUMAN MESENTERIC ARTERIES IS MEDIATED BY THE ENDOTHELIUM, CB_1 AND TRPV1

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Several works, including those in human arteries, have suggested that cannabidiol (CBD) inhibits the vasorelaxant effect of other cannabinoids via antagonism of an as yet unidentified cannabinoid receptor. However, in rat mesenteric arteries CBD has been shown to cause vasorelaxation. The aim of the present study was to investigate any potential vasorelaxant effects of CBD in the human mesenteric artery.

With ethical approval and written informed consent, human mesenteric arteries were taken from patients (27 male, 12 female, 66 ± 14 yrs) undergoing colorectal surgery. Arteries were dissected and mounted on a Mulvany-Halpern myograph and bathed in oxygenated physiological salt solution at 37°C under a set pressure of 90% of 100 mmHg. U46619 and endothelin-1 were added to increase tension by a minimum of 5 mN. Once a stable contraction had been achieved, concentration-responses curves were carried out to CBD. Investigation into mechanisms of CBD action involved antagonism of CB_1, CB_2 and the proposed endothelial bound receptor, desentisation of the TRPV1 receptor, endothelium denudation, inhibition of nitric oxide synthase and cyclooxygenase (COX) and potassium efflux. Controls were carried out in the same patient and comparisons between control and intervention vessels were made using students' *t*-test. Analysis of control responses in patients with a given diagnosis were made using 2-way ANOVA. Significance taken at $P < 0.05$.

CBD causes vasorelaxation of pre-constricted human mesenteric arteries that is significantly different to vehicle control (pEC_{50} = 5.1 ± 0.3 (mean ± s.e.m), R_{max}= 36 ± 7% relaxation, n = 12, $P < 0.05$). Reduced efficacy was observed in vessels pre-treated with CB_1 antagonists (control, R_{max}= 59 ± 3% relaxation; AM251, R_{max}= 34 ± 6% relaxation, n = 12; $P < 0.001$: control, R_{max}= 48 ± 3% relaxation; LY 320135, R_{max}= 27 ± 3% relaxation, n = 7; $P < 0.001$), capsaicin (control, R_{max}= 51% ± 3% relaxation; capsaicin, R_{max}= 26% ± 4.0% relaxation, n = 7; $P < 0.001$) or contracted using KPSS (control, R_{max}= 59 ± 5% relaxation; KPSS contracted, R_{max}= 23 ± 11% relaxation, n = 7; $P < 0.05$). CBD potency was reduced in endothelial-denuded vessels (control, pEC_{50} = 5.8 ± 0.2; endothelium denuded pEC_{50} = 5.0 ± 0.2, n = 8; $P < 0.05$). Incubation with L-NAME inhibited CBD-induced vasorelaxation at low concentrations ($P < 0.01$). AM630, O-1918 and indomethacin had no effect on CBD-induced vasorelaxation. CBD-induced vasorelaxation is reduced in males ($P < 0.05$), hypertension ($P < 0.001$), diabetes ($P < 0.001$), hypercholesterolemia ($P < 0.001$) and in those with ischaemic heart disease ($P < 0.001$).

These data show for the first time that CBD causes vasorelaxation in human mesenteric arteries. This is partially mediated by the CB_1 and TRPV1 receptors, potassium channel efflux, the endothelium and nitric oxide release. Furthermore, CBD-induced vasorelaxation is reduced in patients with cardiovascular disorders.

CYSTOMETRIC EFFECTS OF SPINAL CANNABINOID RECEPTOR ACTIVATION IN NORMAL RATS

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Introduction: Systemic administration of cannabinoid receptor agonists affects bladder function, but whether the main site of action is peripheral tissues or the central nervous system has not been established. Intrathecal cannabinoids have been shown to produce antinociception in several neuropathic pain animal models. Our goal was to determine the effects of cannabinoid receptor agonists administered intrathecally on the bladder function of normal rats, as studied by cystometry.

Methods: Female Sprague-Dawley rats underwent a bladder catheter and a polypropylene intrathecal catheter insertion prior to cystometric evaluation. Urodynamic parameters were recorded in awake animals at baseline, and after sequentially administering vehicle and incremental dosages of drug. The drugs delivered in two different groups were methanandamide (5, 10, 20, 40 μg), a selective CB1 agonist and WIN 55212-2 (10, 20, 40 μg), a non-selective cannabinoid agonist. Urodynamic parameters were compared using one-way ANOVA.

Results: The micturition pressures did not change after vehicle or drug administration in either group. In the methanandamide group, bladder capacity significantly increased from baseline after 40 μg administration (0.62 vs 0.91 mL, $p<0.05$), and frequency decreased (16 vs 11 voids/hour, $p<0.05$). Bladder capacity also significantly increased after administration of 40 μg of WIN 55212-2 when compared to baseline (0.80 vs 1.04 mL, $p<0.01$) and to vehicle (0.82 vs 1.04 mL, $p<0.05$). Frequency decreased after 40 μg of WIN 55212-2 was administered when compared to baseline (12.5 vs 9.6 voids/hour, $p<0.01$) and to vehicle (12.2 vs 9.6 voids/hour, $p<0.05$). Micturition volume increased from baseline after 20 μg administration of WIN 55212-2 (0.76 vs 1.05 mL, $p<0.05$).

Conclusion: Intrathecal cannabinoid receptor agonist administration increases bladder capacity and decreases micturition frequency in normal rats. These effects seem to be mediated primarily via CB1 receptors, since there were no differences between the effects of methanandamide and WIN 55212-2. Both drugs also activate TRPV1 receptors, but the contribution of this action is not known.

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ROLE OF TRPV1 RECEPTORS IN THE SPINAL EFFECTS OF N-ACYL-ETHANOLAMINE ON THE MICTURITION IN THE NORMAL RAT

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Introduction: Intravesical and systemic administration of a fatty acid amide hydrolase (FAAH) inhibitor affects bladder function, but its sites of action in peripheral tissues or the nervous system have not been established. Intrathecal cannabinoids have been shown to produce antinociception in several neuropathic pain animal models. Many of these compounds also act at the TRPV1 receptor. Our goal was to determine the impact of TRPV1 activation on the bladder function of normal rats when the spinal activity of N-acyl-ethanolamine is increased.
Methods: Female Sprague-Dawley rats underwent a bladder catheter and a polypropylene intrathecal catheter insertion prior to cystometric evaluation. Urodynamic parameters were recorded in awake animals at baseline, and after sequentially administering different compounds. The first group of animals first received intrathecal SB366791 (200 nmol), a selective TRPV1 antagonist, followed by intraperitoneal oleoyl ethyl amide (OeTA) (0.75 mg/kg), a FAAH inhibitor, and finally intrathecal methanandamide (100 µg). The second group received 10 µl intrathecally of the vehicle (DMSO) for SB366791, followed by OeTA (0.75 mg/kg), and intrathecal methanandamide (100 µg). Urodynamic parameters were measure in absolute values and calculated as a change from baseline. They were compared using T-test.
Results: The micturition pressures did not change significantly after vehicle or drug administration in either group. In the SB366791 group, there were no significant changes from baseline in the intermicturition contraction interval, bladder capacity or micturition volume following systemic OeTA and intrathecal methanandamide. In the vehicle group, we observed a significant increase from baseline in the intermicturition contraction interval (13.9%), the bladder capacity (13.9%) and micturition volume (31.8%) following systemic OeTA and intrathecal methanandamide ($p<0.05$).
Conclusion: Intrathecal TRPV1 antagonist administration abolished the effects of spinal N-acyl-ethanolamine on intermicturition contraction interval, bladder capacity, and micturition volume. This suggests that spinal TRPV1 activation by endogenous cannabinoids and methanandamide is necessary to cause a change in afferent signaling in micturition.

Acknowledgements: Departmental funding

DYSREGULATION OF HYPOTHALAMIC-PITUITARY-ADRENAL AXIS IN MICE LACKING CB1 RECEPTOR IN ADRENERGIC AND NORADRENERGIC NEURONS

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Noradrenergic neurons in the locus coeruleus of the brain are involved in stress responses and trigger various systemic reactions in response to stress. The changes leading to modulation of these neurons may produce several neuropsychiatric disorders. Endocannabinoids acting through endocannabinoid receptor 1 (CB1) are known to modulate the activity of neurons in the brain. Therefore, in the present study we studied the role of CB1 in dopamine beta hydroxylase (dbh), a key enzyme for norepinephrine synthesis, expressing neurons by generating a mouse line lacking CB1 in dbh expressing neurons (dbh-CB1-KO) by using cre/loxP technology. dbh-CB1-KO mice showed complete deletion of CB1 receptor in all the dbh expressing cells including adrenergic and noradrenergic neurons in the brain, sympathetic neurons and adrenal medulla.

We analyzed the changes in plasma level of stress hormone in basal and stimulated condition. The changes in hypothalamic peptide and changes in behavior were monitored respectively, by qPCR and a series of behavior experiments. Our results showed increased basal and fasting induced corticosterone level in dbh-CB1-KO mice. The plasma level of adrenocorticotropic hormone also showed increased level in dbh-CB1-KO mice. The qPCR analysis of hypothalamic peptide showed increased CRH and NPY mRNA in dbh-CB1-KO mice. The increase in NPY was associated with the increased food intake in these mice. In forced swim test these mice show increased immobility which indicate depressive like behavior. In open field these mice show no difference in total ambulation however spent less time in central zone. In light-dark chamber test for anxiety dbh-CB1-KO mice spent less time in light and rather spent more time in dark chamber. Further, in elevated plus maze dbh-CB1-KO mice showed reduced entry in open arm. In summary, CB1 deletion from dbh expressing neurons in mice produces behavioral disorder such as anxiety and depression and causes dysregulation of hypothalamic-pituitary-adrenal axis.

NOVEL SELECTIVE CB1 ANTAGONIST AGENT WITH ANTI-OBESITY ACTIVITY

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The present work aimed to study the effect of a novel CB1 antagonist agent (NESS06SM) on food intake, weight, molecular expression profile and cardiovascular risk factors, induced by a chronic treatment in C57BL/6N Diet Induced Obesity (DIO) mice.

As in the case of the reference CB1 antagonist compound rimonabant, NESS06SM solubilised in a microemulsion preparation caused a significant weight loss in DIO mice fed with a fat diet, compared to control mice. Despite the use of fat diet, improvement of cardiovascular risk factors was determined in the case of chronic administration of NESS06SM, evidencing interesting properties of this new compound in the treatment of metabolic syndrome.

Analysis of mRNA expression levels of central and peripheral markers by Real Time PCR showed a significant increase of orexigenic peptides and a decrease of anorexigenic peptides in DIO mice fed with normal diet and in NESS06SM treated mice, compared with control mice fed with fat diet. In central tissue, mRNA analysis revealed a positive modulation of monoaminergic trasporters expression in NESS06SM treated animals.
Our results suggest that the new CB1 antagonist compound reduces body weight and can restore the disrupted expression profile of genes linked to the hunger-satiety circuit, without altering monoaminergic transmission.

In conclusion, NESS06SM may represent a useful candidate agent for the treatment of obesity and its metabolic complications.

CANNABIDIOL ATTENUATES ISOLATION-INDUCED AGGRESSION IN MICE BY ACTIVATION OF 5-HT1A RECEPTORS

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Long-term individual housing increases aggressive behavior in mice, a condition termed isolation-induced aggression or territorial aggression. Several classical neurotransmitters have been linked to aggression. The serotonin (5-HT) system generally dampens aggression in animals and violent behavior in humans. Impulsivity and high aggressiveness are correlated with reduced 5-HT levels or turnover in the rodent's brain, thus pharmacological strategies to increase brain 5-HT levels, such as the use 5-HT reuptake inhibitors or 5-HT1A receptor agonists, are able to reduce aggressive behavior in rodents. Cannabidiol (CBD) is a non-psychotomimetic compound from *Cannabis sativa* plant that induces anxiolytic- and antidepressant-like effects and attenuates the responses induced by stressful situations in rodents. Although the mechanisms to these effects are not completely understood, it is proposed that CBD facilitates the endocannabinoid system and activates 5-HT1A receptors. Therefore, the aim of the present study was to verify if CBD could also modulate the aggressive behavior induced by social isolation and if this effect could be due to 5-HT1A activation. Male C57BL/6J mice (8–10 weeks of age when test began) were used in this study. A group of animals (the residents) was housed individually in plexiglass cages (24x17x12 cm) for 4 weeks to induce aggression. After this period of isolation, we tested whether acute treatment with CBD (5, 15, 30 and 60 mg/kg; ip.; n=7-11/group) 30 min prior to test would inhibit isolation-induced aggression against a conspecific animal (intruder; housed in groups of five per cage) introduced into the resident's cage at the test time. We also investigated if the pretreatment with a 5-HT1A antagonist, WAY100635 (3 mg/Kg), administered 30 minutes before CBD would counteract CBD effects. The resident-intruder interaction was videotaped for 10 min and the latency to the first bite against the intruder, the number of attacks and total duration of aggressive encounters were recorded.

CBD 5 and 30 mg/Kg increased the time to initiate aggressive behavior, indicated by greater latencies to attack ($F_{4,40}$=2.5; p=0,05, Duncan). Moreover, CBD in all doses was able to reduce the number of attacks ($F_{4,40}$=4,5; P<0,005, Duncan) and duration of aggressive behavior ($F_{4,40}$=9.4; P<0,0001, Duncan). Next, we choose the dose of 30 mg/Kg to investigate the participation of 5-HT1A receptors. We observed that the greater latency to attack induced by CBD was blocked by pretreatment with WAY ($F_{3,13}$=4.0, p<0.05, Duncan; n=4-5/group). These findings suggest that CBD, by increasing serotonergic neurotransmission via 5-HT1A, could be a useful drug to control heightened aggressiveness, and possibly to treat aggressive behavior associated with psychiatric disorders. We still need to investigate if CBD effects could also be mediated by cannabinoid receptors.

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EFFECTS OF CANNABINOIDS AND ENDOCANNABINOID-HYDROLYSIS INHIBITION ON PENTYLENETETRAZOLE-INDUCED SEIZURES AND ELECTROENCEPHALOGRAPHIC ACTIVITY

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The cannabinoid CB1 receptor may restrain excitatory neurotransmission and epileptiform seizures, indicating that this could be a target for new drugs effective against epileptic syndromes. However, it has remained to be investigated which would be the more appropriate strategy, particularly comparing direct agonists (synthetic cannabinoids) with endocannabinoid (anandamide)-hydrolysis inhibitor (FAAH-inhibitor). In this study we tested the effects of the cannabinoids WIN-55-212-2 (non-selective, 0,3-3 mg/kg) and ACEA (CB1-selective, 1-4 mg/kg) as well as the FAAH-inhibitor URB-597 (0,3-3 mg/kg) against pentylenetetrazole (PTZ)-induced myoclonic seizure and electroencephalographic (EEG) activity in Wistar rats. The animals received intraperitoneal drug injections followed by intravenous injection of PTZ, after which seizure and EEG were recorded simultaneously. WIN-55-212-2 and ACEA actually facilitated PTZ-induced seizure and EEG activity. On the contrary, URB-597 inhibited both parameters. These results suggest that increasing anandamide levels, rather than direct activating CB1 receptors, might be a more promising strategy for the treatment of epilepsy and related syndromes.

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THE ROLE OF INTRA-VISCERAL INSULAR CORTEX 2AG IN CONDITIONED NAUSEA-LIKE BEHAVIOR IN RATS

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Manipulations that elevate the endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol (2AG), have previously been found to interfere with the establishment of lithium chloride (LiCl)-induced conditioned gaping in rats (a selective measure of conditioned nausea; Cross-Mellor *et al.,* 2007; Sticht *et al.,* 2012). Although the precise brain mechanisms underlying nausea have yet to be fully uncovered, the insular cortex (IC) appears to be a key structure in mediating its sensation; ablation of the IC has been found to interfere with conditioned gaping in rats (Kiefer & Orr, 1992), while administration of the anti-emetic drug, ondansetron, resulted in similar impairments when injected directly into the visceral area of the IC (VIC) of rats (VIC; Tuerke *et al.,* 2012). However, at present, the role of the VIC in mediating endocannabinoid-suppression of nausea remains unknown. Therefore, the current study investigated the potential of intra-VIC 2AG administration to interfere with establishment of conditioned gaping in rats.

A series of experiments evaluated the effects of pretreatment with exogenous 2AG prior to administration of the illness-inducing drug, LiCl. Rats received an intraoral infusion of 0.1 % saccharin (3 min) followed immediately by an intra-VIC infusion of 2AG (0, 0.5, 1.0 μg), and an injection of LiCl 15 min later. Rats were subsequently re-exposed to saccharin 72 hr later in a drug-free taste reactivity test, in which conditioned gaping was assessed. It was found that an intra-VIC infusion of 2AG dose-dependently suppressed conditioned gaping in rats. However, conditioned taste avoidance of saccharin was unaffected by intra-VIC 2AG, as measured by a one- or two-bottle consumption test. Interestingly, the ability of intra-VIC 2AG administration to interfere with LiCl-induced conditioned gaping does not appear to be mediated by CB1 receptors, as pretreatment with the CB1 antagonist, AM251 (1 μg, intra-VIC), did not reverse the anti-nausea-like effects of 2AG.

These findings suggest that manipulations that elevate intra-VIC 2AG may have anti-nausea potential, and that, consistent with the effects of systemic administration (Sticht *et al.,* 2012) downstream metabolites of 2AG may be partially responsible for mediating the anti-nausea effects of exogenous administration. Future studies will assess the effects of intra-VIC anandamide administration, and its mechanism of action.

ADULT RATS OVEREXPRESSING THE CB1 RECEPTOR IN THE MPFC DISPLAY ALTERED EMOTIONAL BEHAVIOR AND IMPAIRED EXTINCTION OF FEAR MEMORY

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It is well established that the endocannabinoid (ECB) system modulates emotional behavior and stress-reactivity. In this study we wanted to investigate the specific role of the CB1 receptor in the medial prefrontal cortex (mPFC) in emotional behavior and fear conditioning. The CB1 receptor was therefore overexpressed in the mPFC of adult Wistar rats by adeno-associated virus (AAV) vector-mediated gene transfer. The animals were then tested in different anxiety-related paradigms for emotional reactivity (e.g. elevated plus maze (EPM), light/dark emergence test (EMT), social interaction) and in a tone-cued fear conditioning paradigm. CB1 receptor overexpressing animals (CB1-R) displayed subtle differences in anxiety-related and exploratory behavior compared to empty vector injected controls (Empty) in the EMT and EPM. General locomotor activity did not differ between the groups. In the social interaction test, CB1-R animals displayed less irritation by the unknown conspecific compared to Empty animals and CB1-R animals showed more exploratory behavior towards the social partner. A tone-cued fear conditioning paradigm showed similar acquisition rates of learning the tone-shock pairings for Empty and CB1-R animals as displayed by similar freezing rates during cue-recall. However, there was a significant difference for extinction learning between groups. Empty animals showed a trend for a reduction of freezing from cue recall to extinction recall as expected. Conversely, CB1 animals did not display a reduction in freezing rate from cue recall to extinction recall but displayed a significantly higher freezing rate for extinction recall compared to Empty animals. This implies an impairment of the CB1-R animals for extinction of tone-cued aversive memories.
Altogether the upregulation of the CB1 receptor specifically in the rat mPFC induces differences in emotional reactivity, social behavior and extinction learning. These findings might be relevant for neuropsychiatric disorders, since higher cortical CB1 receptor expression levels have been described post-mortem in schizophrenic patients.

CRH MEDIATES THE EFFECTS OF GLUCOCORTICOIDS ON ENDOCANNABINOID SIGNALING IN THE AMYGDALA

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Exposure to stress produces a divergent regulation of the endocannabinoids 2-AG and AEA in the brain, which are mediated in part by glucocorticoid hormones. However, other mediators of stress, such as corticotropin-releasing hormone (CRH) may also play a role. For instance, within the amygdala, experiential and hormonal stimuli that increase CRH levels are also capable of reducing AEA content. The aim of the current studies was to determine the extent to which CRH regulates endocannabinoid signaling within the amygdala and to determine whether CRH contributes to the effects of sustained glucocorticoid exposure on shifts in endocannabinoid signaling within this region. In experiment 1, male rats were implanted with cannula into the lateral ventricle and infused with either saline or CRH (1 μg), after which the amygdala was harvested and analyzed for AEA and 2-AG content. Intracerebroventricular CRH administration produced a rapid (~10 min) reduction in AEA content and a small increase in 2-AG content within the amygdala. In experiment 2, we then implanted separate groups of rats with a subcutaneous pellet of corticosterone (200 mg) or placebo, as well as a second pellet containing the CRHR1 antagonist antalarmin (60 mg) or placebo. Animals were left for 7 days, after which they were decapitated and the amygdala was harvested for analysis of AEA and 2-AG content as well as FAAH activity. The results of the study revealed that sustained corticosterone exposure produced a reduction in AEA content within the amygdala, coupled to an increase in FAAH activity. Interestingly, both of these effects were prevented by co-treatment antalarmin. Previous data indicates that chronic corticosterone exposure upregulates CRH expression within the amygdala (Schulkin et al., 2005). Together, these data suggest that the increase in CRH signaling within the amygdala following chronic corticosterone exposure is responsible for alterations in AEA metabolism and content, which is corroborated by our data demonstrating that CRH administration alone is sufficient to reduce AEA content in the amygdala.

PLASMA LEVELS OF ENDOCANNABINOIDS AND RELATED PRIMARY FATTY ACID AMIDES ARE INCREASED IN PATIENTS WITH POST-TRAUMATIC STRESS DISORDER

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Background: Endocannabinoids (ECs) and related N-acyl-ethanolamides (NAEs) play important roles in stress response regulation, anxiety and traumatic memories. EC plasma concentrations are influenced by acute and chronic stress, affective disorders and severe mental illness. We therefore hypothesized that individuals with traumatic stress exposure and post-traumatic stress disorder (PTSD) show measurable alterations in plasma EC and NAE concentrations.

Methods: We determined plasma concentrations of the ECs anandamide (ANA) and 2-arachidonoylglycerol (2-AG) and the NAEs palmitoylethanolamide (PEA), oleoylethanolamide (OEA), stearoylethanolamine (SEA), and N-oleoyldopamine (OLDA) by HPLC-MS-MS in patients with PTSD (n=10), trauma-exposed individuals without evidence of PTSD (n=18) and in healthy control subjects (n=20). PTSD was diagnosed using the *Clinician Administered PTSD Scale (CAPS)* which also assesses traumatic events.

Results: Individuals with PTSD showed significantly higher plasma concentrations of ANA (0.48±0.11 vs. 0.32±0.13 ng/ml, $p<0.01$), 2-AG (8.43±3.20 vs. 6.61±2.11 ng/ml, $p<0.01$), PEA (5.15±2.00 vs. 3.21±1.10 ng/ml, $p<0.01$), OEA (5.90±2.10 vs. 3.40±1.42 ng/ml, $p<0.01$), SEA (2.70±3.40 vs. 0.58±0.32, $p=0.02$) and significantly lower plasma levels of OLDA (0.12±0.05 vs. 0.64±0.74 ng/ml, $p=0.02$) than controls. Trauma-exposed individuals without evidence of PTSD had significantly higher plasma concentrations of ANA (0.44±0.10 vs. 0.32±0.13ng/ml, $p<0.01$) and significantly lower levels of OLDA (0.22±0.11 vs. 0.64±0.74ng/ml, $p=0.03$) than controls, without differences in the other investigated compounds.

Conclusions: The experience of highly traumatic events results in long-term changes in plasma ECs/NAEs which persist long after termination of the stressor and are more pronounced in individuals who develop PTSD.

MORPHOLOGICAL AND BEHAVIOURAL EVIDENCE FOR IMPAIRED PREFRONTAL CORTICAL FUNCTION IN FEMALE CB1 RECEPTOR DEFICIENT MICE

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Increasing evidence argues that the endocannabinoid system plays a critical role in higher order tasks requiring cognitive flexibility. Deficits in endocannabinoid activity are known to impair both extinction and reversal learning in the Morris water maze, largely through promoting perseveratory responses. Consistent with these behavioral changes, CB_1 receptor knockout (CB_1KO) mice have been found to exhibit reduced dendritic arborisation in layer II/III neurons in the medial prefrontal cortex (PFC), a brain region critical for optimal performance in these tasks. However, this body of research has been conducted in male subjects and there is no evidence to date as to whether the endocannabinoid system performs a similar role in females. To this extent, we investigated adult CB_1 receptor deficient female mice in both acquisition and reversal learning in the Morris water maze and also examined dendritic morphology of neurons in the pre-limbic and infra-limbic areas of the prefrontal cortex. Similar to what has been reported in male CB_1KO mice, female CB_1KO mice did not differ from WT on acquisition in a fixed-platform version of the Morris water maze task; however, during the reversal learning phase, female CB_1 KO mice perseverated significantly longer than the WT mice. Furthermore, female CB_1 KO mice had significantly lower savings ratios than WT mice, indicating that they were impaired in learning the new location of the platform during reversal learning. Examination of layer II/III neurons in the prelimbic and infralimbic regions of the PFC revealed significantly shorter and less complex dendritic morphology in the prelimbic, but not the infralimbic region of the PFC in female CB_1KO mice. These data indicate that endocannabinoid signaling does not exhibit a sex difference with respect to its role in cognitive flexibility, and supports the findings indicating that a disruption of CB_1 receptor signaling results in compromised structure and function of the PFC.

THE MODULATION OF THE ENDOCANNABINOID SIGNALING MEDIATES THE EXTINCTION OF AVOIDANCE BEHAVIOR BY CONTROLLING SAFETY LEARNING

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Avoidance of unfamiliar places, ultimately leading to fear of leaving home is largely responsible for the decreased quality of life in patients suffering from anxiety disorders. Studies using animal models have contributed to the understanding of the neural mechanisms underlying the acquisition of avoidance behavior to aversive stimuli; however, much less is known about its extinction. Since the endocannabinoid system (ECS) plays a crucial role in the extinction of aversive memories; the present study was aimed to investigate both the protocols of response extinction vs. safety learning in extinction of step-down inhibitory avoidance in C57BL/6N mice and the role of the EC signaling modification in the safety learning and its long-term impact on avoidance behavior. In this task, mice are placed on a platform and received an electric shock as soon as they step down onto the grid floor with all four paws. Memory performance is assessed by an increase in step-down latencies on subsequent encounters. Repeated exposure to the grid (i.e. safety learning), but not repeated step-down from the platform (i.e. response extinction), leads to extinction of avoidance behavior. This process is context-dependent and can be blocked by the pharmacological (i.e. treatment with the cannabinoid CB1 antagonist rimonabant) or genetic (lack of CB1 receptors in neurons expressing dopamine D1 receptors: D1–CB1 knock-out mice) inactivation of CB1 receptors. By contrast, the endocannabinoid reuptake inhibitor AM404 facilitates safety learning through a CB1-mediated activity and attenuates the relapse of avoidance behavior 28 days after conditioning. Here we demonstrate that learning about the safety of an environment is essential for executing actions after an aversive encounter and that it depends on endocannabinoid signaling.

CHANGES IN THE LEVEL OF ENDOCANNABINOIDS AND ENDOCANNABINOID-LIKE MOLECULES AFTER ACUTE AND REPEATED ADMINISTRATION OF IMIPRAMINE AND N-ACETYLCYSTEINE IN DIFFERENT RAT BRAIN STRUCTURES

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Depression as one of the major lifestyle diseases of the twenty-first century is a serious therapeutic problem in modern pharmacotherapy. Different mechanisms of action of antidepressant drugs suggest that these drug interaction with the direct target molecule is not responsible for the therapeutic efficacy but rather neuroadaptive mechanisms have a significance. In recent years there has been highlighted the potential participation of the endocannabinoid system in the pathogenesis of depression and in the action of antidepressants.

The aim of this study was to investigate the effect of imipramine (IMI), classical antidepressant and of N-acetylcysteine (NAC), showing antidepressant activity in preclinical studies, on the level of endocannabinoids anandamide (AEA) and 2-arachidonylglicerole (2-AG) and of endocannabinoid-like molecules palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) in different rat brain structures.

Male Wistar rats received drugs intraperitonerally chronically (daily for 14 days) and on day 14th, followed by 13 days of saline injections, while the control group received only solvent (saline) for 14 days. Twenty four hours after the last administration of drugs the animals were decapitated and the tissue levels of endocannabinoids were determined using the liquid chromatography mass spectrometry (Applied Biosystem: Agilent 1100 and API 2000, column: Thermo Scientific). Data were analyzed by using one-way ANOVA followed by the Dunnett's test.

After administrations of IMI and NAC, most of changes in concentrations of the examinated molecules had a similar direction and they appeared in the same brain structures. Administered acutely IMI (15 mg/kg) or NAC (100 mg/kg) resulted in decreased levels of 2-AG in cerebellum ($p<0.001$). Chronic administration of IMI (15 mg/kg) or NAC (100 mg/kg) resulted in increased levels of AEA in the hippocampus ($p<0.001$) and of PEA in the prefrontal cortex ($p<0.05$). There was also an increase in PEA and OEA concentrations in the striatum ($p<0.001$). Similarly 2-AG concentration was increased in the frontal cortex and striatum ($p<0.01$ and $p<0.001$, respectively) while in the cerebellum the level of the endocannabinoid was decreased.

Our data suggest the engagement of the endocannabinoid system in the effects of IMI and NAC, but the more detailed explanation of this mechanism requires further investigations.

CANNABINOIDS AMELIORATE IMPAIRMENTS INDUCED BY CHRONIC STRESS TO SYNAPTIC PLASTICITY AND SHORT-TERM MEMORY

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Repeated stress is one of the environmental factors that precipitates and exacerbates mental illnesses like depression and anxiety. In rats, repeated restraint stress has been shown to induce depressive and anxious phenotypes as well as cognitive impairments, which are often experienced in depressive illness.

We have recently shown that acute systemic and local administration of cannabinoids reduces the impairing effects of acute stress on learning and plasticity (Abush and Akirav, 2012). Here we aimed to find whether *chronic* cannabinoid treatment would alleviate the effects of exposure to *chronic* restraint stress on memory and plasticity.
Adult rats were exposed to chronic restraint stress for two weeks and injected with vehicle or with the CB1/CB2 receptor agonist WIN55,212-2 (WIN;1.2mg/kg). One month after the last exposure to chronic stress and WIN, the rats were tested for behavioral and electrophysiological measures of cognitive performance, and for glucocorticoid receptors (GRs) levels in the nucleus accumbens (NAc).

Chronic stress exposure impaired long-term potentiation (LTP) in the ventral subiculum (vSub)-NAc pathway, impaired the performance of rats in the prefrontal-dependent object recognition task and the hippocampal-dependent spatial version of this task, and lowered the levels of GRs in the NAc. Chronic administration of WIN prevented the impairing effect of chronic stress on LTP in the vSub-NAc pathway, improved the performance of rats in the recognition and spatial tasks, and raised the levels of GRs in the NAc back to control levels.

Our findings suggest that the cannabinoid system could represent a therapeutic target for the treatment of conditions associated with chronic stress in adults. A better understanding of the interaction between stress and the cannabinoid system could contribute to the clinical applications of cannabinoids in the treatment of anxiety and affective disorders resulting from chronic stress exposure.

CANNABINOID AND GLUCOCORTICOID RECEPTORS IN THE AMYGDALA MODULATE THE STRESS-INDUCED IMPAIRMENT OF LTP IN THE HIPPOCAMPAL-ACCUMBENS PATHWAY

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Stress-induced disturbances in memory are key components of most psychiatric disorders and are not just secondary symptoms. Exposure to stress results in the release of endocannabinoids and glucocorticoids, potent modulators of learning and plasticity. Here we aimed to examine the effects of exposure to acute stress on LTP in the pathway projecting from the ventral subiculum of the hippocampus (vSub) to the nucleus accumbens (NAc), and to test whether modulating cannabinoid and glucocorticoid receptors in the basolateral amygdala (BLA) could alleviate this impairment.

The NAc receives converging input from a number of structures proposed to play a role in affective disorders. In particular, the BLA provides an affective input that overlaps with context-related information derived from the vSub. We found that altering BLA-mediated emotional input by exposing rats to the elevated platform acute stress impairs LTP in the vSub-NAc pathway. This supports other evidence suggesting that the context information supplied by the vSub is crucial for gating the response of NAc neurons to BLA-mediated emotional input. Next we examined whether intra-BLA cannabinoid receptor activation using the CB1/2 receptor agonist WIN 55,212-2 (WIN; 5 μg/side) or blocking glucocorticoid receptors using the antagonist RU-486 (RU; 6ng/side) can alleviate the stress-induced impairment of LTP in the vSub-NAc pathway.

WIN and RU prevented the stress-induced impairment of LTP when microinjected into the BLA bilaterally or to the hemisphere contralateral to the recording and stimulating electrodes. However, bilateral, but not contralateral, intra-BLA RU microinjection without stress exposure impaired LTP in the vSub-NAc pathway. This suggests that intra-BLA WIN/RU modulation of vSub-NAc plasticity is not mediated by means of a direct neural pathway. These findings provide evidence that cannabinoids and glucocorticoid receptors in the BLA can modulate the effects of stress on plasticity in the vSub-NAc pathway, hence being a potential therapeutic target.

ENDOCANNABINOID-MEDIATED SYNAPTIC DEPRESSION IN THE CENTRAL AMYGDALA: CHOLINERGIC-CANNABINOID INTERACTIONS

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The amygdala regulates the processing of autonomic and behavioral reactions to stress. Although the role of endocannabinoids (eCBs) in regulating synaptic signaling in the basolateral amygdala has been extensively studied, eCB signaling in the central amygdala (CeA) has received relatively less attention. The CeA acts as the amygdala's primary "output" nucleus and is regulated by glutamatergic inputs from diverse sensory and autonomic sources. Given this critical role of excitatory CeA afferents in the regulation of amygdala output, it is likely that eCB-mediated modulation of glutamate release, at these terminals, significantly contributes to the eCB system's prevailing role in the stress response. As such, our aim is two-fold: 1) to explore eCB- mediated modulation of glutamatergic CeA_l afferents and 2) to determine whether this modulation is affected by chronic stress (CS) exposure. To carry out this study, *ex vivo* electrophysiological recordings were performed in the CeA_l of male, ICR mice in the presence of the $GABA_A$ receptor antagonist, picrotoxin. Local stimulation was used to evoke excitatory post-synaptic currents (eEPSCs). To determine eCB-mediated neuroadaptations in response to CS exposure, the above experiments were repeated subsequent to a chronic restraint-stress (CRS) paradigm which involved exposing mice to either 0 (control mice) or 10 consecutive days of CRS for 1 hour per day.

CB_1 receptor agonists, WIN 55212-2 (5μM) and CP 55940 (5μM), decreased eEPSCs to 50% of normalized baseline; an effect that was absent in our global CB_1-deficient mice ($CB_1^{-/-}$). Furthermore, this CB_1 receptor-mediated depression had a presynaptic locus as agonist application (CP 55940, 5μM) significantly decreased the frequency but not the amplitude of spontaneous EPSCs. To study short-term eCB synaptic signaling, we evaluated depolarization-induced suppression of excitation (DSE) in CeA neurons. 10 second depolarization induced DSE (eEPSCs transiently depressed to ~81% of normalized baseline) in control animals but not in our $CB_1^{-/-}$ mice. Furthermore, DSE was largely absent in control slices pretreated with either the diacylglycerol lipase (DAGL) inhibitor, tetrahydrolipstatin (THL, 10μM), or the CB_1 antagonist, SR 141716 (5μM). Activation of muscarinic receptors (mAChR), using Oxotremorine-M (OxoM, 1μM), enhanced DSE in control animals by ~20%, an effect largely blocked by SR 141716 (5μM) or THL (10μM) pretreatment. Furthermore, OxoM alone caused a mAChR-driven eCB-mediated depression (~20%) of eEPSCs, as well as, a muscarinic M2 receptor-mediated synaptic depression (~40%). Interestingly, our CRS mice demonstrated enhanced DSE blocked by SR 141716 (5μM) or THL (10μM) pretreatment. These data indicate that CB_1 receptor signaling modulates glutamatergic CeA_l afferents in a stress-responsive manner, and suggest complex interactions between cholinergic and cannabinoid signaling systems in the regulation of glutamatergic drive to the CeA.

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CHRONIC WIN 55,212-2 EXPOSURE AND DENTATE DENDRITIC MORPHOLOGY IN ADULT RATS

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The chronic abuse of drugs can create vast negative repercussions on behavioral and biological systems by altering underlying neurocircuitry permanently. Long-term abuse of cannabinoids in rats leads to neuronal cell death, detrimental cellular changes, and changes in dendritic morphology. Previous studies have found that chronic treatment with delta-9-THC selectively alters dendritic morphology of neurons in certain brain regions decreasing dendritic morphology and spine density in the dentate gyrus of young rats (Rubino et al., Hippocampus 19 (2009):763-772). It remains to be seen if these changes in adolescence hold constant in adulthood, or are specific to a particular developmental age. The following study was conducted to assess if chronic exposure to WIN 55, 212-2, a potent cannabinoid agonist, in adult rats changed dendritic morphology of the hippocampus. Adult rats were given WIN 55,212-2 (i.p., 3.7 mg/kg) or vehicle injections over the course of either 7 or 21 days. Upon completion of treatment, brains were processed for Golgi-Cox staining. Dendritic branching, length, and spine density from segments proximal, medial, and distal to the cell body were measured on granule cells of the upper blade of the hippocampal dentate gyrus. The results indicate there was a significant reduction in spine density (1 spine/10 μm) in WIN 55,212-2 treated rats from both the 7 and 21 day conditions in segments proximal and medial to the cell body. This spine density loss was significant in proximal segments as early as 7 days of treatment. Although there were no significant differences between groups with regards to dendritic branching and length, there was a trend for rats which received WIN 55,212-2 to have increased branching in medial segments and higher 4^{th} order branching at 21 days. When taken together, these results may imply chronic cannabinoid treatment specifically alters commissural/associational afferents and medial perforant path projections from entorhinal cortex but not lateral perforant path projections. The resulting loss of dendritic spine densities in the dentate gyrus may be an important contributing factor underlying behavioral findings of cannabinoid induced memory impairment and memory consolidation deficits.

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CANNABINOIDS ALTER REVERSAL OF BEHAVIORAL CONTINGENCY BY SUPPRESSING MEMORY-RELATED ENCODING

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We have previously shown that activation of endogenous cannabinoid (CB1) receptors by exogenous agonists WIN 55,212-2 and delta-9-THC suppress hippocampal neural encoding of task-relevant information in rats performing a delayed-nonmatch-to-sample (DNMS) task (Hampson et al. Behav Pharm 2007). The CB1 antagonist/inverse agonist Rimonabant blocks the effects of exogenously applied agonists, and when applied alone produces enhanced neural encoding and facilitated DNMS behavior at delays > 10 sec. Over the past few years, this laboratory has demonstrated that the neurons affected by cannabinoid modulation are essential to maintenance of behaviors requiring cognitive processing – in particular, cannabinoids suppress the development of behaviorally-relevant firing correlates of hippocampal neurons during initial training in the DNMS task (Goonawardena et al. Hippocampus, 2010). Thus, a major role for the endocannabinoid system in learning and memory may be to modulate strength of neural encoding regulating the speed and type of memory formation.

We recently extended the analysis of cannabinoid effects on behavioral acquisition by training animals to perform the DNMS task, then reversing the task to the Match contingency – i.e. a Delayed-Match-to-Sample (DMS) task – and testing the effects of exogenously applied cannabinoids. Prior tests with the CB1 antagonist/inverse agonist Rimonabant indicated that the time to achieve criterion (reversal) performance in the DMS task was delayed by approximately 4 days when animals were exposed to Rimonabant during the reversal learning. We attributed the delay to prolonged "extinction" of the DNMS task, and predicted that exposure to the CB1 agonist WIN 55,212-2 may facilitate extinction, but still delay acquisition of the "reversed" task. In fact, we found that WIN exposure resulted in a shift from memory-based performance to strategy-based performance in which the animal always performed the same response in the Match phase (irrespective of trial type). Once the daily WIN injections ceased, most animals quickly learned the DMS contingency within 5 sessions.

These results suggest that CB1 activation does indeed speed extinction, but also impairs acquisition of a new behavioral contingency. Results will be discussed in view of development of task-related correlates of hippocampal neural activity to the reversed task as well as comparison with memory encoding strategies. These results confirm that CB1 receptors alter encoding within specific mnemonic mechanisms, and suggest that endocannabinoids may normally play a role in the gating of "forgetting" and relearning by brain areas that process cognitive information.

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EXTREMELY LOW DOSES OF TETRAHYDROCANNABINOL PROTECT FROM COGNITIVE DEFICITS AND INDUCE LONG-LASTING BIOCHEMICAL CHANGES IN THE MOUSE BRAIN

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We have previously reported that a single injection of an extremely low dose of delta-9-tetrahydrocannabinol (THC) protected the brain from pentylenentetrazole (PTZ)-induced cognitive deficits when applied either 1-7 days before ("preconditioning"), or 1-3 days after ("postconditioning"), the administration of PTZ[1]. Here we expand the protective profile of THC by showing that it protected mice from cognitive deficits that were induced by a variety of other neuronal insults, including pentobarbital-induced deep anesthesia, carbon monoxide (CO)-induced hypoxia and repeated treatment with 3,4 methylenedioxymeth-amphetamine (MDMA; "ecstasy"). The protective effects of THC against the various insults were detected as long as 7 weeks following the experimental procedure. The same extremely low dose of THC (0.002 mg/kg, a dose that is 3–4 orders of magnitude lower than the doses that produce the known acute effects of the drug in mice) induced long-lasting (7 weeks) modifications in the activity of extracellular signal-regulated kinase (ERK) in the hippocampus, frontal cortex and cerebellum of the mice. The alterations in ERK activity paralleled changes in its activating enzyme MEK and its inactivating enzyme MKP-1. In addition, the single treatment with the low dose of THC elevated the activity of CREB in the hippocampus and the level of BDNF in the frontal cortex.

Our more recent experiments indicate that THC similarly protects against long-term lipopolysacharide (LPS)-induced cognitive damage. Furthermore, THC prevented the elevation of cyclooxigenase-2 (COX-2) that was elicited by LPS in the various brain regions.

These long-lasting behavioral and neurochemical effects indicate that extremely low doses of THC can modify brain plasticity and may be beneficial in the treatment of neurodegenerative diseases.

[1]Assaf et al. (2011) Behav. Brain Res. 220, 194-201.

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ALTERATIONS OF CB1 mRNA EXPRESSION IN STARGAZER MUTANT MICE AND IN PENTYLENETETRAZOLE-INDUCED SPIKE AND WAVE DISCHARGES IN RATS

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Absence epilepsy is a generalized non-convulsive form of epilepsy characterized by bilaterally synchronous spike and wave discharges (SWDs) on the electroencephalogram (Blumenfeld, Epilepsia. 46 (2005) 21-33). SWDs are generated within a cortico-thalamo-cortical loop that comprises cortical pyramidal neurons and GABAergic interneurons, thalamic relay neurons and inhibitory neurons of the reticular thalamic nucleus (nRT). Furthermore, neurons of the nRT are interconnected to each other as well, via inhibitory GABAergic synapses together with gap junctions (Polack et al., Cereb Cortex. 19 (2009) 2078-91). The cortex (layer V and VI) sends excitatory glutamatergic projections to the nRT and thalamic relay neurons send excitatory glutamatergic projections to cortical pyramidal neurons and nRT. The nRT neurons send inhibitory GABAergic connections to thalamic relay neurons but not to the cortex (Zhang et al., J Neurophysiol. 91 (2004) 759-66). We have demonstrated that the cortico-thalamo-cortical loop is finely regulated by the endocannabiniod system since, in the WAG/Rij rat model, the cannabinoid type 1 (CB1) receptors undergoes plastic modifications during the development of absence epilepsy and the CB1 receptor responds to pharmacological activation by reducing SWDs (van Rijn et al., Epilepsia. 51 (2010) 1511-21).

In the present study we evaluated the involvement of the endocannabinoid system in two different models of absence epilepsy: a pharmacological model, based on the systemic administration of pentylenetetrazole (PTZ) at a dose (20 mg/kg, i.p.) able to induce absence like seizures and loss of responsiveness in rats (Marescaux et al., Epilepsia. 25 (1984) 326-31), and a genetic model represented by the stargazer mutant mice ($stg^{-/-}$) characterized by cerebellar ataxia, head tossing and spontaneous SWDs with frequency of 5-7 Hz (Noebels et al., Epilepsy Res. 7 (1990) 129-35). In the pharmacological model we investigated whether PTZ treatment can affect CB1 expression in the cortico-thalamo-cortical circuit and whether the cannabinoid agonist WIN 55,212-2 (WIN, 5mg/kg, i.p.) can influence PTZ effects on neuronal activation and CB1 mRNA expression in this circuit. Neuronal activation was evaluated by analyzing the mRNA expression of the early gene c-fos. In the genetic model we analysed the expression of CB1 mRNA receptors in those areas involved in the pathophysiology of absence seizures. c-fos and CB1 mRNA levels were evaluated by *in situ* hybridization of brain coronal sections, using [^{35}S]-labelled riboprobes, followed by quantitative densitometric analysis of the autoradiographic films. Data showed that, in the pharmacological model, PTZ treatment caused a significant reduction of CB1 mRNA expression in nRT and Zona Incerta (ZI), leaving the cortex unaltered. Furthermore, PTZ treatment stimulated c-fos mRNA expression in a particular subregion of the cortex, the S1 forelimb cortex (S1FL), unaffecting c-fos expression in the other brain regions considered. When administered alone, WIN did not affect either CB1 or c-fos mRNA expression. However, when administered after PTZ treatment, WIN reverted the PTZ-induced effect on CB1 mRNA expression in the ZI and increased c-fos mRNA expression in all the brain regions considered. In the genetic mouse model, a decrease of CB1 mRNA levels in the nRT and in the ZI, and an increase of CB1 mRNA expression in the cortex of $stg^{-/-}$ were observed, with respect to wild type mice. Based on these observations, we argue that PTZ-induced seizures might be associated to modifications of CB1 expression within the cortico-thalamo-cortical network and that altered CB1 expression in specific brain nuclei can be involved in the pathogenesis of absence seizures in the $stg^{-/-}$. These data, taken together, confirms the involvement of CB1 receptor on the regulation of SWDs in the cortico-thalamo-cortical network, suggesting that CB1 receptor might be targeted for the development of novel anti-absence drugs.

CANNABIDIOL INHIBITS THC-ELICITED PSYCHOSIS AND MEMORY IMPAIRMENTS IN HUMANS

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Community-based studies suggest that cannabis products that are high in Δ9-tetrahydrocannabinol (THC) but low in cannabidiol (CBD) are particularly hazardous for mental health. Laboratory-based studies are ideal for clarifying this issue because THC and CBD can be administered in pure form, under controlled conditions. In a between-subjects design, we tested the hypothesis that pre-treatment with CBD inhibited THC-elicited psychosis and cognitive impairment. Healthy participants were randomised to receive oral CBD 600mg (n=22) or placebo (n=26), 210 minutes ahead of intravenous (IV) THC (1.5mg). Post-THC, clinically significant positive psychotic symptoms were less likely in the group pre-treated with CBD than in the group pretreated with placebo, odds ratio (OR)=0.24 ($\chi 2=4.74$, $p<0.05$). In agreement, post- THC paranoia, as rated with the State-Social-Paranoia-Scale (SSPS), increased in the placebo pre-treated group ($\chi 2=16.0$, $p<0.000$) but not in the CBD pre-treated group ($\chi 2=2.0$, $p=0.37$). Episodic memory, indexed by scores on the Hopkins verbal learning ask-revised (HVLT-R), was poorer, relative to baseline, in the placebo pre-treated group (-10.7±18.9%) but not in the CBD pre-treated group (-0.4%±9.7 %) ($t=2.39$, $p<0.05$). These findings are consistent with an emerging body of evidence suggesting that highTHC/lowCBD cannabis products are associated with increased risks for mental health.

EFFECTS OF CANNABINOIDS AND ENDOCANNABINOID-HYDROLYSIS INHIBITION ON PENTYLENETETRAZOLE-INDUCED SEIZURES AND ELECTROENCEPHALOGRAPHIC ACTIVITY

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The cannabinoid CB1 receptor may restrain excitatory neurotransmission and epileptiform seizures, indicating that this could be a target for new drugs effective against epileptic syndromes. However, it has remained to be investigated which would be the more appropriate strategy, particularly comparing direct agonists (synthetic cannabinoids) with endocannabinoid (anandamide)-hydrolysis inhibitor (FAAH-inhibitor). In this study we tested the effects of the cannabinoids WIN-55-212-2 (non-selective, 0,3-3 mg/kg) and ACEA (CB1-selective, 1-4 mg/kg) as well as the FAAH-inhibitor URB-597 (0,3-3 mg/kg) against pentylenetetrazole (PTZ)-induced myoclonic seizure and electroencephalographic (EEG) activity in Wistar rats. The animals received intraperitoneal drug injections followed by intravenous injection of PTZ, after which seizure and EEG were recorded simultaneously. WIN-55-212-2 and ACEA actually facilitated PTZ-induced seizure and EEG activity. On the contrary, URB-597 inhibited both parameters. These results suggest that increasing anandamide levels, rather than direct activating CB1 receptors, might be a more promising strategy for the treatment of epilepsy and related syndromes.

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ENDOCANNABINOID SIGNALLING AND FEAR EXTINCTION IN MOUSE LINES FOR GLUTAMATERGIC AND GABAERGIC-SPECIFIC RESCUE FROM CB1 RECEPTOR DEFICIENCY

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The endocannabinoid system (ECS) is known to modulate processes such as feeding behaviour, stress responses, seizure susceptibility, anxiety, and extinction of aversive memories. The available pharmacological and genetic approaches have allowed establishing the *necessary* role of the ECS, but have not provided evidence for its *sufficient* role. To be able to investigate the sufficient role of cannabinoid type 1 (CB1) receptor signalling, we applied the Cre/loxP system to generate a mouse line with a silenced CB1 receptor as the default state (Stop-CB1), but with the possibility to rescue the CB1 receptor in a region- and cell type-specific manner.

A loxP-flanked stop cassette in the CB1 receptor gene locus results in global CB1 receptor deletion throughout the body, including the brain. By crossing this mouse line with a mouse line ubiquitously expressing Cre recombinase (EIIa-Cre), the stop cassette is excised and the CB1 receptor rescued at its endogenous sites and levels. Complete rescue of CB1 receptor protein and functionality was confirmed by histological analysis, electrophysiology, and behavioural paradigms. To address the importance of intact CB1 receptor signalling in distinct neuronal subpopulations, the Stop-CB1 line was crossed with Cre-expressing mouse lines to rescue the CB1 receptor selectively in cortical glutamatergic (Glu-CB1-RS) or forebrain GABAergic (GABA-CB1-RS) neurons.

Depending on the behavioural paradigm chosen, a partial rescue of the Stop-CB1 phenotype was observed in Glu-CB1-RS, GABA-CB1-RS, or both. The presence of CB1 receptor on either glutamatergic or GABAergic neurons appeared to be sufficient for a partial rescue of the anxiogenic phenotype of Stop-CB1 mice, whereas almost full rescue of food intake after starvation and of protection against kainic acid-induced seizures was only found in Glu-CB1-RS mice. Glu-CB1-RS mice did not show a rescue of the impaired extinction after cued fear learning, whereas GABA-CB1-RS animals seemed to differ in the acquisition of fear memories. These first results indicate that there may be another dimension to the ECS that can only be unravelled when not only the necessity of proper ECS signalling in different brain regions and cell populations is investigated, but when also the sufficiency of the different components is taken into account.

EXPLORATORY BEHAVIOR AND RESPONSE TO THC IN MOUSE LINES FOR GLUTAMATERGIC AND GABAERGIC-SPECIFIC RESCUE FROM CB1 RECEPTOR DEFICIENCY

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A balanced behavior pattern is essential for survival and requires controlled neuronal activation. During the last two decades, the endocannabinoid (eCB) system has emerged as a potent regulator of neuron firing and the cannabinoid type 1 (CB1) receptor, one of its central components, has predominantly been detected at synapses of inhibitory GABAergic and excitatory glutamatergic neurons. It has been shown that GABAergic and glutamatergic neurons are mandatory for controlled social and object investigation and, additionally, are required for locomotor, hypothermic, analgesic, and cataleptic effects of Delta(9)-tetrahydrocannabinol (THC). So far, corresponding studies have mainly performed on transgenic mice lacking the CB1 receptor either in GABAergic or glutamatergic forebrain neurons.

Two novel genetic mouse models allow for an additional approach to analyzing the importance of specific CB1 receptor subpopulations. By exploiting the Cre/loxP system, the CB1 receptor can be rescued in distinct neuronal populations from knock-out background. The present study was aimed at investigating the impact of CB1 receptor reactivation either in GABAergic (GABA-CB1-RS) or glutamatergic (Glu-CB1-RS) neurons on exploratory drive and THC treatment. As controls, littermates without a CB1 receptor rescue (Stop-CB1) and animals with a ubiquitous receptor rescue (CB1-RS) were used, therefore, mimicking the knock-out and wild-type situation, respectively.

By testing these animals for social and object exploration in three different paradigms, we could show a partial behavioral rescue in GABA-CB1-RS as well as in Glu-CB1-RS. Similarly, THC effects on locomotion, hypothermia, analgesia, and catalepsy seem to depend on both GABAergic and glutamatergic CB1 receptors, respectively. Thus, we corroborate previous findings and present novel functions of the CB1 receptor in these two neuronal populations.

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CANNABIS IN (NORTHERN) CALIFORNIA, 2011-2012: STATISTICS AND TRENDS FROM THOUSANDS OF INDIVIDUAL SAMPLES TESTED FOR 15 CANNABINOIDS AND 8 TERPENOIDS

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Since April, 2011, data has been collected from about 2,000 individual cannabis samples. The testing of these samples has used liquid chromatography with detection by ultraviolet absorption (with photo diode array detector) and quadrupole mass spectrometry. For the vast majority of samples, 15 cannabinoids (D9-THCA, D9-THC, D8-THC, THCA-C4, CBDA, CBD, CBNA, CBN, CBGA, CBG, THCVA, THCV, CBCA, CBC, CBLA) and eight terpenes (alpha-pinene, linalool, limonene, myrcene, terpinolene, phytol, beta-caryophyllene, caryophyllene oxide) have been quantified. In addition to customer-submitted samples, selected growers have participated in "seedling-to-harvest" tracking studies for several important strains, including high THCA, high CBDA, high THCVA. Statistical reduction of this extensive data set is used to draw conclusions from 95% confidence interval ranges of various qualities as a function of sample type and strain name, as well as specific characteristics of selected strain names.

IMPORTANCE OF REFERENCE GENES IN VALIDATING THE EFFECTS OF CANNABIDIOL ON FIBROTIC PROCESSES

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Introduction: Intestinal fibrosis is an observable phenotype in approximately one third of Crohn's Disease (CD) patients. Fibrocytes, a peripheral blood mononuclear cell (PBMC) with both mesenchymal and haemopoietic markers, have been implicated in models of wound healing and fibrosis in which they are recruited to sites of injury and differentiate into fibroblasts, depositing extracellular matrix (ECM). Furthermore, the process of epithelial – mesenchymal transition (EMT), whereby cells of epithelial origin adopt a mesenchymal phenotype has also been implicated with fibrotic diseases. Typically, these cells exhibit a decreased expression of adhesion molecules such as E-cadherin and an increase in mesenchymal markers such as vimentin and α-smooth muscle actin (α-SMA). The aims of this study were to investigate: 1) whether transforming growth factor (TGF)-β1 and unmethylated CpG induced EMT in an intestinal cell model of fibrosis, 2) whether cannabidiol (CBD) could impact on this process, and 3) whether CBD acted on TGF-β1-induced primary human-derived fibrocyte differentiation.

Methods: Primary human PBMCs were extracted with Lymphoprep™, cultured in DMEM with 20 % serum for four days. Non-adherent cells were removed and the resulting population maintained in (TGF)-β1 (10ng/ml) with or without CBD (10mM) for a further 7 days. Caco-2 cells were maintained in standard culture conditions until differentiated. Cells were treated with unmethylated CpG (2.5μM), TGF-β1 (5-10ng/ml) and CBD (10μM) and incubated for 96 hours. TRI-reagent® was used for RNA extraction and qRT-PCR was performed with vimentin (VIM1), α-SMA (ACTA2) and E-cadherin (CDH1) primers, normalised against four housekeeping genes: hydroxymethylbilane synthase (HMBS), beta-2 microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and human RPLPO (large ribosomal protein) (QIAGEN).

Results: CBD (10μM) increased vimentin and E-cadherin expression in Caco-2 cells after 96 hours compared to untreated controls. However, the fold increases were dependent upon the reference gene to which the Cq values were normalised and some replicates only showed increases when normalised against the HMBS reference gene. As expected, TGF-β1 increased vimentin expression and decreased E-cadherin expression in Caco-2 cells. However, this was not consistent with the HMBS reference gene. Similarly, inconsistent effects of CpG stimulation on Caco-2 cells were evident, dependent on the reference gene. In primary human fibrocytes, TGF-β1 increased α-SMA expression, the extent of which was influenced by reference gene. CBD inhibited TGF-β1-induced α-SMA expression when compared to all housekeeping genes except HMBS.

Conclusions: In primary fibrocytes, CBD could potentially play an antifibrotic role. However, its role in EMT is unclear. Upregulation of E-cadherin fits with its role in epithelial permeability and junction restoration, but the vimentin upregulation implies a pro-fibrotic potential, which remains to be fully explained. Finally, thorough investigations using an array of reference genes are required to validate the role of CBD in fibrotic processes.

VOLATILE OIL FROM HIGH POTENCY *CANNABIS SATIVA* WITH *IN VITRO* BINDING AFFINITY FOR HUMAN CANNABINOID RECEPTORS

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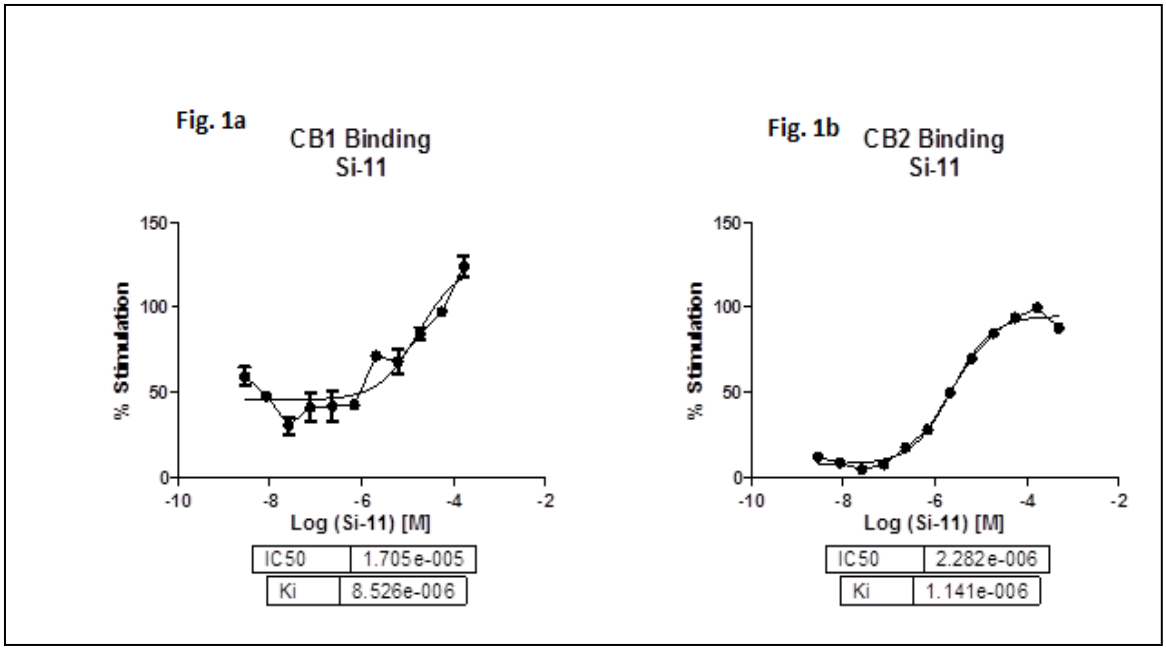
As part of our program to investigate the pharmacological activities of high potency *Cannabis sativa* constituents, its volatile oil displayed strong affinity to human CB1 and CB2 receptors (> 95 %) at 10 ug/mL. Bioassay guided fractionation of freshly prepared volatile oil on both Si gel and Sephadex LH-20 column chromatography led to the isolation of 16 fractions form each column. Each column fraction was tested for their CB1 and CB2 receptor binding affinity followed by GC/FID and GC/MS analysis. Fractions which contained THC showed prominent affinity as expected. However, some non-THC containing fractions (**Si-11, Si-15-16 and L-6**) showed moderate binding affinity to CB1 receptors with K_i values of 8.53, 6.3 and 6.45 µM, respectively, and strong CB2 receptor affinity with K_i values of 1.14 , 2.40 and 4.55 µM, respectively. **Figures 1a** and **1b** show the binding curves for **Si-11** to CB1 and CB2 human receptors respectively. Extensive GC/MS analysis of these fractions to identify the major



components will be carried out followed by testing these components in the CB1 and CB2 binding assay to identify the active compound(s).

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ALTERATIONS OF THEORY OF MIND NETWORK ACTIVATION IN CHRONIC CANNABIS USERS

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Chronic cannabis use is associated with cognitive impairment and has been identified as a risk factor for schizophrenia. Patients with schizophrenia show profound deficits in social cognition such as the ability to attribute mental states to others, referred to as "theory of mind" (ToM). Aberrant activation of the ToM network has been demonstrated across different phases of schizophrenia, including at-risk stages.

Accordingly, we aimed to investigate the ToM network in chronic cannabis users. Fifteen chronic cannabis users received functional brain imaging during performance of a ToM cartoon story task. Findings were compared with 14 non-using control subjects. Cannabis users showed less activation in the left parahippocampal gyrus, the right precuneus and cuneus, but greater activation in the left cuneus and the right anterior cingulate gyrus compared to healthy controls. These activation patterns resemble those found in at-risk populations, suggesting that cannabis use can affect the processing of social information similar to other risk factor constellations for psychosis.

Acknowledgements: This work was supported by a research grant of the Medical Faculty of the Ruhr-University Bochum (FoRUM F622-2008).

CANNABIS USE IN PREGNANCY AND BREASTFEEDING

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Introduction: Cannabis use in pregnancy and breastfeeding is a controversial topic, due to the illegality of cannabis and fear of harm to the fetus. Few studies have been conducted that control for all other medications and drugs. Many studies have shown maternal stress causes structural and functional changes to occur in the fetus, including: impaired learning and coping abilities, depressive-like behavior, pre-term births and low birth weight. Caesarean section is associated with risks of postoperative adhesions, infections and blood loss. Children delivered by caesarean are at risk for Wet Lung, early delivery, and complications. Breastfeeding protects children from a host of conditions, including respiratory illness, allergies, asthma, eczema, SIDS, and childhood lymphoma. Breastfed infants develop higher IQ's, and improved brain and nervous system development. Reducing maternal stress, vaginal birth, and breastfeeding have positive benefits for both mother and neonate.
Aim: To estimate the effect of maternal cannabis use on pregnancy outcomes, desire and ability to breastfeed and effects of maternal cannabis exposure on neonates.
Design: Ethnographic field studies and a questionnaire were employed. A regular and heavy using group experimental group compared to a non-cannabis using control group.
Setting: Participants lived in Humboldt, Sonoma and Mendocino California.
Participants: 45 Northern California women who used cannabis regularly and their cannabis exposed children, and 31 women who did not use cannabis and their non-exposed children. Confounding factors were controlled, such as mother's age, socio-economic factors, relationship status, pre-pregnancy weight, and the self-reported use of tobacco, alcohol, caffeine, other medications and illicit drug use.
Measurements: A questionnaire and in-depth interviews were used to gather information on a wide range of pregnancy and breastfeeding outcomes.
Findings: The experimental group reported fewer serious pregnancy complications, such as preeclampsia, premature delivery, gestational diabetes, and low fetal birth weight. Complications such as back pain, carpal tunnel syndrome, nausea, insomnia, constipation, GERD, hemorrhoids, lower abdominal pain, varicose veins, and stretch marks were just as likely to occur in either group. Experimental mothers gained less weight and lost pregnancy weight significantly faster than the control. Stress was reduced and controlled much better. They were 8 times as likely to breastfeed through pregnancy, tandem nurse, and nursed significantly longer, most more than a year. The experimental group had a lower frequency of cesarean, induced delivery, epidural or other pain control in birth. The majority had natural, vaginal births. Regular users did not experience breastfeeding complications of inadequate milk supply or infant latch issues. 2 mothers developed mastitis, but were able to continue breastfeeding and did not require antibiotics. Experimental mothers reported greater satisfaction with their pregnancy and birth experience. No learning disabilities or major health issues were reported in the experimental group. Experimental infants' weight, height, Apgar scores, developmental milestones and general health were normal.

EFFECT OF CANNABINOIDS (WIN55,212-2) IN ACCELERATION OF WOUND HEALING IN BONE CELL MONOLAYER

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Despite the ongoing political debate regarding the legality of medical marijuana, clinical investigations of the therapeutic use of cannabinoids are now more prevalent than at any time in history. Cannabinoids have been shown to have analgesic, anti-spasmodic, anti-convulsant, anti-tremor, anti-psychotic, anti-inflammatory, anti-oxidant, anti-emetic and appetite-stimulant properties. There are mainly two well known cannabinoid receptors, CB_1 and CB_2, located in the central (CB1) and peripheral (CB2) nervous systems as well as the immune system. More recently, endocannabinoids (ligands) and their receptors have also been found in the skeleton which appear as the main body system and physiologically regulated by CB_2. The purpose of this investigation was to study the rate of wound healing using a scratch assay wound model created on MG63 bone cell-line monolayer and also to investigate proliferation and migration with and without the presence of CB1 and CB2 non-selective receptor agonist WIN55, 212-2. Wounds were made (average scratch width of 300μm±10-30μm SD, 1.7-5μm SEM) on confluent monolayers. After wounding, culture flasks were treated with the synthetic cannabinoid (WIN 55,212-2) diluted in ethanol with concentrations of 500nM, 1μM and 2μM, and compared to non-treated controls and ethanol only. It was found that addition of 1μM synthetic cannabinoid closed the wound completely after 25 hours whereas the control showed no sign of complete wound closure even after 30 hours with ~20% of wound still remained open. Results were then compared with cannabinoid additions to HaCaT cells (Human Keratinocytes monolayer) with only minor effect on wound closure. The rate of wound closure was found to be higher with cannabinoid additions to MG-63 bone cell monolayers. These findings suggest the potential use of synthetic cannabinoid (WIN 55,212-2) for achieving complete wound closure at a faster rate.

EFFECT OF CB_2 SELECTIVE AGONIST ON WOUNDED BONE CELL MONOLAYERS

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More recently, endocannabinoids and their receptors have been found in the skeleton which appears to be physiologically regulated by CB_2 receptor. Previous findings indicated non-selective CB1 and CB2 receptor agonist (WIN55,212-2) accelerated wound healing of MG-63 bone cell monolayers. The purpose of this investigation was to identify which receptor contributes to the process of wound closure with higher acceleration. This was achieved by blocking with either CB1 or CB2 antagonist. Also, in this study CB2 receptor agonist HU308 was used on wounded MG-63 bone cell monolayers to investigate proliferation and migration as compared to WIN55, 212-2 additions using the same scratch assay wound model. It was found that addition of 500nM of CB2 selective agonist (HU308) increased wound healing with 90% of the wound closed after 20 hours whereas the control showed no sign of complete wound closure even after 30 hours. Results indicated that cannabinoids act mainly via CB2 receptors in wounded MG-63 bone cell monolayers. These findings indicate the potential use of cannabinoids in bone repair.

THE EFFECT OF DIFFERENT CONCENTRATIONS OF CANNABINOID (WIN 55,212-2) ON WOUND HEALING OF CHONDROCYTE MONOLAYER

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Numbers of studies have been conducted to highlight the anti-inflammatory and immunosuppressive properties of cannabinoids and their potentials for prevention of cartilage degradation and repair have also been explored. Various wound healing techniques can be used to investigate the mechanisms of chondrocyte repair in monolayer or 3 dimensional tissues. In this study, the effect of two different concentrations (500nM and 1 μM) of synthetic CB1 and CB2 receptor agonist WIN55, 212-2 (WIN- 2) on the wound healing of chondrocyte monolayers using a simple scratch assay wound model have been investigated. DMSO and ethanol were used as solvents and it was found that DMSO decreased the rate of wound closure for chondrocyte monolayers. On the other hand, using absolute ethanol at a concentration of 1μM was found to greatly increase both migration and proliferation of chondrocytes cultured in a chondrogenic media with an increase in the wound closure rate. Further increase in the rate of wound closure was observed by treating the cells with 500nM of the CB1 antagonist LY-320,135. These findings suggest the potential use of the synthetic cannabinoid for improving the rate of chondrocyte monolayer wound closure, which could be used to enhance cartilage repair.

THE EFFECT OF DIFFERENT CONCENTRATIONS OF THE SYNTHETIC CANNABINOIDS WIN 55,212-2, URB602 AND HU-308 ON WOUND HEALING OF CHONDROCYTE MONOLAYER

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Our previous study showed that the rate of wound closure for chondrocyte monolayer was increased by treating the chondrocytes with 1μM of the non-specific CB1 and CB2 agonist (WIN-2). In this study, the roles of different cannabinoid agonists on the rate of chondrocyte wound closure have been investigated. Specifically, the effect of different concentrations of WIN55, 212-2 (WIN-2), CB1 receptor agonist URB602 and CB2 receptor agonist HU-308 with and without their antagonists on the wound healing of chondrocyte monolayers using a simple scratch assay wound model have been investigated. These synthetic cannabinoids were found to increase the rate of wound healing of chondrocyte monolayers at different rates as compared to controls. WIN55, 212-2 at a concentration of 1μM was found to increase both migration and proliferation of chondrocytes cultured in a chondrogenic media, with increase in the wound closure at the highest rate amongst the three cannabinoids used in work. It was also found that treating the cells with 2μM of any of the cannabinoids lead to a significant decrease in cell proliferation and the rate of wound closure. These findings indicate the potential use of the specific and synthetic cannabinoids for improving the rate of wound closure which could be used to enhance cartilage repair.

PROTEOMIC IDENTIFICATION OF THE RAT CANNABINOID CB2 RECEPTOR USING MASS SPECTROMETRY AND IMMUNOBLOTTING

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Expression of cannabinoid CB2 receptor protein has often been measured with immunohistochemistry and Immunoblotting, employing unvalidated CB2 directed antibodies. We therefore used an approach, combining Western blot with mass spectrometry to detect and identify CB2 receptor protein in overexpressing cells, and to develop a validated protocol for measuring CB2 expression with a commonly used antibody. We report the detection and identification of the rat CB2 receptor in overexpressing CHO cells at 37 kDa, the weight expected for unmodified rat CB2. A band was observed at this weight in spleen tissue, in addition to two bands with higher molecular weights (44 and 59 kDa), but not in spinal cord tissue where a single intense band was detected at 44 kDa. It has recently been suggested that CB2 may be an intracellular entity rather than plasma membrane bound as previously assumed. We therefore assessed the subcellular localization of the CB2 receptor by a differential extraction of cytosolic and membrane proteins. We report that in spleen tissue a membrane enrichment and cytosol exclusion resulted in an increased intensity of the 37 kDa band, which has been confirmed as CB2 receptor protein in CHO cells. In spinal tissue a very faint 37 kDa band was detected only in the membrane fraction. This finding suggests that the CB2 receptor is associated with the plasma membrane and not localized in the cytosol as recently suggested. The 44 kDa band has been used in several publications to assess CB2 expression, but our results suggest that the abolition of the 44 and 59 kDa spleen bands and the 44 kDa spinal band with cytosol exclusion indicates that these bands represent unspecified intracellular proteins and not CB2.

Acknowledgements: Funded by the Marsden Fund of New Zealand. We would like to thank Torsten Kleffmann for his assistance with mass spectrometry.

BRAIN REGIONAL DESENSITIZATION OF CANNABINOID CB1 RECEPTOR SIGNALING IN MICE WITH GLOBAL GENETIC KNOCKOUT OF MONOACYLGLYCEROL LIPASE

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Monoacylglycerol lipase (MAGL) is a serine hydrolase mainly responsible for the termination of 2-arachidonoylglycerol (2-AG) signaling in the central nervous system. Previous studies indicate that both genetic and pharmacological inactivation of MAGL result in massive 2-AG accumulation in brain tissue and desensitization of cannabinoid CB1 receptors (CB1R), leading to functional and behavioral tolerance.

Previously, brain region-specific desensitization of CB1R-$G_{i/o}$ signaling axis has been observed in mice with chronic JZL184 administration but it is not known whether similar changes take place in brains of global MAGL knockout animals. In this study, functional autoradiography was used to explore this issue in detail by analyzing basal as well as CP55,940-stimulated, CB1R-dependent $G_{i/o}$ protein activity in multiple brain regions of MAGL knockout mice in comparison to their wild-type littermates. In addition, we assessed the capacity of the broadly-acting serine hydrolase inhibitor methylarachidonoylfluorophosphonate (MAFP) to elevate brain tissue levels of 2-AG to boost CB1R activity in various brain regions. Tissue and buffer contents of 2-AG and anandamide (AEA) were analyzed using LC/MS/MS.

The results of these studies will be presented in this meeting.

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CANNABINOID RECEPTOR 1 REGULATES PROTEIN SYNTHESIS AND TRANSLATION IN HUMAN SKELETAL MUSCLE

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The endocannabinoid system plays an important role in health and disease, but its role in skeletal muscle protein synthesis and translation remains to be defined. We investigated the effects of single resistance exercise (RE) bout with or without whey protein ingestion on cannabinoid receptor 1 (CB1R) expression and protein synthesis in skeletal muscle, and further aimed to establish the mechanism by which the CB1R modulates protein synthesis and translation in skeletal muscle cells. Previously untrained young men were randomized into protein (n = 9) or placebo (n = 9) groups. Protein (15 g of whey) or non-energetic placebo was ingested before and after a single RE bout. Vastus lateralis (VL) muscle biopsies were taken before and 1 h and 48 h after a leg press of 5 x 10 repetitions. Cultured skeletal muscle cells were exposed to CB1R agonist (WIN-55,212-2) and antagonist (AM251), and the resulting effects on protein synthesis/translation machinery were investigated.

The results showed that CB1R protein levels increased significantly at 1 hour post-exercise, and were dramatically decreased at 48 hours post-exercise, the effects being greater with protein ingestion. Changes in CB1R expression was associated with p70(S6K) and phosphorylated eukaryotic initiation factor 4E binding protein 1 (4E-BP1) but not with mammalian target of rapamycin (mTOR). Consistently, WIN-55,212-2 alone had no effect, while AM251 treatment induced extracellular signal-regulated kinase (ERK1/2) and 4E-BP1 phosphorylation independent of mTOR activation. These results provide evidence that signaling via CB1R can significantly modulate protein synthesis and translation in skeletal muscle in response to RE and protein ingestion through ERK ½ pathway independent of mTOR.

CB_1 AND CB_2 ANTAGONIST SIGNALING IN OSTEOBLASTS

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In MC3T3-E1 osteoblastic cells, the CB_1 antagonist, AM251, has been reported to induce increases in Runx2 mRNA, mineralized bone nodule formation, and activation of signaling molecules such as ERK and AKT (Wu et al. Bone 49 (2011) 1255-63). Studies from our lab characterizing mice in which both CB_1 and CB_2 receptors were inactivated by homologous recombination have demonstrated increased bone mass coupled with enhanced osteoblast differentiation of bone marrow stromal cells in culture (manuscript in preparation). We explored the effect of antagonizing CB_1 and CB_2 cannabinoid receptors in osteoblastic cells to gain insights into molecular pathways that may help to explain the effects of the endocannabinoid system (ECS) in bone development.

Our data was generated by running time course experiments with MC3T3-E1 cells under the influence of SR141716A, SR144528 or both in combination. The cells were harvested with a lysis buffer at specific time points and analyzed by western blot analysis. Quantification of protein activation was calculated using LiCor imaging equipment and software. Within 15 minutes, treatment with the CB_1 receptor antagonist SR141716A resulted in several fold increases in pERK, pSMAD158, and pAKT. SR144528, a CB_2 receptor antagonist, caused increases in pERK and pSMAD158, but not pAKT. When both antagonists were applied together, pERK and pSMAD158 levels increased, while pAKT signaling was diminished compared to SR141716A alone. The finding that cannabinoid receptor antagonists alter the activity of the SMAD158 complex is a novel finding, which suggests that cannabinoids can influence bone morphogenic signaling pathways, and therefore play a significant role in osteoblast differentiation and function.

The results of our investigation suggest dynamic signaling events occur during CB_1 and CB_2 receptor antagonism, which can alter the activity of ERK, AKT, SMADs, and potentially affecting Runx2 expression. MC3T3-E1 cells express all the genetic information necessary for the ECS, but their precise roles remain to be defined. The coupling of certain G-proteins in bone may be specific to this tissue and different from other organs or cell types. Hence the effect of CB_1 and CB_2 antagonism in osteoblastic cells may be distinct from that of a neuron or another cell type within the body, due to differences in receptor expression, ligand production, G-protein coupling, and the role of the ECS within a specific environment.

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SIGNALLING TRAFFICKING AT THE CANNABINOID CB_1 RECEPTOR BY THE CB_1 ALLOSTERIC MODULATOR, ORG 27569

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Org 27569, an allosteric modulator of the cannabinoid CB_1 receptor (Price et al, 2005), increases the binding affinity of agonists for CB_1 receptors but reduces the signalling efficacy of these receptors. It has been suggested that allosteric modulators can have differential effects on various signalling pathways (Zhang et al, 2005). The aim of the present study was to investigate how Org 27569, affects different signalling pathways linked to the CB_1 receptor. The effect of Org 27569 on CP55940- or WIN55212-2-induced CB_1 receptor signalling was investigated using the [^{35}S]GTPγS binding assay in mouse brain membranes and cyclic AMP, beta arrestin and pERK assays in CB_1 transfected cells. Table 1 summarises the effect of 100nM Org 27569 on maximal responses to CP55940 and WIN55212-2 (E_{max}) in all of these assays.

Table 1: E_{max} values with 95% confidence limits for the effect of Org 27569 on the CB_1 receptor agonists, CP55940 and WIN55212-2, in each assay type.

Assay used	Agonist	Vehicle/modulator	E_{max} (95% C.L)
GTPγS binding	CP55940	DMSO	61 % (47 – 75)
		100nM Org 27569	18 % (14 – 22)
	WIN55212-2	DMSO	98 % (90 – 107)
		100nM Org 27569	90 % (75 – 105)
Beta-Arrestin	CP55940	DMSO	99 % (95 – 103)
		100nM Org 27569	-
	WIN55212-2	DMSO	84 % (70 – 97)
		100nM Org 27569	3.4 % (0.3 – 7)
Cyclic AMP	CP55940	DMSO	87 % (75 – 97)
		100nM Org 27569	-
	WIN55212-2	DMSO	79 % (64 – 93)
		100nM Org 27569	65 % (51 – 78)
pERK	CP55940	DMSO	50 % (44 – 56)
		100nM Org 27569	72 % (64 – 80)
	WIN55212-2	DMSO	40 % (27 – 54)
		100nM Org 27569	51 % (35 – 66)

Responses to CP55940 and WIN55212-2 are affected differently by Org 27569 in some assays. In the [^{35}S]GTPγS binding, cyclic AMP and ß arestin assay, Org 27569 is more potent as an inhibitor of CP55940 than WIN55212, Org 25769 reduces the E_{max} of CP55940 at lower concentrations than are required to affect WIN55212. In the pERK assay Org 27569 fails to inhibit either agonist.

These data provide evidence that when the allosteric modulator, Org 27569, binds to the cannabinoid CB_1 receptor, it preferentially impacts signalling through some pathways and not others. This suggests that allosteric modulators could be used to alter a desired or undesired response to receptor activation without affecting others.

Price, M.R *et al*. (2005) *Mol Pharmacol* 68, 1484-1495
Zhang, Y *et al*. (2005) *J. Pharmacol Exp Ther* 315 (3), 1212-1219

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LEELAMINE, A NOVEL DITERPENE, EXHIBITS CANNABIMIMETIC EFFECTS IN CB_1 RECEPTOR KNOCKOUT MICE

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While searching for new ligands for the cannabinoid$_1$ (CB_1) receptor, we discovered that leelamine (Fig. 1) displayed a weak affinity for the rat brain CB_1 receptor. We tested leelamine for cannabinoid-like effects in biochemical and behavioural paradigms.

Leelamine displayed a Ki of ~5 μM at the transfected human CB_1 receptor using [^{3}H]SR141716A. Similar results were obtained using rat brain homogenates and [^{3}H]HU-243 as the radioligand. Unlike typical cannabinoid receptor agonists, leelamine failed to stimulate G-protein activity as measured by the binding of [^{35}S]GTPγS to rat cerebellar and striatal homogenates.

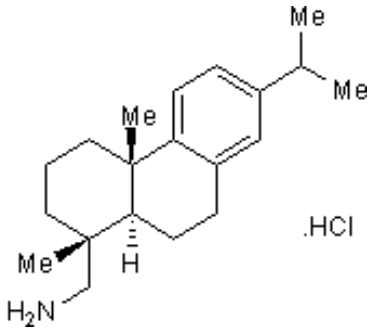

Fig. 1 Structure of leelamine

Leelamine (3-125mg/kg i.p.) was tested in the cannabinoid tetrad tests using C57 mice (25-40g). Leelamine was more potent and efficacious than Δ-9-THC at inhibiting spontaneous motor activity and decreasing rectal temperature, but less potent and efficacious than Δ-9-THC in the ring immobility and tail-flick tests. The CB_1 receptor antagonist SR141716A was able to reverse the behavioural effects of leelamine, but not as potently or efficaciously as its reversal of THC's effects. When the tetrad tests were performed using C57-$CB_1^{-/-}$ knockout mice, leelamine was almost as efficacious in all tests as in wild-type C57 mice; Δ-9-THC was relatively inactive in CB_1 knockout mice, except for inhibiting spontaneous activity.

It was investigated whether leelamine is present in brain using LC-MS technology. Leelamine was not found in rat or porcine brain using assays with detection limits of 0.5-2 fmol.

Lastly, we are investigating the binding of [^{3}H]leelamine to CB_1 receptor knockout mouse brain membranes. The kinetics of binding are unusual. More [^{3}H]leelamine is usually bound when 1-2 μM of unlabelled leelamine is present as compared with vehicle alone. A competition binding curve bottoms out with 200-300μM of unlabelled leelamine present, with an IC_{50} around 50 μM. Binding of [^{3}H]leelamine was inhibited by both $CaCl_2$ and $MgCl_2$ in a concentration-dependent manner. 300 μM of rosiglitazone (a PPARγ agonist) failed to inhibit the binding of [^{3}H]leelamine to CB_1 receptor knockout mouse brain membranes, whereas 300 μM of CP55,940 inhibited specific binding by about 25%. Displaceable binding of [^{3}H]leelamine by unlabelled leelamine was also observed in rat liver and heart.

It is currently unknown if the behavioural effects of leelamine are mediated via a receptor, an ion channel, a transporter, or perhaps an enzyme.

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EARLY AND LATE PHASE ERK RESPONSES REVEAL A NOVEL FUNCTIONALLY SELECTIVE AGONIST PROFILE AT CANNABINOID TYPE 1 RECEPTORS

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The cannabinoid type 1 (CB_1) receptor is a seven-transmembrane G protein-coupled receptor (GPCR), which is known to activate a number of intracellular signalling pathways, including the phosphorylation of mitogen-activated protein (MAP) kinases. Activation of these pathways via the CB_1 receptor can occur in an agonist-dependent manner, including the activation of specific MAP kinases.

In the present study, we compare the activation of the MAP kinase extracellular signal-regulated kinase (ERK) 1/2 signalling pathway by several structurally distinct cannabinoid receptor agonists using the In-Cell Western assay technique. Chinese Hamster Ovary (CHO) cells stably transfected with the human CB_1 receptor were cultured in 96-well plates for 2 days prior to experimentation. Cells were then treated with stated concentrations of the cannabinoid agonists CP 55,940, WIN 55,212-2 and methanandamide over a time course of 6 hours.

All three agonists (10 μM) produced an initial ERK response with a rapid on- and off-rate (2 - 10 min, peak 4 min) of similar magnitude (~250% of basal phospho-ERK). CP produced a second, late-phase ERK response (peak 60 – 90 min) of approximately half the magnitude of the initial response (~180 % of basal phospho-ERK), which returned to near basal levels after 6 hours (fig.1). In complete contrast, WIN produced a significant sustained decrease in phosphorylated ERK with a distinct profile from the CP response, reaching a plateau at 4 hours (~35% basal phospho-ERK) (fig.1). Methanandamide appeared to lack any second phase response (fig. 1). Both phases of the CP response were concentration-dependent; however, the compound was markedly less potent in provoking the second phase response compared with the initial phase response.

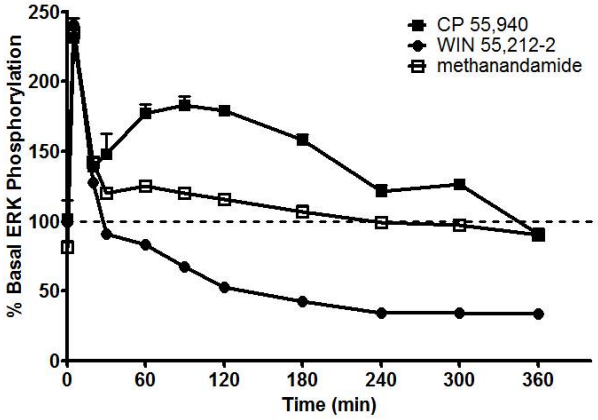

Fig 1. Effect of cannabinoid agonists on ERK 1/2 phosphorylation in CHO-hCB_1 cells.

In conclusion, CP and WIN exhibit agonist bias when comparing late and early-phase ERK responses, providing an interesting example of functional selectivity at the CB_1 receptor. These profiles may have important implications in relation to agonist-directed downstream functional responses.

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BETA-ARRESTIN1 MEDIATION OF THE EFFECTS OF CANNABINOID LIGANDS

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Primarily from *in vitro* studies, beta-arrestin1 and 2 have been reported to couple G-protein couple receptors (GPCRs) causing: 1) uncoupling from G-proteins, 2) internalization of GPCRs, and/or 3) activation of non-G-protein-mediated signaling pathways. To determine the role of the beta-arrestins, knock-out mice for each were administered THC, CP55940 or vehicle and tested for antinociceptive effects (via latency to tail withdrawal from a 53ºC water bath) and rectal temperature after both single doses and chronic daily administration. *In vitro* studies utilized brain membrane preparations from drug-naïve mice and examined receptor density via [^{3}H]SR141716A saturation analysis or agonist-mediated activation of G-proteins via [^{35}S]GTPγS binding.

In *in vivo* studies, CP55940 produced greater overall antinociception and temperature depression in beta-arrestin1+/+ (wild-type) mice than in beta-arrestin1-/- mice. There was no difference between the genotypes in their response to THC. *In vitro* receptor binding to cortex membrane preparations from drug-naïve mice indicated no difference in CB_1 density between beta-arrestin1+/+ and -/-. However, in brain membranes from beta-arrestin1-/- mice, CP55940 efficacy for stimulating [^{35}S]GTPγS binding was increased by 40% (compared to beta-arrestin1+/+). Chronic treatments with THC, CP55940 or vehicle showed that tolerance did develop to the acute effects of these drugs, but that deletion of beta-arrestin1 had no effect on the development of tolerance.

Thus, beta-arrestin1 appears to mediate some of the acute in vivo effects of CP55940, but not THC, and is not essential for the development of tolerance. It appears that the presence of beta-arrestin1 suppresses G-protein activation. Thus, the observed effects of CP55940 may be mediated to a large degree by beta-arrestin1, rather than G-proteins.

EVIDENCE FOR A FUNCTIONAL ROLE OF CB1 CANNABINOID RECEPTORS IN THE MAMMALIAN CONE PATHWAY

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We have previously shown that many components of the cannabinoid signaling system are present in the vertebrate retina. CB_1 cannabinoid receptors and MGL are present in both rod and cone photoreceptor terminals whereas DGLa, the principal likely source of 2-AG in the outer retina is chiefly expressed in a specific subpopulation of cone bipolar cells. This arrangement suggests that Type I OFF cone bipolar cells participate in a retrograde cannabinoid signaling circuit with cone photoreceptors. Our previous finding that calcium currents in cone photoreceptors are inhibited by CB1 activation suggests a specific model of cannabinoid signaling. To investigate this further we used a combination of electroretinograms (ERGs) in assorted cannabinoid mutants along with endocannabinoid measurements under specific lighting conditions.

ERG waveforms of WT, CB_1-/- and MGL-/- showed a- and b-waves that increased in amplitude with flash intensity. For the 171 (P) cd.s/m^2 test flash, the average a-wave implicit time of MGL-/- was 12.8 ms and shorter than those of WT (19 ms, $p<0.0013$) and CB_1-/- (21.8 ms, $p<0.00079$). The average b-wave implicit time of CB_1-/- was 87.3 ms and delayed relative to WT (54.3 ms, $p<0.00037$) and MGL-/- (32ms, $p<0.000072$). The b-wave of CB_1-/- also took a longer time to recover as indicated by the ½ band width of 129 ms relative to those of the WT (37 ms) and MGL -/- (27 ms). The b-wave of MGL-/- was followed by a large negative potential. The a- and b-wave amplitudes were not significantly different for the 3 groups. However, the intensity response function of the b-wave amplitude indicated a smaller semi-saturation constant [14 (P) cd.s/m^2] with a slope of 1.75 for CB1 -/- relative to WT [67 (P) cd.s/m^2, slope 0.83] and MGL -/- [20 (P) cd.s/m^2, slope 2.1]. We additionally found that 2-AG levels were much higher under dark-adapted conditions and that exposure to light dramatically reduced levels of retinal 2-AG.

The significant light-dependent reduction in 2-AG levels taken together with the distinct genotype-specific differences in the photopic flash ERG responses of CB1-/- and MGL-/- transgenic mice suggest that cannabinoids are well-placed to play an active role at the first synapse of the visual system.

CALCIUM MOBILIZATION AND MAPK ACTIVITY VIA GPR18

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Since the discovery of GPR18, elucidation of the pharmacology of this cannabinoid receptor candidate has been sluggish. Previous studies have shown that GPR18 is activated by N-arachidonoyl glycine, (NAGly), abnormal cannabidiol (AbnCbd) and the cannabidiol analog, GPR55 agonist, O-1602 in migration as well as in MAPK assays (McHugh et al., BMC Neurosci. (2010) 11:44; Console-Bram et al., Soc. Neurosci Abst. (2011) 38.17; McHugh et al., BJP (2012) 165:2414). With the identification of NAGly as an endogenous ligand for GPR18, and the finding that NAGly activation of GPR18 mediates increases in intracellular calcium concentration (Kohno et al., Biochem Biophs Res. Comm 347 (2006) 827—832), we investigated calcium mobilization by several GPR18 agonists. In addition, examination into the inhibition of intracellular calcium increases by ligands reported to attenuate GPR18 activity was also accomplished. Findings from these studies indicate that the "AbnCbd receptor" antagonist O-1918 does not antagonize AbnCbd, NAGly, nor O-1602 mediated increases in MAPK in HEK293-GPR18 cells. Rather an increase in MAPK activity in HEK293-GPR18 cells was demonstrated in the presence of O-1918. In addition, concentration dependent increases in intracellular calcium observed following activation of GPR18 in HEK293-GPR18 cells by NAGly, AbnCbd, and O-1602 were also demonstrated in the presence of O-1918. Together these findings indicate that O-1918 behaves as an agonist at GPR18 in MAPK and calcium mobilization assays. Results presented herein suggest the possibility that O-1918 is a biased ligand

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THE IMPORTANCE OF THE CB2 HOMODIMER TO THE CB2 CATALYZED ACTIVATION OF Gi PROTEIN

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In previous work, we have used molecular dynamics simulations to study the activation of the CB2 receptor by the endogenous ligand, 2-AG via the lipid bilayer (Hurst et al., *J. Biol. Chem.* 285 (2010) 17954-17964). In order to study the next step in the CB2 signaling cascade, we have used a combination of cysteine and homobifuctional cross-linking studies along with molecular modeling to determine the nature and geometry of the CB2/Gi protein signaling complex. Using a homobifuctional Lys-Lys linker, disuccinimidyl suberate, we identified two crosslinks between CB2 and Gi protein: (1) Gi: K349 (i-5 C term Gα / CB2: K6.35(245); and, (2) Gi: K317 ($\alpha 4\beta 6$ loop region G-alpha)/ CB2: K5.64 (215). In addition, using $HgCl_2$, we identified a disulfide bridge between Gi: C351 (i-3 C term Gα)/ CB2: C3.53(134). This data was then used in modeling studies to identify the orientation of Gi protein at CB2. Our model suggested that all three crosslinks can be achieved at the same time only by a dimeric CB2 complex. Substituted cysteine cross-linking experiments, then showed that in the absence of $HgCl_2$, an A6.60(270)C mutant spontaneously can form a CB2 dimer. In the presence of $HgCl_2$, a H6.57(267)C mutant also could form a CB2 dimer. These experiments suggest that A6.60 and H6.57 are part of the CB2 dimer interface. Modeling studies showed that both the Gi/CB2 and CB2/CB2 data could be satisfied by a dimeric complex that uses a TMH5/TMH6 interface (Singh et al., ICRS 2011). This result is consistent with the x-ray crystal structure of the chemokine CXCR4 receptor (Wu et al., *Science* 330 (2010) 1066-1071).

The next step in the CB2 activation process involves the coupling of the Gi protein to an activated CB2 and the subsequent release of GDP via the opening of the helical and Ras domains of Gα-i (Eps et al., *PNAS* 108(23) (2011) 9420-9424). To probe the structural determinants for this next step, we undertook NPT NAMD (Phillips et al., *J. Comp. Chem.* 26 (2005) 1781-1802) molecular dynamics simulations of the 2-AG/CB2R/R*/Gi protein complex in a fully hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer. The CHARMM27 (MacKerell et al., *J. Phys. Chem .B* 102 (1998) 3586-3616) and CHARMM36 (Klauda et al., *J. Phys. Chem .B* 114(23) (2010) 7830-7843) force fields were implemented for proteins and lipids respectively. The system was equilibrated with backbone and sidechain restraints by warming to 310K in 10K increments. After each increment step, 500steps minimization was performed followed by 20ps of dynamics run. At 310K, the restraints were slowly released by bringing the force constants to 0 in 6 steps of 50ps each. Initial NAMD production runs (150 ns) suggest a correlation between the degree of insertion of the C-terminus of Gα-i into the intracellular domain of R* in the CB2 R/R* complex and the distance between the helical and Ras domains of Gα-i (R90-E238) (Eps et al., *PNAS* 108(23) (2011) 9420-9424) These preliminary results suggest the next phase of CB2 catalyzed Gi protein activation. [Support: DA011551 (ZHS) and DA003934 and DA021358 (PHR)].

TCTEX1 - A PUTATIVE CB2 RECEPTOR REGULATING PROTEIN

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Introduction: Tctex1 is a dynein-light chain and belongs to the dynein motor complex responsible for transporting cargo-proteins along microtubules. Importantly, it binds e.g. to „classical" class A GPCR rhodopsin (Tai et al., Cell. 97 (1999) 877-87) or intracellular G-beta proteins (Sachdev et al., EMBO J. 26 (2007) 2621-32). As a binding motif of tctex1 the R/KR/KXXR/K sequence has been identified. Because human CB2 receptors have a KKCVR motif at the C-terminus, we studied a possible interaction of both proteins.

Methods: To test our hypothesis we transiently over-expressed CB2 receptors in HEK293 cells and studied a direct interaction of tctex1 and CB2 receptors using co-immunoprecipitation (co-IP). Additionally, we performed in vitro pull-down experiments using the C-terminal region of the CB2 receptor containing the putative binding domain fused to glutathion-sulfotransferase (GST). The expression levels of CB2 receptors and tctex1 were analysed by Western blot technique using protein specific and anti-flag and anti-myc antibodies. To study a possible regulatory effect of tctex1 on CB2 receptor signalling we also analysed Erk 1/2 phosphorylation after ligand stimulation using Western blot and the LI-COR Odyssey system.

Results: We could not observe a direct interaction of both proteins using pull-down and co-IP experiments but our results demonstrated a strong down regulation of tctex1 protein when co-expressed with CB2 receptors. This down regulation was dose dependent and independent of the promoter used in the expression construct. Cells with a high CB2 receptor expression show only weak tctex1 levels and vice versa. This result is also of interest and in line with former experiments showing that tctex1 mRNA levels were highly up-regulated in CB1/CB2 double knockout tissues (Karsak et al., Science. 316 (2007) 1494-7). To test whether the amount of tctex1 influences CB2 receptor signalling we quantified the levels of Erk 1/2 phosphorylation after HU-210 stimulation (100 nM) in cells transfected with increasing amounts of tctex1 (0.2 µg – 2 µg) together with a constant amount of CB2 receptor (1 µg) plasmid. Our results showed that tctex1 co-transfection indeed modulates Erk 1/2 phosphorylation levels after CB2 receptor stimulation by HU-210, which could be blocked by the CB2 receptor antagonist AM630.

Summary: We identified tctex1 as a CB2 receptor regulating protein, which probably is not directly interacting but which modulates the efficacy on CB2 receptor signalling. Interestingly, we observed this regulation although the tctex1 protein expression is strongly down-regulated by CB2 receptor co-expression.

THE CB2-PREFERRING AGONIST JWH015 ALSO POTENTLY AND EFFICACIOUSLY ACTIVATES CB1 IN AUTAPTIC HIPPOCAMPAL NEURONS

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The G protein coupled receptors CB_1 and CB_2 are targets for the psychoactive constituents of cannabis, chief among them Δ^9-THC. They are also key components of the multifunctional endogenous cannabinoid signaling system. CB_1 and CB_2 receptors modulate a wide variety of physiological systems including analgesia, memory, mood, reward, appetite and immunity. Identification and characterization of selective CB_1 and CB_2 receptor agonists and antagonists will facilitate understanding the precise physiological and pathophysiological roles of cannabinoid receptors in these systems. This is particularly necessary in the case of CB_2 because these receptors are sparsely expressed and problematic to detect using traditional immunocytochemical approaches.
1-propyl-2-methyl-3-(1-naphthoyl)indole (JWH015) is an aminoalkylindole that has been employed as a "CB_2-selective" agonist in more than 40 published papers. However, we have found that JWH015 potently and efficaciously activates CB_1 receptors in neurons. Using murine autaptic hippocampal neurons, which express CB_1, but not CB_2 receptors, we find that JWH015 inhibits excitatory postsynaptic currents with an EC50 of 216 nM. JWH015 inhibition is absent in neurons from CB_1-/- cultures and is reversed by the CB_1 antagonist, SR141716 [200nM]. Furthermore, JWH015 partially occludes CB_1-mediated DSE (~35% remaining), an action reversed by the CB_2 antagonist, AM630 [1 and 3μM], suggesting that high concentrations of AM630 also antagonize CB_1 receptors.

We conclude that while JWH015 is a CB_2-preferring agonist, it also activates CB_1 receptors at experimentally encountered concentrations. Thus, CB_1 agonism of JWH015 needs to be considered in the design and interpretation of experiments that use JWH015 to probe CB_2-signaling.

PUTATIVE CANNABINOID RECEPTOR GPR18 IS PRODUCED IN RAW 264.7 MACROPHAGE CELLS

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Macrophage cells play a critical role in immunity and thus macrophages represent an important cellular tool for studying human disease. Macrophages digest pathogens, present antigens in response to pathogen presence, and produce monokines (e.g. TNFα, IL1) that play a wide variety of roles in cellular functioning and immune response. In particular, macrophages are important with respect to vascular disease. They are known to undergo a phenotypic switch in the presence of excessive cholesterol, becoming "foam cells" that embed in the walls of blood vessels, causing atherosclerosis (Howell et al., 2011). An understanding of macrophage activity is vital when considering suitable therapies for such diseases.

Recently, macrophages have been important targets in the growing field of lipidomics. Due to their versatile nature and importance in immune response, macrophages present an ideal cell type for generating a lipid map of molecular signaling molecules. Perusal of LIPID MAPS, a consortium for lipidomics publications, reveals that RAW 264.7 macrophage cells (RAW cells) are frequently used in this effort.

Cannabinoids represent a subset of lipids that serve an important role in immune functioning in mammals. RAW cells are known to express the traditional cannabinoid receptor CB_2 but not CB_1 (Jeon et al., 1996). The so-called "abnormal cannabidiol receptor" GPR18 was initially reported in BV2 murine microglial cells (McHugh et al., 2010). As microglial cells are the brain's analog to somatic macrophage cells, we tested the hypothesis that GPR18 would also be present in macrophages. Using an immunohistochemical staining technique with a monoclonal antibody to GPR18, we demonstrate the robust presence of GPR18 in RAW cell membranes, whereas immunohistochemical staining of C6 Glioma cells resulted in negligible detection of GPR18, suggesting that microglia and macrophage cells continue to share phenotypic similarities that are not present in other cell types.

The exogenous cannabinoid Δ^9-tetrahydrocannabinol (THC) is recognized as a full agonist for GPR18, as is *N*-arachidonyl glycine (NAGly), a lipid that is structurally similar to the endocannabinoid anandamide (AEA) (McHugh et al., 2012). Previously, NAGly was shown to be generated by AEA in RAW cells (Bradshaw et al., 2009), which is further evidence that these macrophage cells are regulated by endogenous cannabinoids and therefore have the potential to be regulated by phytocannabinoids as well. GPR18 is emerging as a cannabimimetic therapeutic target in humans, and its presence in both microglial and peripheral macrophage cells underscores this potential.

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N-ARACHIDONOYL GLYCINE ACTIVATION OF p44/42 MAPK IN HUMAN ENDOMETRIAL CARCINOMA CELLS

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Recently, we have shown that *N*-arachidonoyl glycine (NAGly) is synthesized in uterine tissue and its levels vary across the hormonal cycle (Bradshaw and Allard, 2011). In addition, we have demonstrated that human endometrial carcinoma (HEC-1B) cells migrate in response to anandamide, delta-9-tetrahydrocannabinol, and NAGly through CB_1-independent GPR18-dependent mechanisms (McHugh et al., 2011). NAGly was found to be more potent than both anandamide and delta-9-tetrahydrocannabinol at inducing endometrial cell migration. Additionally, the same study showed that NAGly is more potent than anandamide at activating p44/42 MAPK through GPR18. As the p44/42 MAPK pathway is associated with cell proliferation and migration, this study investigated NAGly's effect upon the activation of p44/42 MAPK in HEC-1B cells. Using in-cell Western assays, we determined the relative amount of p44/42 MAPK activation in HEC-1B cells challenged by different concentrations of NAGly. Additionally, the time-dependency of p44/42 MAPK activation by NAGly in HEC-1B cells was analyzed by incubating the cells with NAGly at different time points. Experimental technique was confirmed through assays conducted on HEK293 GPR18 cells incubated with NAGly as well as estradiol as a known p44/42 MAPK activator in HEC-1B cells (Keshamouni et al., 2002).

In all experiments, activation of p44/42 MAPK increased with increasing concentrations of NAGly. HEC-1B cells incubated with estradiol for 5 minutes had a more robust p44/42 MAPK activation than HEC-1B cells incubated with the same concentrations (10 nM – 10 μM) of NAGly for 5 minutes. However, NAGly-induced p44/42 MAPK activation was 4 fold higher with 10 minute incubation. After 20 minutes, p44/42 MAPK activation was negligible for these same NAGly concentrations. It is concluded that NAGly drives HEC-1B p44/42 MAPK activation both in a concentration- and time-dependent manner. The time-dependency of NAGly on p44/42 activation in this study closely mimics the rapid inactivation of p44/42 during stimulation of CB_1 (Daigle et al., 2007). Thus, receptor desensitization and/or cell metabolism may dictate NAGly's MAPK signaling action in endometrial tissue.

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TUNING CANNABINOID RECEPTOR SIGNALING BY RGS12 (REGULATORS OF G-PROTEIN SIGNALING) PROTEIN

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RGS proteins are classically known for turning off Gi and Gq-coupled receptor signaling. Agonist-directed differential signaling via preferential coupling with Gi subtypes is known for CB1 and CB2 cannabinoid receptors but very little is known about how these signals are regulated in cannabinoid receptor signalosomes. In the current study we investigate differential role of RGS12 in the regulation of CB1 vs. CB2 receptor signaling.

Previously we showed that CB1 and CB2 receptors activation inhibits adenylyl cyclase and stimulates nitric oxide synthase in a pertussis-toxin sensitive manner. In the current study using real-time PCR, we screened for native expression of individual RGS proteins in CB1 and CB2 receptor expressing neuronal and endothelial cells lines. We found that RGS12 protein is present in both N18TG2 and EAhy926 cells. We then selectively knock down (using SiRNA) RGS12 proteins in N18TG2 and EAhy926 cells and measured CB1/CB2 cannabinoid receptor-mediated cAMP and nitric oxide production.

Our preliminary findings shows that knocking down RGS12 protein results differential effect on CB1/CB2 mediated nitric oxide production and inhibition of forskolin-stimulated cAMP production. RGS12 knockdown significantly increases CB2 receptor-mediated nitric oxide production but does not alter CB1 receptor-mediated nitric oxide production. On the contrary, RGS12 knockdown produce larger inhibition of CB1 receptor mediated forskolin-stimulated cAMP production compared to CB2 receptor-mediated inhibition of cAMP production. These results showed for the first time that CB1/CB2 receptor mediated different signaling pathways are differentially regulated by RGS12 protein and therefore can be targeted to dissect those signaling.

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SEX DEPENDENT BEHAVIORAL AND MOLECULAR EFFECTS OF THC AND MDMA IN AN ANIMAL MODEL OF ADOLESCENT DRUG CONSUMPTION

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Ecstasy is a club drug with psychostimulant properties that is usually consumed in the week-end in combination with other drugs of abuse, mostly cannabis. Although cannabis and psychostimulants (apart from cocaine) are mainly consumed by adolescents, only a few studies have analyzed their long-lasting effects after a chronic treatment during adolescence. Long-term physiological and behavioral consequences of a chronic treatment with cannabis (delta-9-tetrahydrocannabinol, THC) and an animal model of week-end consumption of ecstasy (3,4-methylenedioxymethamphetamine, MDMA) during adolescence were analyzed in male and female Wistar rats. Animals received increasing doses of THC (2.5, 5 and 10 mg/kg i.p.) from postnatal day (pnd) 28 to 45 together with two daily doses of MDMA (10 mg/kg s.c.) every 5 days from pnd 30. Animals were exposed to the holeboard (HB) and the elevated plus maze (EPM) one day after the end of the treatment (pnd 46), and to the novel object test (NOT) and the prepulse inhibition of the startle response (PPI) at adulthood (pnd 75 and 84, respectively). In addition, Arc and pERK1/2 expression were measured in hippocampus and prefrontal cortex as well as the expression of prepro-orexin in hypothalamic mRNA pools in adult animals (pnd 87-91).

MDMA induced a reduction in directed exploration in the HB and an increase of time of exploration of the open-arms in the EPM, maybe indicative of increased impulsivity. In the long-term, a severe disruption in cognitive function has been observed due to the THC treatment in female animals, but not in males. Adolescent exposure to MDMA induced a disruption in PPI at the highest intensity of prepulse studied (80 dB) whereas only its combination with the THC treatment induced a decrease of the percentage of PPI at 75 dB. Arc expression was reduced in the hippocampus due to the chronic treatment with THC although it only reached statistical significance in THC+MDMA females. Both THC and MDMA induced a reduction of pERK1/2 expression in the prefrontal cortex which was significant in THC+Sal and THC+MDMA males as well as in THC+Sal females when compared to their corresponding control groups. THC+Sal males showed a reduction of prepro-orexin mRNA expression in the hypothalamus when compared with control male animals while no effects were observed among females. The present results show long-term sex-dependent psychophysiological effects of adolescent exposure to THC and MDMA and highlight the potential deleterious impact of their combined consumption in adolescents.

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PHARMACOKINETIC AND PHARMACODYNAMIC EFFECTS OF SUPRA-THERAPEUTIC Δ^9-THC DOSES IN CANNABIS USERS

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The purpose of the present study was to characterize the pharmacokinetic and pharmacodynamic effects of oral Δ^9-THC in cannabis users at doses higher than those tested previously in humans. Dose escalation procedures were used to assess increasingly larger doses of Δ^9-THC, up to a maximum of 90 mg or until side effects occurred that contraindicated the administration of higher doses. We hypothesized that peak Δ^9-THC concentrations and time would vary considerably within and across subjects; that Δ^9-THC would have a flat dose-effect function on cannabinoid-sensitive measures; and that Δ^9-THC would be well tolerated in cannabis users. Seven subjects who reported at least weekly cannabis use participated in session activities from 1600-2300 h and remained in the hospital overnight; sessions were separated by at least 72 h. Data were collected prior to, and hourly for 6 h following drug administration. Blood was drawn at these times, and also 12 h post-dose. Five subjects received all doses, and two subjects were discontinued after experiencing adverse GI effects. Data were analyzed using linear mixed models. Plasma Δ^9-THC concentrations were generally a function of dose, but a wide range of C_{max} values were observed across subjects for a given dose (e.g., 9.0 vs. 127.1 ng/mL at 90 mg). Peak plasma levels for both cannabinoids emerged at 4 h post-dose on average, but T_{max} ranged from 1-12 h. The behavioral effects of Δ^9-THC increased as a function of dose and blood concentration at low doses, but were not consistently related to dose or blood concentration at higher doses. Further, peak abuse-related self-reported effects occurred at low Δ^9-THC plasma concentrations. These data demonstrate the variable pharmacokinetic profile of oral Δ^9-THC, are consistent with a partial agonist classification, and demonstrate that acute doses of Δ^9-THC higher than those tested previously can be safely administered to cannabis users.

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ABSTRACT WITHDRAWN

MATERNAL IMMUNE ACTIVATION DISRUPTS THE FUNCTIONALITY OF MESOLIMBIC DOPAMINE NEURONS: INVOLVEMENT OF CANNABINOIDS

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Exposure to maternal immune products is believed a prenatal risk factor for the development of severe neuropsychiatric disorders like psychoses (Boksa, Brain Behav Immun, 24(2010):881-97). On the other hand, several human and animal studies consider *Cannabis* consumption during the adolescence to increase from two to five-fold the odds to be affected by schizophrenia or major depression in adulthood (Rubino et al., J Psychopharmacol, 26(2012):177-88). The possibility that these two factors interact in the development of psychotic-like dysfunctions is still unclear, and this interaction might induce detrimental (i.e. synergistic) effects. Furthermore, beside the investigation of anatomical, molecular and behavioral aberrations triggered by the exposure to either maternal immune products or cannabis, whether these factors might induce abnormal functioning on ventral tegmental area dopamine (VTA DA) neurons is yet unknown. Since VTA DA neurons play a prominent role in the pathophysiology of psychoses, in this study we asked whether these pre-and postnatal risk factors affect the normal VTA DA neuronal activity and functionality.

To this aim, we carried out in vivo single cell extracellular electrophysiological recordings in adult anaesthetized rats, which were divided into experimental groups according to the prenatal treatment. To mimic the exposure to maternal immune products we administered the proinflammatory cytokine inductor polyriboinosilic-polyribocitidylic acid (poly I:C, 4mg/kg iv) to pregnant dams at gestational day 14 and to mimic a model of adolescent cannabis consumption we treated adolescent rats (from postnatal day 45 to 55) with increasing doses of the active principle of *Cannabis* Δ^9 tetrahydrocannabinol (THC) (2.5-10 mg/kg i.p.).

Rats exposed in utero to Poly I:C (Poly I:C rats) showed reduced mean frequency (n=109; Student's t-test; $p<0.01$ vs. controls) and number of spontaneously active VTA DA neurons (n=19; Student's t-test; $p<0.01$ vs. controls). No differences were detected in other parameters such as the percent of spikes in bursts and coefficient of variation (CV) (Student's t-test; $p>0.05$ vs. controls). In contrast, the number of active VTA DA neurons and their mean frequency were not altered in rats, which underwent only THC treatment in adolescence (Student's t-test; $p>0.05$ vs. controls). However, analysis of both percent of spikes in burst and CV revealed a reduction in THC exposed animals compared to controls (n=96; Student's t-test; $p<0.05$). Interestingly, co-exposure to prenatal Poly I:C and adolescent THC normalized rather than further disrupted several electrophysiological parameters. Hence, THC treatment normalized to control values the number of spontaneously active VTA DA neurons (n=10; two-way ANOVA and Tukey's test; $p<0.05$ vs. Poly I:C) and restored the reduction of firing rate observed in Poly I:C exposed rats (n=105; two-way ANOVA and Tukey's test).

Overall, our findings provide the first electrophysiological evidence of disrupted activity and functionality of DA neurons following exposure to pre- and postnatal risk factors predictive of psychoses. Dysfunctions in structures projecting to VTA DA neurons (e.g. prefrontal cortex) and an imbalance of GABA and glutamate release to DA cells by either up-regulation or down-regulation of cannabinoid receptor (e.g. CB1) might be taken into account to explain Poly I:C- and/or THC-induced aberrations.

CHANGES IN CONCENTRATION OF ENDOCANNABINOIDS AND N-ACYL ETHANOLAMIDES IN BRAIN STRUCTURES OF RATS UNDERWENT COCAINE SELF-ADMINISTRATION AND ITS WITHDRAWAL

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Some recent preclinical reports indicate that naturally occurring lipid signaling molecules derived from arachidonic acid may alter reward and seeking behavior related to abused drug [Adamczyk et al., Pharmacol Rep. 2009; Arnold, Pharmacol Biochem Behav 2005; Melis et al., J Neurosci. 2008] These endogenous molecules include endocannabinoids, such as anandamide (AEA) or 2-arachidonoylglycerol (2-AG), and N-acyl ethanolamides including N-oleoylethanoloamine (OEA) and N-palmitoylethanoloamine (PEA). AEA and 2-AG are ligands for cannabinoid receptors while OEA and PEA activate peroxisome proliferator-activated receptors alpha. By substrate competition, N-acyl ethanolamides reduce AEA metabolism while PEA also inhibits rate of AEA metabolism by suppression of the fatty acid amide hydrolase (an enzyme blocking the degradation of AEA, OEA and PEA) expression, too [Fowler, Brain Res Brain Res Rev. 2003; Vandevoorde et al. J Med Chem. 2003]

The aim of this project was to evaluate the changes on the tissue levels of AEA, 2-AG, OEA and PEA in several brain regions in rats underwent cocaine self-administration and extinction training. We used a triad-yoked procedure to separate motivation vs. pharmacological actions of cocaine.

Male Wistar rats (280–300 g) were trained to self-administered cocaine (0.5 mg/kg/infusion); some groups of rats underwent 10-day extinction training also [Frankowska M. et al. Pharmacol Rep. 2009]. After completion of behavioral experiments the following brain structures were isolated: the nucleus accumbens (NAC), dorsal striatum (STR), prefrontal cortex (PFC), frontal cortex (FC), hippocampus (HIP) and cerebellum (CER). The concentrations of AEA, 2-AG, OEA and PEA were evaluated with using LC/MS-MS.
We found a statistically significant ($p<0.05$) decrease of the AEA level in the FC and CER in animals self-administered cocaine while yoked cocaine controls showed decreases in the NAC and CER. Moreover, during maintenance of cocaine self-administration, a reduction in the 2-AG level was seen in the STR and HIP and an increase in the CER while animals passively administered cocaine displayed an increase in the 2-AG level in the HIP and FC. Following 10-day extinction, there was a potent decrease in the AEA level in almost all limbic and subcortical areas in rats previously self-administered cocaine; less potent decreases in these brain areas were seen in the "yoked" cocaine group. During extinction, the level of 2-AG either increased (in the NAC and PFC of rats self-administered cocaine, or in the FC in the "yoked" cocaine group) or decreased (in the STR and CER of rats self-administered cocaine, or in the CER in the "yoked" cocaine group). Moreover, a potent increase ($p<0.001$) in levels of OEA and PEA was observed in the PFC and HIP during both maintenance and extinction while a decrease in the STR was noted in the extinction phase only.

To summarize, the present findings support a role for endocannabinoids and endogenous N-acyl ethanolamines to control reward and seeking behavior related to cocaine in rats. Further studies focusing on other endocannabinoids/endovanilloids as well as CB1 receptor proteins allow to better explain the role of these system in the mechanism of drug addiction.

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CANNABIS POTENCY IN NEW SOUTH WALES AUSTRALIA: AN ANALYSIS OF POLICE SEIZED STREET SAMPLES

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There is concern in many countries that contemporary cannabis cultivation is biased towards producing plants with high THC and negligible CBD content. Consumption of high THC/low CBD cannabis strains may predispose users towards greater adverse psychiatric and cognitive effects than consumption of moderate THC or balanced THC/CBD strains. While excellent studies of cannabis potency have been published from the UK, the Netherlands and the USA, there are no equivalent data from Australia, which has one of the highest rates of international cannabis use. This study therefore analysed 207 cannabis seizures made by police from urban and rural locations in New South Wales (NSW) under the NSW Cannabis Cautioning Scheme. Based on the methods of Theunis L et al (2009) (J. Chromatography B, 877: 4115-24), cannabinoids were extracted from a portion of dried flowering heads (200 mg) using methanol/chloroform, and quantified using HPLC with UV detection relative to standards (GW Pharm, UK) for THC, THCA, CBD, CBDA, CBG, CBGA, THCV, CBC and CBN.

Results showed a high mean THC+THC-A content (w/w%) of 1.92+13.96 = **15.88%** and a very low mean CBD+CBD-A content of 0.08 + 0.08 = **0.16%.** Other cannabinoids featuring at low levels included THC-V (**0.05%),** CBC (**0.06%)** and CBN (**0.07%).** CBG and CBG-A content were at intermediate levels of **0.79%** and **0.24%** respectively. Correlational analysis showed a significant clustering of CBD-A, CBD and THC-V content across samples and a second cluster of THC, CBN, CBC and (negatively) THC-A. Urban seizures tended to have higher THC content and lower CBD content than rural seizures. Ongoing work is now examining "known provenance" seizures where police have confiscated cannabis from known hydroponic manufacturing facilities or known bush plantations. We hypothesise that hydroponically grown cannabis will have higher THC and lower CBD levels than bush grown cannabis. At this stage, our results confirm international data revealing the dominance of THC over CBD in Australian street cannabis, most likely reflecting the widespread consumption of "skunk" and "hydro" in users.

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DUAL FAAH/MAGL INHIBITION ATTENUATES SOMATIC SIGNS OF SPONTANEOUS MORPHINE WITHDRAWAL IN ICR MICE

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Our lab has recently reported that elevation of the endocannabinoids arachidonoylethanolamide (AEA) or 2-arachidonylglycerol (2-AG), through inhibition of their respective hydrolytic enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), reduces naloxone-precipitated withdrawal signs in morphine-dependent mice. The FAAH inhibitor PF-3845 (10 mg/kg) produced a partial attenuation of somatic withdrawal signs (e.g., jumping and paw flutters), but did not reduce diarrhea or weight loss. In contrast, a high dose of the MAGL inhibitor JZL184 (40 mg/kg) elicited powerful anti-withdrawal effects, though it also produced a subset of cannabimimetic effects (i.e., catalepsy and hypothermia).

In the present study, we tested whether dual FAAH and MAGL inhibition would block morphine withdrawal, with minimal cannabimimetic effects. Male ICR mice were implanted subcutaneously with a 75 mg morphine pellet to be rendered opioid dependent. At 72 h the pellets were removed to evoke spontaneous withdrawal and endocannabinoid catabolic enzyme inhibitors were administered 1 h later. Mice were assessed for 15 min every 2 h following pellet removal for paw flutters, head shakes, platform jumping, the presence of diarrhea, and changes to body weight. Combination of low dose of JZL184 (4mg/kg) and a high dose of the FAAH inhibitor PF-3845 (10 mg/kg) significantly blocked spontaneous withdrawal signs in morphine-dependent mice, but did not produce THC-like side effects. Additionally, we tested whether the novel dual FAAH-MAGL inhibitor SA-57 (1.25, 2.5, or 5 mg/kg), which possesses greater potency at inhibiting FAAH than MAGL, would attenuate somatic withdrawal signs. SA-57 (2.5 and 5 mg/kg) significantly reduced jumping behavior at 4, 6 and 8 h, total number of paw flutters, and expression of diarrhea. Additionally, the high dose (5 mg/kg) reduced the total number of head shakes and loss of body weight. These doses did not produce cannabimimetic effects in the tetrad. All doses of SA-57 resulted in maximal elevation of AEA (approximately 10-fold). In addition, this compound significantly raised 2-AG levels approximately 3-fold at 2.5 mg/kg, 6-fold at 5 mg/kg, and 10-fold at 12.5 mg/kg.

The results of the present study suggest that the dual FAAH-MAGL inhibitor SA-57 is a promising treatment for opioid withdrawal. Future studies will evaluate its ability to maintain anti-withdrawal effects following sub-chronic treatment and additionally assess its efficacy at attenuating affective aspects of opioid withdrawal as well as opioid self administration.

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ROLE OF ΔFOSB IN CB_1R DESENSITIZATION IN THE BASAL GANGLIA

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Chronic administration of Δ^9-tetrahydrocannabinol (THC) produces tolerance to the in vivo effects of THC and desensitization and downregulation of CB_1 receptors (CB_1Rs). Previous studies in our laboratory and others have shown that the magnitude of desensitization and downregulation differs across brain regions, with a high magnitude of adaptation in the hippocampus and lower magnitude in the caudate-putamen and its projection areas (globus pallidus and substantia nigra). Similarly, tolerance to antinociception and hypothermia develops at lower THC treatment doses than tolerance to catalepsy and hypolocomotion. More recently, our laboratory found that there is a brain region specific inverse correlation between induction of the stable transcription factor ΔFosB and CB_1R desensitization. This result led to the hypothesis that ΔFosB might contribute to reduced desensitization in areas like the caudate-putamen and its projection areas and attenuate the development of tolerance to catalepsy and hypolocomotion that involve CB_1Rs in these regions. To test this hypothesis, mice overexpressing ΔFosB in the D1/dynorphin medium spiny neurons of the striatum (nucleus accumbens and caudate-putamen) under the control of the tetracycline promoter were treated with THC or vehicle and assessed. With this model, mice overexpress ΔFosB in the absence of doxycycline. Therefore mice were either maintained on doxycycline (100 μg/ml) (On-Dox) throughout the experiment as controls or taken off doxycycline (Off-Dox) to overexpress ΔFosB for 8 weeks before injections. Mice were then treated with THC (b.i.d) for 6.5 days (10-30-60 mg/kg with dose increasing every two days) or vehicle (1:1:18; ethanol:emulphor:saline) producing four conditions: On-Dox/vehicle, On-Dox/THC, Off-Dox/vehicle, Off-Dox/THC. One group (n = 10/condition) of mice was sacrificed 24 hours after the last injection, and brain sections were processed for CP55,940-stimulated [^{35}S]GTPγS autoradiography to determine whether CB_1R desensitization occurred in the caudate-putamen and its projection regions. Another group (n = 8/condition) was tested for antinociception, hypothermia and catalepsy using a cumulative within-session dosing paradigm, and locomotor activity was measured with a single dose of THC. Results showed that significant CB_1R desensitization occurred in ΔFosB overexpressing and control mice in all regions tested. However, there was significantly less CB_1R desensitization in the substantia nigra of ΔFosB overexpressing mice (47% On-Dox vs. 27%, Off-Dox, $p < 0.01$). Significant tolerance developed for both THC-treated groups in the measures tested. These results suggest that ΔFosB expression attenuates CB_1R desensitization because overexpression of ΔFosB in D1R/dynorphin neurons of the caudate-putamen, which project to the substantia nigra, reduced desensitization in that region.

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EFFECTS OF CANNABINOIDS ON THE DEVELOPMENT OF CHICK EMBRYOS *IN OVO*

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We have examined the effects of the synthetic cannabinoids HU 210 and HU 211, the plant-derived cannabidiol and the endogenous cannabinoid anandamide on the viability and development of chick embryos.

Methods: Fertilized White Leghorn chicken eggs were injected with the test compounds or carrier vehicle, via a drilled small hole in the blunt end of the egg, directly into the egg yolk. After nine days of exposure, the embryonal viability and development stages were assessed according to the Hamburger and Hamilton (HH) scale.

Results: The potent synthetic cannabinoid receptor agonist HU 210 and the non-psychotropic cannabidiol were embryotoxic at the highest concentrations examined (10 μM and 50 μM, respectively), with no viable embryos (average HH stage 19 ± 3.5 compared to 34 ± 1.1 in the DMSO-treated control group) after the HU 210 injection, and 22% viability (average HH stage 11 ± 4.9) after cannabidiol injections.
The effects of HU 210 on the chick embryo were attenuated by 100 μM of the antioxidant α-tocopherol and the cannabinoid receptor antagonist AM251 (1 μM), whereas only α-tocopherol gave a statistically significant protection against the embryotoxic effects of cannabidiol.
HU 211, an enantiomer to HU 210 without cannabinoid receptor activity, and anandamide were without any significant effects on the viability and development of the chick embryo at the concentrations examined (up to 10 μM and 50 μM, respectively).

Conclusion: This study shows that exposure to, both plant-derived and synthetic, cannabinoids during early embryonal development decrease embryonal viability. Extrapolation of data across species is of course difficult, but the data would argue against the use of cannabinoids, be it recreationally or therapeutically, during pregnancy.

TARGETED LIPIDOMICS PROFILING OF INJURED RAT BRAIN: POSSIBLE IMPLICATIONS FOR NICOTINE DEPENDENCE

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Traumatic brain injury (TBI) is the leading cause of death in the young age group and the most commonly identified cause of epilepsy in adult populations older than 35 years. At present, there are no effective drugs to treat brain injury, although it is well established that the injury triggers both the accumulation of harmful mediators that lead to secondary damage and the initiation of neuroprotective processes (see Mechoulam and Shohami, *Mol. Neurobiol*. 2007 for review). The role of the endocannabinoid system in neuroprotection is well established. It was reported that cannabinoid receptor agonists protect cultured rat hippocampal neurons from neurotoxicity, and several groups reported enhanced levels of anandamide (AEA) after acute injury. Moreover, in response to TBI there is local and transient accumulation of 2-arachidonoylglycerol (2-AG) at the site of injury, peaking at 4 h and sustained up to at least 24 h (see Shohami et al., *Br. J. Pharmacol.* 2011 for review). Furthermore, very recently, Naqvi and co-workers (*Science,* 2011) found that cigarette smokers presenting with TBI, with damage at the level of the insula, experience a cessation of smoking. Given the reinforcing role of endocannabinoids and CB_1 receptors in nicotine self-administration, it is possible that TBI is accompanied by reduced endocannabinoid levels in the insula. The aim of this study was, therefore, to investigate further the alterations of endocannabinoid levels in a model of rat TBI. We also aimed at discovering new endocannabinoid molecules possibly involved in this process through the use of very sensitive and specific "targeted lipidomics" methods involving high resolution LC-ESI-IT-ToF (Liquid Chromatography-ElectroSpray Ionization-Ion Trap-Time of Flight).

Rats underwent TBI using the lateral fluid percussion model (LFP) and were decapitated one day after injury. The prefrontal cortex, hippocampus and insular cortex, both ipsi- and contra-lateral to the injury were dissected, and lipids extracted. In these areas, which were not directly affected by TBI, levels of AEA and other *N*-acylethanolamines as well as 2-AG were measured by liquid chromatography mass spectrometry. In addition, we developed new methods for the simultaneous identification and quantification in tissues of low (< 1 pmol) amounts of *N*-acylglycines, *N*-acyldopamines and monoacylglycerols.

Surprisingly, TBI led to decreased 2-AG and/or AEA levels, also in contralateral brain areas. On the other hand, we observed that the levels of two *N*-acylethanolamines with neuroprotective and anti-inflammatory properties, *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA), tended to increase only in ipsilateral areas. Finally, we identified *N*-oleoylglycine in the prefrontal cortex and hippocampus of the injured hemisphere. The intriguing possibility that the reduced levels of endocannabinoids following TBI might be also accompanied by decreased nicotine self-administration, and that *N*-oleoylglycine, OEA and PEA might instead play a role in neuroprotection is currently being investigated.

CANNABINOID RECEPTORS: GENE STRUCTURES, SNPS, CNVS, CPG ISLANDS MICRORNA REGULATION AND VARIATION IN NEUROPSYCHIATRIC DISORDERS

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Smoking marijuana, phytocannabinoids, synthetic cannabinoids, and the naturally occurring endocannabinoids (eCBs) in mammals activate two well-characterized cannabinoid receptors-(CBRs), CB1Rs and CB2Rs. Many studies have shown that *CNR1* and *FAAH* SNPs may contribute to drug addiction, depression, eating disorders, schizophrenia, and multiple sclerosis. Previous investigations have defined a number of features of the *CNR1* gene's structure, regulation and variation. However, there are still controversies over the CNS functional neuronal characterization and expression of the *CNR2* gene. Nevertheless, our studies have provided the first evidence for neuronal CNS effects of CB2Rs and its possible role in drug addiction, eating disorders, psychosis, depression and autism spectrum disorders (ASDs. We have continued studies on cannabinoid genomic variants in humans and animal models. The *CNR1* and *CNR2* genes are in human chromosome 6q15 and 1p36.11 respectively. Although *CNR1* gene has more CPG islands than *CNR2* gene, both have CPG islands less than 300 bases, but they may be regulated by DNA methylation. MicroRNA binding to the 3′ untranlated region of the *CNR1* gene with two polyadenylation site may also potentially regulate CB1R expression.

CNR1 gene has 4 exons and there are 135 SNPs reported in more than 1% of the population with no common SNP that changes amino acids of CB1R currently known or reported. A copy number variant (CNV) which is 19.5kb found in 4 out of 2026 people covers exons 3 and 4 and codes amino acid that could alter the expression of CB1Rs. *CNR2* on the other hand also has 4 exons with CB2A with 3 exons and CB2B with 2 exons; and there are about 100 SNPs found in more than 1% of the population, which include common cSNPs that change amino acids of the CB2R, including R63Q, Q66R and H316Y. CNVs in Asian and Yoruba population have been reported. We report that there is association between polymorphisms of *CNR2* gene and psychosis, eating disorders, depression and alcoholics in the human populations investigated. The ubiquitous CBRs - probably the most abundant binding sites in the CNS- are known to be involved in a number of neuropsychiatric disturbances and have become major targets of investigation for their impact in neuropsychiatry. Therefore understanding the CBR genomic structure, it's polymorphic nature, subtype specificity, their variants and associated regulatory elements that confer vulnerabilities to a number of health disturbances may unravel the underlining mechanisms.

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ACUTE INTOXICATIONS FOLLOWING CONSUMPTION OF HERBAL PRODUCTS CONTAINING SYNTHETIC CANNABINOIDS

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Introduction. Products from *Cannabis sativa* have long been used by humans for medical and recreational purposes. The psychoactive ingredient in these products is Δ^9-tetrahydrocannabinol (Δ^9-THC), and its primary target in the brain is the G-protein coupled CB_1 cannabinoid receptor (Pertwee et al., Pharmacol Rev 62: 588–631, 2010). The recreational use of Δ^9-THC and products from *Cannabis sativa* is prohibited in most countries. In order to circumvent this prohibition, synthetic cannabinoids were recently introduced to the drug market: harmless herbal mixtures for smoking ("Spice", "Yucatan Fire", "Sence", 'Smoke'…) are "enriched" with such synthetic cannabinoids. Our aim was to characterize intoxications following consumption of herbal mixtures containing synthetic cannabinoids. We also studied the effect of one of the synthetic cannabinoids added to the herbal mixtures on synaptic transmission in brain slices.

Methods. The patients were treated in emergency departments after recreational use of herbal mixtures enriched with synthetic cannnabinoids. Their clinical reports were evaluated and synthetic cannabinoids in their blood and urine were analytically determined. For studying synaptic transmission, GABAergic spontaneous inhibitory synaptic currents (sIPSCs) were recorded in Purkinje cells in mouse cerebellar slices with patch-clamp techniques (see Kovacs et al., Brit J Pharmacol 162: 974–988, 2011).

Results. Twenty nine patients were included in the study, and the following synthetic cannabinoids were detected in their blood: CP-47,497-C8 (1 patient), JWH-018 (4 patients), JWH-081 (4 patients), JWH-122 (9 patients) and JWH-210 (11 patients). The following intoxication symptoms were observed: tachycardia, hypertension, chest pain, myoclonia, seizure, rhabdomyolysis, agitation, restlessness, hallucination, acute psychosis, vomiting, minor elevation of blood glucose and hypokalemia.

In brain slices, JWH-018 (0.1 and 1 μM) had no effects on the amplitude, frequency and cumulative amplitude of sIPSCs recorded in cerebellar Purkinje cells. JWH-018 (5 μM) did not change the amplitude of sIPSCs, but significantly decreased the frequency (by 39 ± 8 %) and cumulative amplitude (50 ± 8 %) of sIPSCs. The effects of JWH-018 (5 μM) on synaptic transmission were very similar to the effects of the reference cannabinoid receptor agonist WIN55212-2 (5 μM).

Conclusions. Many of the intoxication symptoms are also observed after intake of high doses of *Cannabis*. The symptoms agitation, seizures, hypertension, emesis and hypokalemia seem to be characteristic to the synthetic cannabinoids. Since the synthetic cannabinoids are high affinity and high efficacy agonists of the CB_1 receptor, these effects are probably due to a strong stimulation of the CB_1 receptors. The pattern of effects of JWH-018 on sIPSCs indicate that JWH-018 inhibited GABAergic synaptic transmission with a presynaptic mechanism, which is the typical neuronal effect of cannabinoids.

ANANDAMIDE ATTENUATES THE INFLAMMATORY PHENOTYPE OF RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS BY ACTIVATING MULTIPLE RECEPTOR PATHWAYS

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Background: In rheumatoid arthritis (RA), synovial fibroblasts (SF) secrete large amounts of IL-6, IL-8 and several matrixmetalloproteinases which are crucial for cartilage destruction. RASF are sensitive to the action of cannabinoids and they express cannabinoid receptors type I and II as well as endocannabinoid degrading enzymes. Cannabinoids are regarded as antiinflammatory and since anandamide is found in RA synovial fluid we investigated how this endocannabinoid affects adhesion, proliferation and the production of inflammatory mediators of RASF.

Methods: Adhesion was assessed by Roches *xcelligence* system. Proliferation was quantified by the amount of incorporated fluorescent dye into cellular DNA. MMP-3 and cytokines were detected by ELISA. Cannabinoid receptors were visualized by immunofluorescence.

Results: Anandamide dose-dependently decreased the production of IL-6, IL-8 and MMP-3 by activating classical cannabinoid receptors. Adhesion was increased in a cannabinoid receptor-dependent manner. Proliferation was inhibited by anandamide and this was reverted by blocking cyclooxygenase-2. Furthermore, proliferation was negatively correlated with CB1 receptor expression. Additionally, the expression of GPR18 and GPR55 was verified in SFs.

Conclusion: Anandamide promotes an antiinflammatory phenotype in RASFs by activating classical cannabinoid receptors. Additionally, cyclooxygenase-2 metabolites of anandamide exert their anti-proliferative effects independent of cannabinoid receptors 1 and 2. The identification of GPR18 and GPR55 in RASFs present novel sites of action for anandamide and its metabolites and provide attractive targets for future therapeuticals in RA.

CORTISOL-INDUCED ADHESION OF SYNOVIAL FIBROBLASTS IS MEDIATED BY THE ENDOCANNABINOID SYSTEM

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Objective: Glucocorticoids partly regulate their action via a negative feedback loop by activating the endocannabinoid system. While this has been described only on a neuronal level, we provide evidence that glucocorticoids also employ the endocannabinoid system in the periphery by modulating complex events such as integrin-mediated adhesion in synovial fibroblasts.

Methods: We used the XCelligence system to measure adhesion of synovial fibroblasts to fibronectin. Immunocytochemistry was employed for the detection of FAAH and cox-2. Anandamide was detected by mass spectrometry.

Results: Cortisol dose-dependently increased adhesion of RA and OA synovial fibroblasts to fibronectin with an a maximum ~ 200 % at 10^{-7}M and 10^{-8}M. When cortisol was incubated with either rimonabant (100nM), a cannabinoid receptor 1 antagonist or capsazepine (1μM), a TRPV1 antagonist, adhesion was reduced below control level. Similar reductions were obtained by partly blocking endocannabinoid degradation by targeting either FAAH or cox-2. Complete inhibition of endocannabinoid degradation by blocking both enzymes reversed the effects. Mass spectrometry revealed the presence of intracellular anandamide in synovial fibroblasts.

Conclusion: Glucocorticoid treatment of synovial fibroblasts leads to the generation of anandamide which levels are regulated by cox-2 and FAAH. Our observations indicate that a combination of FAAH and cox-2 inhibition along with low-dose glucocorticoids may provide a therapeutic option to maximally boost the endocannabinoid system in RA and other autoimmune disorders.

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CANNABINOIDS INDUCE PANCREATIC BETA-CELL DEATH BY DIRECTLY INHIBITING INSULIN RECEPTOR ACTIVATION

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The absolute number of pancreatic β cells reflects a dynamic balance between β-cell proliferation and death. An inadequate expansion of β-cell mass to compensate for increased insulin demand, associated with eventual loss of β cells due to apoptosis, is a hallmark of diabetes mellitus (DM). Insulin receptor (IR) signaling through IRS-PI3K-AKT pathway positively regulates survival and growth of most mammalian cells, including β cells, whereas growing interest is currently focused on the role of cannabinoid 1 receptors (CB1Rs) in the regulation of cell death and survival. Their pro-apoptotic and anti-proliferative effects have been reported in various cancer cells and results from inhibition of the PI3K-AKT cascade. However, the mechanism by which CB1R does this and its role, if any, in β-cell survival are unknown.

We previously reported that CB1Rs are expressed in pancreatic β cells, where they influence insulin action. We now report that CB1Rs form a heteromeric complex with IR and Gαi that inhibits IR kinase activity on β cells when activated. Gαi mediates the inhibitory effects of CB1Rs on IR kinase activity by direct binding to the activation loop in the tyrosine kinase domain of IR, in turn reducing phosphorylation of pro-apoptotic protein BAD and inducing β-cell death. Consistently, pharmacological and genetic blockade of CB1Rs in mouse models of DM leads to reduced blood glucose and increased β-cell survival and growth because of enhanced IR signaling through the IRS2-AKT-BAD and -p27 pathways. This effect was not unique to pancreatic β cells because activation of CB1Rs also impeded insulin-stimulated IR autophosphorylation and BAD phosphorylation in non-insulin-secreting cells. These findings provide the first evidence of direct physical and functional interaction between CB1R and IR signaling and provide a mechanism whereby peripherally acting CB1R antagonists improve IR activity in insulin-sensitive tissues, independent of other metabolic effects of CB1Rs.

Acknowledgements: Supported by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. R.N.K. is supported by NIH RO1 DK 67536 and 68721.

PHARMACOLOGICAL INHIBITION OF MONOACYLGLYCEROL LIPASE ATTENUATES LPS-INDUCED INCREASES IN CYTOKINE EXPRESSION IN THE RAT FRONTAL CORTEX AND PLASMA: DIFFERENTIAL MECHANISMS OF ACTION

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The endocannabinoid system is an important novel therapeutic target for the treatment of neuroinflammatory disorders. This study determined the effect of JZL184, a selective inhibitor of monoacylglycerol lipase (MAGL), the enzyme which preferentially catabolises 2-AG, on inflammatory cytokines in the brain and plasma following an acute immune challenge. The receptor and molecular mechanisms involved were also investigated. JZL184 (10mg/kg i.p.) and/or AM251 (CB_1 antagonist; 1mg/kg i.p.) or AM630 (CB_2 antagonist: 1mg/kg i.p.) were administered to male Sprague Dawley rats 30 minutes prior to the administration of lipopolysaccharide (LPS: 100 µg/kg i.p.). Animals were sacrificed at 2 hrs post LPS, plasma, spleen and frontal cortex taken and stored at -80°C. The expression or levels of TNFα, IL-1β, IL-6 and IL-10 were determined using quantitative RT-PCR or ELISA respectively. Concentrations of the endocannabinoids, anandamide and 2-AG, were determined in frontal cortical and spleen tissue using LC-MS-MS and arachidonic acid measured by HPLC coupled to UV detection. Data were analysed using ANOVA followed by Fisher's LSD *post-hoc* test when appropriate. $P<0.05$ was deemed significant.

JZL184 attenuated LPS-induced increases in IL-1β, IL-6, TNF-α and IL-10, but not IκBα expression in the rat frontal cortex. AM251 attenuated the JZL184-induced suppression of frontal cortical IL-6 expression. Although arachidonic acid levels in the frontal cortex were reduced in JZL184-treated rats, 2-AG levels remained unchanged. In comparison, 2-AG levels were enhanced in the spleen following JZL184 administration, with no change in arachidonic acid levels. In the plasma, LPS-induced increases in TNF-α and IL-10 levels were attenuated by JZL184. AM251 attenuated the JZL184-induced suppression of plasma IL-10 levels. In comparison, AM630 blocked LPS-induced increases in plasma IL-1β in the presence, but not absence, of JZL184. Inhibition of MAGL in the rat by JZL184 differentially suppresses LPS-induced frontal cortical and circulating cytokines. The data provide further evidence for the therapeutic potential of targeting the endocannabinoid system for the treatment of central and peripheral inflammatory disorders.

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N-ARACHIDONOYL GLYCINE REGULATES THE PHENOTYPIC MORPHOLOGY OF BV-2 MICROGLIA

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Following CNS injury or inflammation, the local microenvironment governs the morphological and phenotypic characteristics microglia manifest. Walter *et al* (2003) reported the involvement of the endogenous cannabinoid signalling in recruiting microglia toward dying neurons *in vitro*. They demonstrated that pathological stimulation of both neurons and microglia triggered microglial cell migration by engaging CB_2 and abnormal cannabidiol (Abn-CBD) receptors. Recently *N*-arachidonoyl glycine (NAGly) signalling via GPR18 has been introduced as an important 'new player' in microglial-neuronal communication (McHugh *et al*., 2010). NAGly is synthesized in the CNS primarily from AEA via a fatty acid amide hydrolase (FAAH) dependent pathway that can be prevented by URB597, an irreversible inhibitor of FAAH (Bradshaw *et al*., 2009). It is ineffective as an agonist at either CB_1 or CB_2 receptors despite the obvious structural overlap with AEA. Our hypothesis is that NAGly-GPR18 signalling regulates phenotypic change in BV-2 microglia.

To test this, BV-2 microglia were plated on 25x75 mm glass microscope slides and simultaneously exposed to 10 nM concentrations of NAGly or Vh control (0.1% DMSO) for twelve hours. Phorbol 12- myristate 13- acetate (PMA) was used as control. Cells were then fixed and images from 10 random fields of view were collected at x40 magnification using a light microscope. Cell morphology was categorized (amoeboid, unipolar, bipolar, tripolar, multipolar) by multiple investigators. The morphology of the BV-2 microglia population exposed to NAGly was significantly different compared to Vh control (one-way ANOVA; $p<0.05$); the number of amoeboid and multipolar cells decreased and increased, respectively.

The coupling between the death of neurons and their degradation by microglia is both striking and swift. This remarkable relationship suggests a fast-acting communication between neurons and microglia, such that the microglia are forewarned of the specific task (*i.e.* apoptosis, infection, or damage). A better understanding of how NAGly and related lipid signaling can both actively recruit and instruct microglia to adapt their phenotype selectively will provide insights regarding their possible contribution to neurodegenerative diseases.

McHugh, D., Hu, S. S-J., Rimmerman, N., Juknat. A., Vogel, Z., Walker, J.M., and Bradshaw, H. B. (2010). "*N*-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor." *BMC Neuroscience*, 11:44.

Bradshaw HB, Rimmerman N, Hu SS-J, Burstein S, Walker JM (2009). Novel endogenous N-acyl glycines: Identification and Characterization. *Vitamins and Hormones* **81**: 191-205.

Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, *et al* (2003). Nonpsychotropic Cannabinoid Receptors Regulate Microglial Cell Migration. *J Neurosci* **23**: 1398-1405.

NOVEL ENDOGENOUS *N*-ACYL AMIDES INDUCE CALCIUM MOBILIZATION IN BV-2 MICROGLIA

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In both developmental and post-developmental contexts, microglia discriminately engulf and eliminate dead or dying neurons. These processes must be tightly controlled in order to sustain the least possible collateral damage to adjacent neurons. This relationship suggests a fast-acting communication between neurons and microglia. Endogenous cannabinoid signaling has been shown to recruit microglia toward dying neurons *in vitro*. Recently *N*-arachidonoyl glycine (NAGly) signaling via GPR18 has been introduced as an important 'new player' in microglial-neuronal communication (McHugh *et al*., 2010). Both NAGly and AEA are members of a large family of endogenous, novel *N*-acyl amide lipids discovered in the brain and peripheral nervous system. Receptor-mediated calcium signals are a common transduction mechanism in all living cells, including microglia. We have recently shown that 15 additional members of the *N*-acyl amide family of molecules activate the transient receptor potential channels, TRPV1, TRPV2, and TRPV4 causing calcium mobilization. Our hypothesis is that novel *N*-acyl amides, likewise, induce calcium mobilization in BV-2 microglia.

BV-2 microglia were cultured to 90% confluency, in T-75 cm^3 flasks with DMEM with 5% FBS and 1% penicillin-streptomycin, before being plated in poly-D-lysine coated 96-well plates. Calcium mobilization assays were performed using Flexstation II and the calcium-sensitive fluorescent dye, Fura 2AM. BV-2 microglia were exposed to 15 individual *N*-acyl amide family mixtures (alanines, β-alanines, aspartic acids, GABAs, glycines, leucines, isoleucines, methionines, phenylalanines, prolines, serotonins, threonines, tyrosines, tryptophans, valines conjugated to palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid) at concentrations of 1 μM and 10 μM or vehicle control (1% DMSO). 100 nM platelet-activating factor (PAF) was also used as a positive control. The 340/380 fluorescence ratio of Fura-2 was collected every 5 seconds for a total scan time of 200 sec. Calcium influx as the area under the curve was quantified using Softmax software.

Of the 15 *N*-acyl amide family mixtures tested, 5 caused a significant increase in the intracellular calcium concentration of BV-2 microglia (one-way ANOVA; $p<0.05$). The order of potency being: GABAs, prolines, alanines, valines, and isoleucines. 10 μM of the *N*-acyl phenylalanine family significantly reduced the amount of intracellular calcium when compared to vehicle control without any loss of cell viability (one-way ANOVA; $p<0.05$).

McHugh, D., *Hu, S. S-J., Rimmerman, N., Juknat. A., Vogel, Z., Walker, J.M., and Bradshaw, H. B.* (2010). "*N*-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor." *BMC Neuroscience*, 11:44.

N-ARACHIDONOYL GLYCINE AND Δ^9-THC REGULATE THE CYTOKINE PRODUCTION OF BV-2 MICROGLIA

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Following CNS injury or inflammation, the local microenvironment governs the morphological and phenotypic characteristics microglia manifest. Indeed, the particular signalling molecules released by neurons regulate production and secretion of cytokines in activated microglia. Walter *et al* (2003) reported the involvement of the endogenous cannabinoid signalling in recruiting microglia toward dying neurons *in vitro*. Recently *N*-arachidonoyl glycine (NAGly) signalling via GPR18 has been introduced as an important 'new player' in microglial-neuronal communication (McHugh *et al*., 2010). NAGly is synthesized in the CNS primarily from AEA via a fatty acid amide hydrolase (FAAH) dependent pathway and can be prevented by URB597, an irreversible inhibitor of FAAH (Bradshaw *et al*., 2009). It is ineffective as an agonist at either CB_1 or CB_2 receptors despite the obvious structural overlap with AEA. Our hypothesis is that NAGly-GPR18 signalling regulates phenotypic cytokine production in BV-2 microglia.

BV-2 microglia were cultured to 90% confluency in T-75 cm^3 flasks with DMEM with 5% FBS and 1% penicillin-streptomycin. Media was replaced with phenol red and serum free DMEM 12 hours prior to the start of the experiment. Cells were exposed to 10 and 100 nM concentrations of NAGly and Δ^9-THC or vehicle control (0.1% DMSO) for 3 hours. 1 μg/ml LPS was used as positive control. Culture media was then collected and the presence of 40 cytokines was quantified using Quantibody Mouse Cytokine Array 4 (RayBiotech, Inc) and Odyssey infra-red imager (Li-Cor Biosciences). The production of 5 cytokines by BV-2 microglia was significantly altered by NAGly and Δ^9-THC (One-way ANOVA, $p<0.05$): Axl, CD40, IGF-I, OPN and Pro-MM9-9.

The coupling between the death of neurons and their degradation by microglia is both striking and swift. This remarkable relationship suggests a fast-acting communication between neurons and microglia, such that the microglia are forewarned of the specific task (*i.e.* apoptosis, infection, or damage). A better understanding of how NAGly and related lipid signaling can both actively recruit and instruct microglia to adapt their phenotype (pro- or anti-inflammatory) selectively will provide insights regarding their

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Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, *et al* (2003). Nonpsychotropic Cannabinoid Receptors Regulate Microglial Cell Migration. *J Neurosci* **23**: 1398-1405.

CANNABINOID RECEPTOR-INDEPENDENT ANTI-PROLIFERATIVE EFFECT OF DOCOSAHEXAENOYL ETHANOLAMIDE IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CELLS

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It has been suggested that endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), might be the promising anti-cancer agents in clinical fields of cancer treatment. We previously observed that AEA inhibited effectively cell proliferation of head and neck squamous cell carcinoma (HNSCC) cell lines by their receptors-independent action. In this study, we tried to check the anti-cancer effects of omega-3 ethanolamides in three HNSCC cell lines (SNU-1041, 1066 and 1076). Docosahexaenoyl ethanolamide (DHEA) and Eicosapentaenoyl ethanolamide (EPEA) are polyunsaturated fatty acid-based ethanolamides like AEA and are known to be increased in human body by dietary supplement with omega-3 fatty acids (DHA and EPA).

Similarly to AEA, DHEA inhibited more effectively cell proliferation of HNSCC cells than DHA but EPEA did not. The anti-cancer effect of DHEA seemed to be mediated by cannabinoid receptor-independent action since the antagonist of CB1 and VR1 (AM251, cay10448 and capsazepine) did not reverse DHEA-inhibited cell proliferation (no CB2 was detected in our cell model). Instead, we observed the increase of reactive oxygen species (ROS) and 8-isoprostane production by DHEA. Antioxidants (NAC and ebselen) and over-expression of glutathione peroxidase-4 reversed DHEA-inhibited cell proliferation partially. From these findings, lipid peroxidation provoked by DHEA seems to play a partial role in anti-cancer effect of DHEA in HNSCC cells. Also, our observations suggest the possibility that DHEA induced by dietary supplement of DHA might mediate the anti-cancer effect of DHA in some cancers such as HNSCC.

FUNCTIONAL ANALYSIS OF THE HUMAN CB2 RECEPTOR VARIANTS

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Anne Zimmer[1] and Andreas Zimmer [1]

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Mammalian tissues contain two types of cannabinoid receptor, CB1 and CB2, which belong to the family of G protein coupled receptors. Binding of endocannabinoid and exogenous agonists to these receptors activates several cellular signalling pathways including the inhibition of the adenylyl cyclase–cyclic AMP–protein kinase A activity, activation of mitogen-activated protein kinase cascades (extracellular-signal-regulated kinase (ERK), JUN amino-terminal kinase (JNK) and p38), and activation of the phosphatidylinositol-3-kinase–AKT pathway. In general, CB1 receptors are expressed at high levels in the central nervous system, whereas CB2 receptors are found predominantly in peripheral tissues e.g. on bone and immune cells. Our team has recently identified two different variants of the human CB2 receptor: hCB2Gln63 and hCB2Arg63. Interestingly, the hCB2Arg63 variant has been associated with reduced bone density, osteoporosis (Karsak et al. 2005), psychiatric disorders and autoimmune diseases in different human population samples. These findings are in concordance with the demonstration of a reduced signaling of the hCB2Arg63 variant.

The aim of this study is to analyze the functional consequences of the two CB2 gene variants *in vitro* and *in vivo*. In order to assess the difference *in vitro*, we generated two lentiviruses containing one of the human variants coupled to an internal ribosome entry site (IRES2) and enhanced green fluorescent protein (eGFP). We will transduce the lentiviruses into bone marrow-derived macrophages from CB2 receptor knockout mice and measure phagocytic activity as well as activation states of these transduced cells. To study the functional consequences of the gene variants *in vivo*, we are generating humanized mice harboring either one of the CB2 receptor variants. These mice will be a useful tool to study the *in vivo* effects of selective CB2 receptor agonists and CB2 receptor-mediated pathomechanisms in pain, osteoporosis, psychiatric and immune diseases. In addition, these mice will be helpful for the development of new drugs, which will more precisely target the human CB2 receptor.

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- DFG Research Unit FOR 926

CANNABINOIDS IN PROSTATE CANCER: AN UPDATE

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Endocannabinoid Research Group, National Research Council, Italy: [1]Istituto di Cibernetica, [2]Istituto di Chimica Biomolecolare, [3]Istituto di Biochimica delle Proteine; [4]GW Pharma Ltd., UK.

THC induces prostate carcinoma cell (PCC) apoptosis. We previously investigated the effect of eleven non-THC cannabinoids (CBD, CBG, CBC, THC, THCV, THCVA, CBN, CBGA, CBGV, CBDA, CBDV and CBN) as pure compounds and/or botanical extracts from *Cannabis* strains enriched in these compounds (BDSs), on androgen receptor-positive (e.g. LNCaP) and -negative PCCs. We found that both pure compounds and BDS reduce PCC viability (as assessed by MTT assay) with high potency and efficacy in absence of serum proteins, regardless of the presence of hormones in the medium and of the androgen dependency of the PCC line under study. We also showed that CBD is the most efficacious inducer of apoptosis (as assessed by FACS scan analyses, chemoluminescence-based assay of caspase 3/7 activity, DNA fragmentation and TUNEL). CBD pro-apoptotic effect was accompanied by a remarkable transcriptional up-regulation of the pro-apoptotic marker PUMA (p53 upregulated modulator of apoptosis, a pro-apoptotic member of the Bcl-2 protein family) and a considerable elevation of intracellular Ca^{2+}, and was due only in part to antagonism of transient receptor potential melastatin-8 (TRPM8) channels Here, we investigated further the pro-apoptotic effect of CBD on LNCaP cells and its molecular mechanisms.

Apart from p53 phosphorylation, CBD-induced increase of PUMA expression was correlated also to the expression increase of the transcription factor CHOP (CCAAT/enhancer binding protein), which has been described to activate PUMA expression in the absence of p53 activation and to be overexpressed following endoplasmatic reticulum (ER) stress. These data strengthen the suggestion of the involvement of an apoptotic intrinsic pathway mediated by mitochondrial and/or ER stress in the effects of CBD. Furthermore, we studied the involvement of nuclear oestrogen receptors 1 and 2 (ERα and ERβ) and of GPER, an intracellular transmembrane G protein–coupled oestrogen receptor. GPER resides in the ER, where it activates multiple intracellular signalling pathways including intracellular Ca^{2+} mobilization, occurring also concomitantly with CBD pro-apoptotic effects. We found high expression of GPER in LNCaP cells, whereas ERα and ERβ were poorly expressed, if at all. G15, a specific GPER antagonist with minimal binding binding to ERα or ERβ, counteracted CBD activation of caspase 3/7 and calcium mobilization from intracellular stores.
These findings indicate that CBD causes apoptosis in PCCs via the activation of intrinsic pathways and following interactions with several receptors, and support the clinical testing of CBD against various types of prostate carcinoma.

REGULATION OF INFLAMMATORY PROCESSES BY THE ENDOCANNABINOID SYSTEM

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The endocannabinoid system (ECS) is involved in many different neurodegenerative disorders, in which extensive neuronal damage is caused by an inflammatory response of the central nervous system (CNS). Microglia, the resident immune cells of the CNS, are one of the key players in neuroinflammation. These cells are able to adopt different states of activation in order to coordinate an immune response. In the healthy brain microglia are constantly surveilling their environment, a status commonly referred to as "resting state". In case of neuronal damage caused for example by toxins, pathogens or injury, microglia become activated and display a more pro-inflammatory phenotype, tailored to kill pathogens or to remove toxins. In order to limit self-damage in the course of an inflammation, they can also adopt a rather anti-inflammatory phenotype, which supports tissue repair and removal of apoptotic cells.

Microglia express all main components of the ECS. They are capable of producing and effectively inactivating the endocannabinoids N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG). Moreover, activated microglia express the cannabinoid receptor 2 (CB2) and can react on elevated endocannabinoid levels by migration towards the lesion sites and by increased proliferation. Activation of the CB2 receptor on microglia influences their phenotype towards an anti-inflammatory, less harmful character and seems to support tissue repair and dampening of the immune response.

How the ECS is exactly regulated in microglia cells is currently a hot topic of research. In the present study we have established the culture of primary microglia from neonatal (p1-p5) C57BL/6 mice. In order to mimic different phases of an inflammatory response, we have shifted the phenotype of these cells into different activation states, using LPS/IFNγ for the induction of a pro-inflammatory phenotype and IL-4, TGFβ and IL-10 to induce different activation states with anti-inflammatory properties. A set of activation markers tailored for each phenotype facilitates the identification of the different subtypes by FACS analysis, ELISA and RT-PCR. Subsequent gene expression analysis will provide new insights into the regulation of the ECS in microglia cells on a transcriptional level and facilitates a better understanding of its role in neuroinflammatory processes.

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NOVEL CANNABIDIOL DERIVATIVES AND THEIR USE AS ANTI-INFLAMMATORY AGENTS

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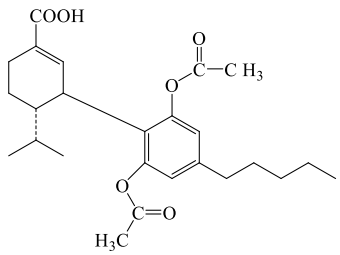
Cannabidiol (CBD) is a nonpsychoactive component of cannabis with a high potential for use in several therapeutic areas. It binds very weakly to the CB1 and CB2 cannabinoid receptors. It has been evaluated both *in vitro* and *in vivo* in anti-inflammatory assays. Thus, it lowers the formation of TNF-α, a proinflammatory cytokine and was found to be an oral anti-arthritic therapeutic in murine collagen-induced arthritis (CIA) *in vivo*. Its numerous potentially therapeutic pharmacological effects may be due in part to its metabolites. Indeed, a derivative of such a metabolite, CBD-dimethylheptyl (DMH)-7-oic-acid (HU-320) is more potent than CBD as an anti-arthritic agent in the above model of CIA. The mechanism of the cannabinoid anti-inflammatory effect is not fully understood, but most probably it has to do with its anti-oxidative action, its ability to enhance adenosine signaling through inhibition of adenosine uptake and by lowering of the levels of proinflammatory cytokines and the enhancement of Lipoxin A4 (LXA4) levels. We synthesized a novel CBD derivative, based on an active metabolite of CBD, which cannot be converted into a THC-like compound. This derivative, HU-444, was evaluated, in vitro, for anti-inflammatory activity, such as decrease of reactive oxygen substances (ROS), suppression of NO formation, inhibition of monocytic cell migration and inhibition of TNFa production by macrophages. In vivo we looked for suppression of production of TNFα in mice and amelioration of liver damage. HU-444 was also evaluated in a mouse collagen-induced arthritis (CIA) assay by both intraperitoneal and oral administration. HU-444 was synthesized from CBD. It showed potent activity in all the above tests. HU-444 ameliorates arthritis in mice when administered either intraperitoneally or orally as shown in a collagen-induced arthritis assay.

We believe that HU-444 represents a potential novel rheumatoid arthritis drug.



HU-444

CANNABINOID MODULATION OF LEUKOCYTE-ENDOTHELIAL INTERACTIONS IN AN ENDOTOXIN-INDUCED UVEITIS MODEL

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Introduction: Uveitis is a disease that involves inflammation of the uvea, and can lead to decreased vision. Experimental endotoxin-induced uveitis (EIU) can be generated with an intravitreal injection (IVTA) of lipopolysaccharide (LPS). LPS induces leukocyte activation and leukocyte-endothelial interactions, resulting in the release of proinflammatory mediators that cause tissue damage. The endocannabinoid system (ECS) is involved in immune cell activation. Furthermore, cannabinoid drugs that interact with the endogenous cannabinoid 2 receptors (CB2R) have immunomodulatory effects. The purpose of this study was to investigate the effects of CB2R activation on leukocyte-endothelial interactions in the rat iridal microcirculation, using intravital microscopy (IVM).

Methods: Four groups of Lewis rats (n=5-8 in each group) were studied: control, EIU (100 ng LPS/ 5 µL IVTA), EIU + 5 µL vehicle (Tocrisolve®), and EIU + CB2R agonist HU308 (1.5 µg/µL in 5 µL Tocrisolve®). All drug treatments were given 15 min after IVTA of LPS. IVM of the iridal microcirculation was carried out at 1, 2, 3, 4, 5, and 6 hrs post-LPS administration. Leukocyte adhesion was measured offline in a blinded manner.

Results: A baseline for leukocyte-endothelial adhesion was initially established for all groups at 1 hr. In comparison to the control group, the LPS-treated group had a significant increase in leukocyte adhesion by 6 hr after induction of endotoxemia in all iridal microvessels ($p<0.001$ in vessels >25 µm and $p<0.05$ in vessels <25 µm). Application of vehicle to EIU did not result in a significant change in leukocyte-endothelial adhesion. Administration of the selective CB2R agonist, HU308 (1.5 µg/ µL Tocrisolve®), significantly attenuated leukocyte adhesion in vessels <25 µm and >25 µm in EIU animals ($p<0.001$ and $p<0.05$, respectively). There was no significant decrease in leukocyte rolling in vessels <25 µm or >25 µm, in any of the drug treatment groups ($p>0.05$).

Conclusion: These data demonstrate cannabinoid modulation of leukocyte adhesion in the iridal microcirculation after EIU. Activation of CB2R by HU308 significantly attenuates leukocyte adhesion in the iridal microcirculation in all vessels 6 h after EIU. These results are consistent with an immunosuppressive action of CB2R agonists and indicates that future drugs targeting CB2R could aid in the treatment of ocular inflammatory diseases, such as uveitis.

THE ROLE OF CANNABINOID RECEPTOR 2 IN THE EXPERIMENTAL AUTOIMMUNE UVEORETINITIS

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Introduction: Experimental autoimmune uveoretinitis (EAU) is an experimental model of a sight-threatening inflammatory disease that affects the neural retina and related tissues. Microglia, a resident population of macrophages in the central nervous system (CNS), play an important role in host defense and tissue repair. Paradoxically, microglia activation also contributes to neuronal damage in a number of CNS diseases. In the EAU, microglia migration toward the photoreceptor layer and its activation contributes to the pathological changes seen in the retina. The endocannabinoid system is a potent regulator of immune response; cannabinoid receptor 2 (CBR2) is expressed on all immune cells including the microglia. Furthermore, activation of CB2R, by cannabinoid agonists, has been shown to attenuate the inflammatory response that leads to tissue damage associated with a number of autoimmune and inflammatory diseases. In the EAU, a selective CB2R agonist, JWX 133, has been reported to decrease the induction and severity of the disease. However, the mechanisms of action of cannabinoids in mitigating disease severity in the eye are not fully understood. The aim of the current study was to examine the role of CBR2 on the activation and motility of retinal microglia in EAU pathology using a genetic knock-out of CB2R.

Methods: EAU was induced in $CBR2^{-/-}$ and C57BL/6 mice by intraperitoneal injections of interphotoreceptor retinoid-binding protein (IRBP) peptide. The morphology of the eyes was evaluated under the light microscope at 7, 14, and 21 days after immunization, and the clinical scores were recorded. Three weeks following the immunization procedure mice were sacrificed; the eyes were enucleated and processed for histological grading and immunohistochemical staining for activated microglia.

Results: The induction of the EAU resulted in more pronounced pathological changes in retinas of $CB2R^{-/-}$ mice, compared to control C57BL/6 animals. In both the knockout and control animals immunized with the IRBP peptide, there was evidence of retinal folds, retinal detachment, choroiditis (inflammation of the choroid) and exudation. However the severity of these symptoms in control C57BL/mice was milder than in mice lacking the CB2R, and corresponded with average clinical scores. In addition, we found a significant increase ($p<0.05$) in the number of activated microglia in the $CB2R^{-/-}$ animals, as compared to control mice.

Conclusion: Mice lacking CB2R showed increased susceptibility to retinal damage in the EAU, together with increased numbers of activated MG. This data is supportive of an immunosuppressive role for CB2R in autoimmune disorders.

CANNABIDIOL AFFECTS MITOCHONDRIAL TARGETS IN BV-2 MICROGLIAL CELLS

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Cannabidiol (CBD) is a non-psychoactive plant cannabinoid that is clinically used (as Sativex) in a 1:1 mixture with the psychoactive cannabinoid Δ^9- tetrahydrocannabinol (THC) for the treatment of neuropathic pain and spasticity in multiple sclerosis. In animal models of multiple sclerosis such as the experimental autoimmune encephalomyelitis model, CBD ameliorates the pathological symptoms and significantly reduces the disease-induced immune cell infiltration and microglial activation. The mechanisms by which CBD acts to reduce inflammation remain unclear. Among other cellular pathways, CBD may affect mitochondrial molecular targets in immune cells including microglia.

Here, we studied the effects of CBD on various mitochondrial functions. These included: formation of reactive oxygen species (ROS), and mitochondrial permeability transition pore opening (tested using live cell imaging flow cytometry), changes in mitochondrial morphology (studied using electron microscopy), and CBD interaction with mitochondrial proteins (studied using density gradients and Western blotting). We found that CBD treatment led to significant changes in mitochondrial morphology (mainly swelling). In addition, density gradient analysis of detergent-free BV-2 microglial membrane fractions showed co-localization of CBD with mitochondrial proteins. Furthermore, CBD increased ROS production in a dose dependent manner. Finally, CBD interacted with the outer-mitochondrial membrane protein, the voltage-dependent anion channel (VDAC1) and decreased its channel conductance.

To conclude, CBD shows affinity to BV-2 mitochondrial membrane fractions and affects mitochondrial function and morphology. The functions of CBD at specific targets will be discussed.

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FISHING FOR CANNABINOIDS FROM FISH; IMMUNE-MODULATORY MECHANISMS OF AMIDES DERIVED FROM *N*-3 FATTY ACIDS

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Increasing evidence indicates that fatty acid amide (FAA) metabolites from the dietary fish oil DHA (22 : 6*n*-3) contribute to the beneficial effects of long chain omega-3 poly-unsaturated fatty acids (*n*-3 PUFAs). In previous studies we showed that the ethanolamine conjugate of DHA, DHEA (docosahexaenoylethanolamine) can function as 'fish oil derived modulator of inflammation'. DHEA was found to be highly present in animal tissues after a diet rich in DHA. It possesses anti-inflammatory properties as shown by reducing several cytokines and nitric oxide levels in LPS-stimulated macrophages (Meijerink et al., Br. J. Nutr. 105 (2011) 1798-1807) and in adipocytes. Furthermore, being structurally related to AEA, DHEA was shown to bind to the CB2 receptor. Here, we studied the key inflammatory mediators and pathways involved in the immune-modulatory action of DHEA. Additionally, other 'novel' DHA-derived fatty acid amides with potential biological relevance were investigated. Tests were performed with LPS stimulated RAW264.7 macrophages by measuring cytokine, IFN-β, PGE2 and nitric oxide production using ELISA, LC-MS/MS or Griess assay. NF-κB activity was measured using a HEK 293 NF-κB lacZ luciferase reporter assay.

It was found that DHEA exhibits it's modulatory effects via both the MyD88-dependent (TLR4-stimulated) and the MyD88-independent pathway (TLR3-stimulated). However, a concentration series of DHEA (0.1- 10 μM) did not inhibit activity of two prominent key inflammatory mediators of these pathways, namely NF-κB and INF-β. Remarkably, DHEA dose-dependently inhibited COX-2 derived prostaglandin E2 (PGE2) production. A comparison with structurally related fatty acid conjugates of serotonin showed that the conjugate of DHA had the largest immune-modulatory potency. Concluding, DHEA did not inhibit activity of NF-κB and INF-β, but strongly reduced the production of the COX-2 derived key inflammatory mediator PGE2. Our studies and those of others imply that there may be many more DHA-derived fatty acid amides which could function as 'fish oil-derived modulators of inflammation'. If so, it is intriguing to elucidate whether their mechanism of action is similar.

CANNABIDIOL AFFECTS CELLULAR BIOENERGETICS IN COLON CANCER

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The phytocannabinoid cannabidiol (CBD) has therapeutic potential for a number of diseases, including as an anti-cancer agent. CBD has anti-proliferative effects in many cancer cell types and in animal models, reducing invasiveness and metastasis (reviewed by Massi *et al*., 2012). However, the mechanisms through which CBD act are recognised to be wide-ranging and may include the CB1 receptor. Using standard proliferation assays (XTT and Presto Blue, Invitrogen) in 1% serum conditions, CBD dose-dependently (1-10 mM) inhibits the viability of human colon adenocarcinoma cell line, Caco-2, with complete cell death at 10µM after 24-48hr. To assess whether this effect was due to an action at the CB1 receptor, viability of CBD was assessed in the presence of AM251, an inverse agonist of the CB1 receptor and O-2050, a high affinity cannabinoid CB1 silent antagonist. These compounds did not prevent the anti-proliferative effect seen in CBD treated Caco-2 cells, suggesting the effect is not CB1 mediated, despite an early enhancement at 24h. Flow cytometric analysis using propidium iodide (PI) indicated that CBD does not act on cell cycle, but rather can be attributed to reactive oxygen species (ROS) production, as assessed by DCF/DA analysis.

Since ROS production primarily originates from the mitochondria, an XF Flux analyser from Seahorse Bioscience was used to assess the metabolic action of CBD on proliferating Caco-2 cells over 2 hours. The analyser measures the oxygen consumption rate (OCR) of cells simultaneously with the extracellular acidification rate (ECAR), a measure of glycolysis. CBD dose-dependently decreases the OCR, which is not inhibited by AM251 (100nm), injected 30 minutes before CBD. The effect of CBD is mirrored by an increase in ECAR, suggesting that the reduction of mitochondrial respiratory capacity could lead to an early cellular compensation and activation of aerobic glycolysis. However, under mitochondrial stress conditions, CBD inhibits this capability, an effect not mediated by CB1.

These results imply that under low serum conditions, CBD acts on cellular bioenergetic processes that limit the cells' respiratory capacity and ability to generate ATP, which leads to cell death, rather than cell cycle inhibition. This action is not mediated by the CB1 receptor. Interestingly, deeper analysis of the mitochondrial stress tests using oligomycin and FCCP, indicate that CBD (10mM) may prevent FCCP-induced mitochondrial uncoupling. However, this effect requires further analysis, particularly with fully differentiated Caco-2 cells, a model of normal enterocytic epithelium. Overall, the therapeutic window for this effect is rather small, an issue which may impact on its potential use as an anti-cancer medicine.

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ANTI-TUMOR EFFECTS OF AJULEMIC ACID ON THE EWING'S SARCOMA FAMILY OF TUMORS

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Ewing's Sarcoma is a pediatric bone cancer that is highly aggressive, leading to a five-year survival rate of only 30% even with multi-modal treatment protocols. Improved therapeutic options are desperately needed. Our research, completed primarily by undergraduate students, has focused on the ability of the non-psychoactive cannabinoid, ajulemic acid, to induce apoptosis and decrease metastatic potential in cells from members of the Ewing's sarcoma family of tumors. Our data show this compound can successfully decrease cell viability, migration and invasion *in vitro*. Further, we demonstrate its ability to inhibit endothelial cell migration and angiogenesis in a VEGF-independent manner. In order to test the efficacy of this compound in a more realistic model of human cancer, we developed a novel bioluminescent mouse model of Ewing's sarcoma in which engineered tumor cells were injected into the tibiae of mice and the growth of tumors in control and ajulemic acid treated mice was tracked using specific imaging techniques. We believe these findings support a role for ajulemic acid as a potential cancer therapeutic.

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MAPPING OF THE ENDOCANNABINOID SYSTEM IN MANTLE CELL LYMPHOMA

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Mantle cell lymphoma (MCL) is a Non-Hodgkin's B cell with a poor response to therapy and poor survival. New treatment options are clearly needed. MCL express cannabinoid receptors and undergo apoptosis if exposed to cannabinoids. To further study the role of the endocannabinoid system in MCL, levels of cannabinoid receptors and enzymes involved in the biosynthesis and degradation of the endocannabinoid anandamide (AEA) were analysed in a panel of primary lymphomas from MCL from patients and in MCL cell lines and compared to non-maliganant B-lymphocytes. We show that N-arachidonoyl phosphatidylethanolamine phospholipase D (NAPE–PLD), the enzyme participating in the biosynthesis of anandamide, is upregulated in MCL, while fatty acid amide hydrolase (FAAH), an enzyme participating in the degradation of endocannabinoids, is downregulated. The majority of the primary MCL samples expressed higher levels of cannabinoid receptors type 1 and type 2 (CB_1 and CB_2) than non-malignant lymphocytes. Moreover, we show that SR141716 and SR144528, potent antagonists to CB_1 and CB_2 respectively, induce cell death by caspase-3 dependent apoptosis. Dose-response analysis (viability curves) suggests that the CB1/CB2 antagonists induce cell death in MCL *via* an alternative route than AEA.

In conclusion, our data show that MCL is characterized by high expression of components involved in AEA biosynthesis and signaling, while AEA degradation is limited. Also, CB_1 and CB_2 antagonists lead to apoptotic cell death independently of cannabinoid receptor expression in MCL. Our data suggest a potentially important role of AEA and/or signaling *via* CB_1 and/or CB_2 in MCL pathogenesis.

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CANNABINOID RECEPTOR CB_2 CROSSTALK WITH CHEMOKINE RECEPTOR CXCR4 TO MODULATE BREAST CANCER GROWTH AND METASTASIS

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Synthetic cannabinoids which bind to cannabinoid receptor 2 (CB2) have been reported to possess anti-tumorogenic activity. However, not much is known about the effects and mechanism of action of synthetic non-psychotic cannabinoids on breast cancer growth and metastasis. We have shown that CB2 is overexpressed in primary human breast tumors compared to normal breast tissue. We also observed that breast cancer cell lines express CB2 receptors. Furthermore, we have shown that CB2 synthetic agonist JWH-133 inhibit cell proliferation and migration under *in vitro* conditions. These results were confirmed *in vivo* in various mouse model systems. Mice treated with JWH-133 showed a 40-50% reduction in tumor growth and 65-80% reduction in lung metastasis. These effects were reversed by CB2 antagonists SR144528, suggesting involvement of CB2 receptors. In addition, JWH-133 was shown to delay and reduce mammary gland tumors in PyMT transgenic mouse model systems. Furthermore, we have shown that JWH-015 significantly inhibits orthotopic tumor growth in syngenic mice *in vivo* using mammary tumor cell line.

Elucidation of mechanisms revealed that CB_2 may modulate breast tumor growth and metastasis by inhibiting signaling of the chemokine receptor CXCR4 and its ligand CXCL12. This signaling pathway has been shown to play an important role in regulating breast cancer progression and metastasis. We found that CB_2-specific agonist JWH-015 inhibits the CXCL12-induced chemotaxis and wound healing of various breast cancer cells. Further studies revealed that JWH-015 treatment inhibited CXCL12-induced P44/P42 ERK activation, cytoskeletal focal adhesion and stress fiber formation, which play a critical role in breast cancer invasion and metastasis. We also observed that JWH-015 significantly inhibited phosphorylation of CXCR4 and its downstream signaling *in vivo* in orthotopic and spontaneous breast cancer MMTV-PyMT mouse model systems. This study provides novel insights into the crosstalk between CB_2 and CXCR4/CXCL12-signaling pathways in the modulation of breast tumor growth and metastasis. Furthermore, these studies indicate that CB_2 receptors could be used for developing innovative therapeutic strategies against breast cancer.

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CB2 RECEPTOR-MEDIATED REGULATION OF I2PP2A (INHIBITOR 2 OF PROTEIN PHOSPHATASE 2A): A NEW TARGET FOR PROSTATE CANCER TREATMENT

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Prostate Cancer (PC) is the second highest fatal cancer among males worldwide and the prognosis for the advanced PC is very poor due to non-responsive hormonal androgen therapy. Therapeutic options for treating advanced/ recurrent PC are limited due to poor understanding of molecular mechanism of disease relapse. A validated drug design can be aimed if the possible key pathways, involved in the prostate cancer progression are well understood. Previous results from our laboratory and others have showed that activation of CB2 receptors inhibit PC cell proliferation, viability and in vivo tumor growth. In the current study we examined the role of an oncoprotein, I2PP2A (Inihibitor 2 of Protein Phosphatase 2A; overexpressed in PC cells) in CB2 receptor-mediated inhibition of PC growth through modulation of. ceramide.

We found that I2PP2A is highly overexpressed in all PC cells in LNCaP, and DU145, than normal prostate epithelial cells (PrEC). Treatment with ceramide or CB2 receptor agonist JWH015 induces cell death in PC cells whereas no effect was found in normal PrEC cells. We also found that activation of CB2 receptor reduced I2PP2A expression, decreased c-Myc accumulation but increases histone acetylation in PC cells in a time and dose-dependent manner. Overexpression and knockdown of I2PP2A confirmed the role of I2PP2A in CB2 receptor-mediated decrease in PC cell proliferation. Collectively these results highlight the role of I2PP2A in CB2R-mediated regulation of PC progression.

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EFFECTS OF PRO-INFLAMMATORY CYTOKINES AND CANNABINOIDS ON INTESTINAL EPITHELIAL PERMEABILITY

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Proinflammatory cytokines such as TNF-α, IL-1β and IL-17 are associated with the pathology of inflammatory bowel disease. These cytokines have detrimental effects on colonic epithelium, increase epithelial permeability and allow entry of luminal antigens into the lamina propria, facilitating inflammation. Cannabinoids reduce inflammation in animal colitis models, but it is unclear whether this occurs via any direct influence on epithelial permeability. We tested whether cannabinoid ligands could modulate any epithelial permeability changes induced by such cytokine treatments *in vitro*. Caco-2 cells were grown on Transwell permeable supports for up to 25 days to allow monolayer formation and differentiation. TNF-α, IL-1β, IL-17 and a combination of TNF-α and IL-1β (all applied to the basolateral compartment at 10 and 100 ng/mL each) were added to confluent Caco-2 cells. Integrity of the monolayer was measured via trans-epithelial electrical resistance (TEER) recordings. Readings were taken before treatment and at 5, 24, 48 and 72 hours post treatment. Anandamide (AEA), the CB1 agonist arachidonyl-2'-chloroethylamide (ACEA), the CB2 agonist JWH-015 and cannabidiol (CBD) all applied basolaterally at 1μM were tested against the TNF-α and IL-1β cytokine combination (10 ng/mL each and 100 ng/mL each). IL-1β, TNF-α or IL-17 (10 ng/mL) alone caused no significant changes in epithelial permeability. TNF-α and IL-1β together at both 10 and 100 ng/mL caused increased Caco-2 monolayer permeability; at 100 ng/mL they elicited an increase in permeability at 24 ($p<0.05$), 48 ($p<0.0001$) and 72 ($p<0.05$) hours. The endocannabinoid AEA had no effect on monolayer permeability induced by 10 ng/mL or 100 ng/mL TNF-α and IL-1β. Similarly, ACEA, JWH-015 or CBD treatments did not altered monolayer permeability in response to these cytokines. This study demonstrates that TNF-α and IL-1β directly increase epithelial permeability which was not attenuated by cannabinoids. The anti-inflammatory effects of cannabinoids observed in animal models of colitis may occur via immune cell modulation rather than direct effects on barrier function, as previous studies in our lab have demonstrated AEA and JWH015 reduce mucosal damage from TNF-α and IL-1β incubation in human colonic explant culture.

L-α-LYSOPHOSPHATIDYLINOSITOL AND AM251 PROMOTE CYTOSKELETAL REORGANISATION IN CANCER CELL LINES THAT EXPRESS GPR55

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Cytoskeletal reorganisation is important for cell detachment and migration, processes that are critical for metastasis from the site of primary tumours to the development of secondary growth in new tissue. The putative cannabinoid receptor G-protein coupled receptor 55 (GPR55) is known to signal via pathways that regulate the cytoskeleton and is thought to be expressed in a variety of different cancer cell lines. Moreover, increased expression of GPR55 correlates with aggressiveness in several different cancers. Emerging data suggests, the endogenous lipid L-α-lysophosphatidylinositol (LPI), is a natural ligand for GPR55 and the synthetic cannabinoid antagonist AM251 is also known to activate GPR55 in recombinant systems. Interestingly, LPI is reported to be synthesised in DU145 prostate cancer cells and may activate GPR55 via an autocrine loop further exacerbating oncogenic pathways in these cells.

In the present study we investigated GPR55 ligand-mediated cytoskeletal rearrangement in two cancer cell lines that are reported to express endogenous GPR55: the DU145 prostate cancer cell line and the T98 glioblastoma cell line. Cells were exposed to either 3 μM LPI or 3 μM AM251 and effects on the actin cytoskeleton were evaluated. We find that these ligands induce the formation of stress fibres in these cells and a change in morphology which are characteristic of activation of RhoA and ROCK signalling pathway. Indeed, the effects of LPI and AM251 were blocked in the presence of 1 μM H1152 dihydrochloride, a Rho kinase (ROCK) inhibitor.

These data suggest that activation of GPR55 can influence the actin cytoskeleton in DU145 and T98 cancer cell lines and raise the possibility that the endogenous ligand LPI may be an intrinsic regulator of cancer metastasis.

DEVELOPMENT OF LM-4131, A SUBSTRATE-SELECTIVE INHIBITOR OF CYCLOOXYGENASE-2

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We recently reported that rapid-reversible inhibitors of cyclooxygenase-2 (COX-2) are substrate-selective inhibitors, while slow, tight-binding inhibitors of COX-2 are non-substrate-selective inhibitors. In particular, we found that (*R*)-profens, which had previously been classified as inactive toward COX-2, are actually substrate-selective inhibitors of COX-2. We also found that (*R*)-profens increased the levels of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) while decreasing the levels of prostaglandin ethanolamides (PG-EAs) and prostaglandin glycerol esters (PG-Gs) in primary murine dorsal root ganglia cells stimulated to express COX-2. However, *in vivo*, (*R*)-profens undergo a one way stereoisomerization to (*S*)-profens, which are non-substrate-selective inhibitors of COX-2.

In the present study, we sought to develop new substrate-selective inhibitors of COX-2 for use *in vivo*. Site-directed mutagenesis indicated that disruption of the hydrogen bonding and ion-pairing network at the base of the active site can cause slow, tight-binding, non-substrate-selective inhibitors to become rapid-reversible, substrate-selective inhibitors. As suggested by the site-directed mutagenesis data, we found that tertiary amides of indomethacin are potent inhibitors of endocannabinoid oxygenation by COX-2. In particular, the morpholino amide of indomethacin, LM-4131, is a potent substrate-selective inhibitor with an IC_{50} of 620 nM for inhibition of 2-AG oxygenation by COX-2. LM-4131 also inhibited PG-G production in stimulated RAW 264.7 macrophages with an IC_{50} of 660 nM while increasing the levels of 2-AG. LM-4131 did not inhibit fatty acid amide hydrolase activity (FAAH) or monoacylglycerol lipase (MAGL) *in vitro*.

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STRUCTURE-ACTIVITY RELATIONSHIPS OF LIGANDS FOR GPR119 – A GPCR THAT IS RELATED TO CANNABINOID RECEPTORS

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GPR119, a G protein-coupled receptor, is a promising therapeutic target for both type 2 diabetes and obesity. GPR119 belongs to the MECA (melanocortin, endothelial differentiation gene, cannabinoid, adenosine) group of receptors. GPR119 is phylogenetically related to the cannabinoid receptors and has been shown to be activated by fatty acid amides. The first purpose of the current study was to validate a cell-based assay appropriate for discovering novel ligands for GPR119. The second purpose of this study was to apply the assay to investigate the structure-activity relationships of the acyl side chains and the charged head groups in fatty acid amides for activating GPR119.

A cell-based, homogenous time resolved fluorescence (HTRF) method was used for measuring cAMP levels in this study. The determined Z'factor for the assay was 0.68. The assays can tolerate up to 1% of DMSO. The known GPR119 agonists exhibited the expected rank order of potency and efficacy. Among the fatty acid ethanolamides tested, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), and linoleoyl ethanolamide (LEA), which contain zero, one, and two double bonds, respectively, increased cAMP accumulation in cells stably expressing GPR119. In contrast, dihomo-gamma-linolenoyl ethanolamide (DLEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), arachidonoyl ethanolamide (AEA), eicosapentaenoyl ethanolamide (EPEA), and docosahexaenoyl ethanolamide (DHEA), which contain three to six double bonds in their acyl side chains, failed to activate GPR119. Furthermore, both oleoyl dopamine (OLDA) and oleamide activated GPR119, with similar potency and efficacy as OEA. However, oleoyl alanine, oleoyl glycine, and oleoyl gamma-aminobutyric acid (GABA) were unable to activate GPR119. In conclusion, we have validated that the cell-based HTRF cAMP assay is a robust and suitable technology for screening ligands that may act on GPR119. In addition, our data provided direct evidence to further support the hypothesis that degree of saturation in the acyl chain of fatty acid ethanolamides affects the ability of these compounds to activate GPR119. Furthermore, our results demonstrated that in order to activate GPR119, there are certain structural requirements for the charged head groups of the fatty acid amides.

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3,4-DISUBSTITUTED THIADIAZOLES AS FAAH AND/OR MGL INHIBITORS

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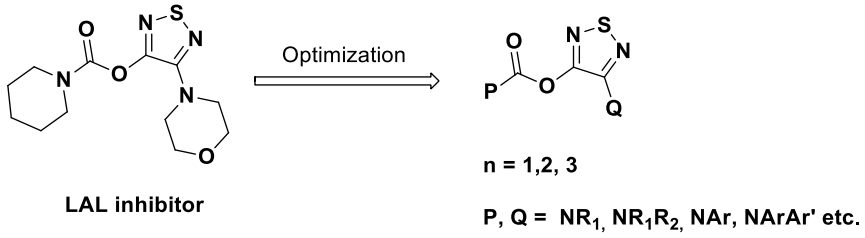
The fatty acid amide hydrolase (FAAH) knockout mice shows elevated levels of arachidonoyl ethanolamine (anandamide, AEA) thereby suggesting its role in degradation while monoglyceride lipase (MGL) along with two more supplementary enzymes α/β hydrolase-6 (ABHD6) and α/β hydrolase-12 (ABHD12) were found to be responsible for the 2-arachidonoyl glycerol (2-AG) degradation. These lead developement of FAAH and/or MGL inhibitors mainly based on known serine hydrolase inhibitors and substrate analogues.

Earlier our colleagues have identified various hormone-sensitive lipase (HSL) inhibitors as FAAH and/or MGL inhibitors[1-3]. In further pursuing our search for potent and selective FAAH and/or MGL inhibitors, herein, we report FAAH and MGL inhibitory activities of thiadiazole based lysosomal acid lipase (LAL) inhibitors[3](Figure 1). Desired 3,4-disubstituted thiadiazoles are synthesized conveniently as per the reported method[4] and their FAAH and MGL inhibitory activity were measured by the procedures described previously.[5,6]



LAL inhibitor

Fig. 1. Thiadiazole derivatives

According to our preliminary findings, 3,4-disubstituted thiadiazole carbamates can be utilized as FAAH and/or MGL inhibitors and further structural modification is under progress in order to achieve higher potency and better selectivity.

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EVIDENCE FOR A BIDIRECTIONAL ENDOCANNABINOID TRANSPORT ACROSS CELL MEMBRANES AND IDENTIFICATION OF SELECTIVE INHIBITORS

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Endocannabinoids are lipid mediators involved in many physiological and pathophysiological conditions in the CNS as well as in peripheral tissues where they exert biological activities by interacting with extracellular and intracellular targets. The effects of endocannabinoids are regulated by the biosynthesis, release, re-uptake, trafficking and enzymatic metabolism. Unlike the clear knowledge about the biosynthetic and metabolic pathways of endocannabinoids, their release and cellular re-uptake are still debated issues with several mechanisms proposed. Moreover, despite an extensive research on the trafficking of anandamide (AEA) across cell membranes little is known about membrane transport of other endocannabinoids, including 2-arachidonoylglycerol (2-AG). Previous studies have provided data both in favor and against a cell membrane carrier-mediated transport of endocannabinoids, using different methodological approaches. Since AEA and 2-AG undergo rapid and almost complete intracellular hydrolysis, we employed a combination of radioligand assays and absolute quantification of cellular and extracellular endocannabinoid levels. In human U937 leukemia cells, 100 nM of AEA and 1 μM of 2-AG were taken up through a fast and saturable process reaching a plateau after 5 min. Employing differential pharmacological blockage of endocannabinoid uptake, breakdown and interaction with intracellular binding proteins, we show that endocannabinoids harbouring an arachidonoyl chain compete for a common membrane target that regulates their cell membrane transport. Intriguingly, other *N*-acylethanolamines did not interfere with AEA and 2-AG uptake. By combining fatty acid amide hydrolase (FAAH) or monoacyl glycerol lipase (MAGL) inhibitors at low concentrations (≤10 nM) with hydrolase inactive concentrations of the AEA transport inhibitors UCM707 (1 μM) and OMDM-2 (5 μM) we observed a superadditive effect on cellular AEA and 2-AG uptake. Intriguingly, structurally unrelated AEA uptake inhibitors also blocked the cellular release of AEA and 2-AG. We show, for the first time, that a membrane target is involved in the bidirectional movement of AEA and 2-AG across cell membranes. Our findings confirm that the elusive endocannabinoid membrane transporter (EMT) is the major bottle neck in cellular AEA and 2-AG trafficking and metabolism. Our results support the EMT as a unique drug target which can modulate endocannabinoid levels and activity. We are currently screening natural products and synthetic analogs for the identification and development of new chemical scaffolds as inhibitors of the EMT activity. Two novel endocannabinoid-unrelated lead structures have been obtained which show high potency (low nanomolar range) and high selectivity towards EMT over other intracellular targets involved in the AEA trafficking and degradation (i.e. AEA intracellular binding proteins and FAAH).

SYNTHESIS AND EVALUATION OF NOVEL CANNABINOID TYPE 2 RECEPTOR TRACERS FOR PET IMAGING

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The cannabinoid receptor type 2 (CB2) has very low expression level in brain tissue under basal conditions. However, it is up regulated in cerebellum, cortex and brainstem in pathological conditions such as neuroinflammation and neurodegenerative diseases including ALS, MS, Parkinson's and Alzheimer`s disease. As part of our program to develop a brain PET tracer towards CB2, we evaluated the potential of KD-2 – a promising lead structure from literature [1] - as an imaging agent for the CB2. *In vitro* competitive binding assay was performed using [^{3}H]CP-55940 on human CB2 and CB1 membranes. Permeation of KD-2 across P-glycoprotein transfected MDCK epithelial cells was moderate but in the range of blood-brain barrier permeating compounds. No efflux of KD-2 by P-glycoprotein was detected. In vitro serum albumin binding was very high with a free fraction of 7×10^{-5}. KD-2 has been successfully labeled with ^{11}C-isotope and *in vitro* autoradiography of rat and mouse spleen slices demonstrated high specific binding to CB2, which was blocked by WIN 55212-2. Preliminary PET studies with rats showed that radiolabeled [^{11}C]KD-2 accumulated in liver, spleen and intestine. Uptake in pons and cerebellum was higher than in hippocampus and caudate/putamen, which is in agreement with the expression pattern of CB2 in the rat brain. The accumulation in spleen was displaced by 1.5 mg/kg GW405833, indicating specific binding.

Furthermore, eight new derivatives of KD-2 were designed and synthesized in our laboratory. Their binding affinities towards hCB2 and hCB1 were determined and the Ki values for the CB2 receptor ranged from 0.7 – 1220 nM, with a selectivity hCB2 over hCB1 from 13 to >10'000. The most promising candidates will be radiolabeled with ^{11}C or ^{11}F for further in vitro and in vivo evaluation.

[1] Pasquini et al., *J. Med. Chem.*, 54 (15), 5444-53 (2011)

Acknowledgements: Funded by Swiss ALS Foundation

2,4,6-TRISUBSTITUTED 1,3,5-TRIAZINES AS SELECTIVE CB2 AGONISTS

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In this report, we describe a series of novel 2,4,6-trisubstituted 1,3,5-triazines as cannabinoid receptor 2 (CB2) agonists. The compounds are highly selective for CB2 over CB1, and the 1,3,5-triazine scaffold enables easy structural modification due to very feasible synthetic routes and fairly affordable starting materials.

The hit structure, N-cyclopentyl-4-ethoxy-6-(4-methylpiperidin-1-yl)-1,3,5-triazin-2-amine (EC value 35 nM, hCB2-CHO), was found by ligand-based virtual screening where BRUTUS virtual screening software was used to search compounds with steric and electrostatic fields similar to those of SR141716A (Rimonabant). The hit compound was selected based on its potency, selectivity, small molecular weight and synthetic feasibility.

A small library of analogs was synthesized by displacement of chlorine atoms in cyanuric chloride by various substituents to get 2,4,6-substituted 1,3,5-triazines. Altogether, around 50 synthesized and commercially available compounds were tested for their CB1/CB2 receptor activities by using the [^{35}S]GTPγS binding assay[1,2]. Half of the compounds showed CB2 agonist activity with EC50 values ranging from low nanomolar to low micromolar concentrations.

Currently, we utilize the information gathered from the structure-activity relationship studies to develop even more potent CB2 agonists while maintaining selectivity and enhancing the water solubility of the compounds.

The research was supported by Academy of Finland (grant 128056) and National Graduate School of Organic Chemistry and Chemical Biology.

[1] Savinainen, J. R.; Kokkola, T.; Salo, O. M. H.; Poso, A.; Järvinen, T.; Laitinen, J. T. Br. J. Pharmacol. **2005**, 145, 636.
[2] Savinainen, J. R.; Saario, S. M.; Niemi, R.; Järvinen, T.; Laitinen, J. T. Br. J. Pharmacol. **2003**, 140, 1451.

[^{18}F]-LABELED HETEROCYCLIC DERIVATIVES FOR PET IMAGING OF CANNABINOID CB2 RECEPTOR

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The cannabinoid receptor type 2 (CB2R) is an important target for development of drugs for several diseases, such as neuroinflammation, neurodegeneration and cancer. Although CB2R was initially considered to be expressed primarily by the immune system, it is now well accepted that CB2R is expressed by activated microglia and other macrophages in the brain. Furthermore, recent studies have demonstrated an over-expression in various neuroinflammatory events caused by neurodegenerative (e.g. Alzheimer's) or autoimmune disorders (multiple sclerosis), stroke, trauma or brain neoplasia. Hence, radioligands for *in vivo* Positron Emission Tomography (PET) imaging of the CB2R expression can be a valuable research tool to explore the role and relevance of CB2R in neuroinflammation and to evaluate the therapeutic value of new CB2R-related drugs.

In a research program designed with the goal of obtaining CB2R selective ligands we recently described the synthesis and pharmacological characterization of 1,8-naphthyridin-, quinolin- and pyridine-3-carboxamide derivatives (C. Manera et al., J. Med. Chem. 52 (2009) 3644-3651; C. Manera et al., Eur. J. Med. Chem. (2012), doi:10.1016/j.ejmech.2012.03.031) which demonstrated high affinity and selectivity towards CB2R and anticancer properties in different type of tumor cells. In particular some of them are characterized by a *p*-fluoro-benzyl group in position 1 of the heterocyclic nucleus (Figure 1).

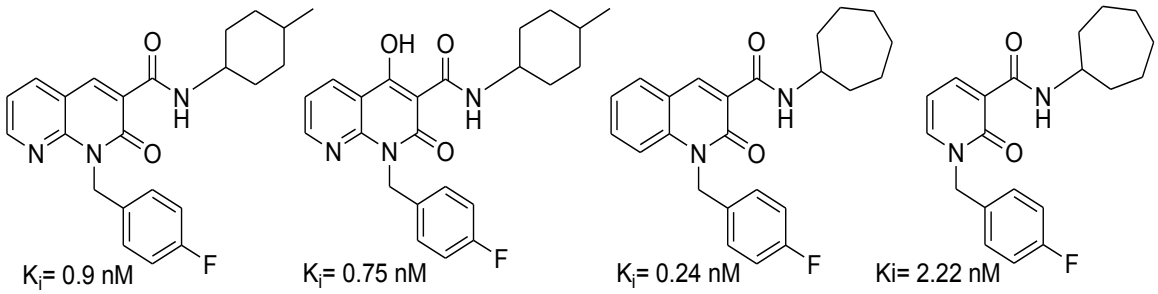

Figure 1.Binding affinities (Ki values) on CB2 receptor.

To evaluate their *in vivo* biodistribution by PET and assess their potential use as therapeutic or diagnostic tools, the synthesis of [^{18}F]-labeled analogues of the aforementioned ligands was developed. [^{18}F]fluorination experiments on aryliodonium salts synthesized as suitable precursors were conducted using the automated system Nanotek of Advion, which involves the use of microfluidic approach to obtain high yields and reproducibility of the labeling procedures. The results from *in vivo* studies performed using microPET/microCT suggest that these [^{18}F]-labelled compounds may be good candidates for *in vivo* PET imaging of the CB2R expression.

Acknowledgment: This work is supported by AriSLA- The Agency for Research on Amyotrophic Lateral Sclerosis (grant "PETALS II")

4-OXO-1,4-DIHYDROQUINOLINE-3-CARBOXAMIDE DERIVATIVES AS FUNCTIONAL SELECTIVE LIGANDS AT THE CB2 CANNABINOID RECEPTOR

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Cannabinoid compounds (both synthetic and endogenous) exert their effects through at least two different G protein-coupled receptors, the CB_1 and CB_2 cannabinoid receptors. While the development of a large variety of ligands has permit to improve our knowledge on the pharmacological modulation of the CB_1 receptor, the CB_2 receptor is far less well-characterised. Particularly, in the context of ligand-selective activation of specific signalling pathways (functional selectivity) much less is described regarding CB_2 than CB_1 cannabinoid receptors.

In a previous study, our group has reported on the development of a series of 4-oxo-1,4-dihydroquinoline-3-carboxamide derivatives showing high affinity and selectivity at CB_2 receptor. We herein report on the functional characterisation of these ligands in [^{35}S]-GTPγS assay which reveals that this original class of drugs contains agonists, antagonists and inverse agonists. Besides, the complexity of the CB_2-mediated signalling pathways was further characterised using eight ligands selected amongst this series. The measurements of cAMP and MAPK phosphorylation levels demonstrated that several of these derivatives displayed preference for the regulation of distinct signalling cascades therefore supporting functional selectivity at CB_2 cannabinoid receptor.

Considering the growing interest for the CB_2 cannabinoid receptor in the development of new drugs, the discovery and the functional characterisation of ligands displaying selective intracellular responses could provide valuable tools to extend the pharmacological comprehension of this receptor.

NOVEL CB2 AGONIST HU-433 MODULATES SKELETAL REMODELING AND BONE MASS

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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. We have previously reported that the HU-308 enantiomer HU-433 binds specifically to CB2 and potently stimulates osteoblast number by targeting Gi protein-Erk1/2 pathway. Here we show that HU-433 also inhibits bone resorption in *ex vivo* osteoclast cultures by promoting osteoclast apoptosis through the inhibition of Erk1/2 activation. In intact mice, HU-433 at a 0.02-0.002 mg/Kg/day for 6 weeks moderately increases bone volume density by stimulating bone formation and inhibiting bone resorption, reflected in increased serum osteocalcin (a bone formation marker) and decreased TRAP5b (a bone resorption marker), respectively. In a mouse ovariectomy (OVX) model for osteoporosis, HU-433 had a similar effect on these serum markers of bone remodeling. These changes in bone remodeling led to rescue of the OVX-induced bone loss. The reversal of bone loss in the distal femoral metaphyses and body of L3 vertebrae was differentially induced at 0.02 and 0.002 mg/Kg/day, respectively, presumably reflecting site variation in CB2 responsiveness. These data portray HU-433 as a highly potent lead to antiosteoporotic drug discovery, advantageous to currently available therapies, which are essentially either proformative or antiresorptive.

SYNTHESIS AND EVALUATION OF NOVEL CB2 CANNABINOID LIGANDS

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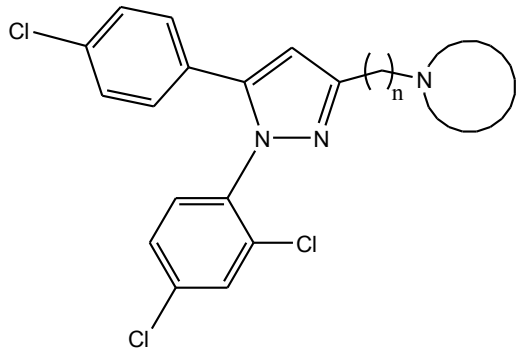
Continuing with our ongoing research on the design and synthesis of cannabinoid structures,[1,2] we wish to report a series of novel aminoalkylpyrazoles of general formula:



The compounds have been prepared from the corresponding α,β-insaturated ketones and phenylhydrazine. Improved yields and reaction times were obtained when the reaction was carried out under microwave irradiation.

The newly synthesized compounds have been evaluated as cannabinoid ligands by means of radioligand displacement experiments. Preliminary results indicate CB2 selectivity over CB1 in some derivatives.

Support contributed by: Spanish Grant SAF2009-12422-C02-02 and CANNAB-CM S2011/BMD-2308

References:

[1] ChemMedChem **2012**, *7*, 452-463

[2] ACS Med Chem Lett **2011**, *2*, 793-797

INITIAL HTS HIT OPTIMIZATION: NOVEL, HIGHLY POTENT AND SELECTIVE CB2 AGONISTS

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Cannabinoid Receptor 2 (CB2) agonists are considered to be a useful modality for the treatment of numerous diseases. A wide variety of potential applications is described, amongst others, treatment of pain, atherosclerosis, inflammation, fibrosis and cancerous tumors. A high-throughput screen was performed which led to the identification of a structurally novel class of highly potent CB2 agonists which exhibited a high (functional and binding) selectivity against the CB1 receptor. The initial structure activity relationship as well as molecular modeling interrogations supported optimization of the hit cluster. The agonists in depth *in vitro* profiling including binding, cAMP and beta arrestin recruitment data will be discussed. In addition, data on the CB2 Q63R variant, physicochemical properties and early ADME profiles of more advanced compounds will be highlighted.

INVESTIGATION ON 1,8-NAPHTHYRIDIN-2(1*H*)-ON-3-CARBOXAMIDES A CLASS OF POTENT CB_2 SELECTIVE CANNABINOID RECEPTOR LIGANDS: SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIPS AND FUNCTIONAL ACTIVITY

Valentina Lucchesi,[a] Haleli Sharir,[b] Giuseppe Saccomanni,[a] Alessia Ligresti,[c] Vincenzo Di Marzo,[c] Marco Macchia,[a] Mary E. Abood[b] and Clementina Manera[a]

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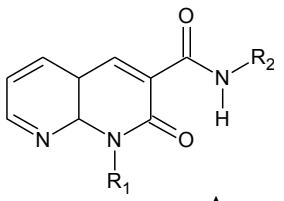
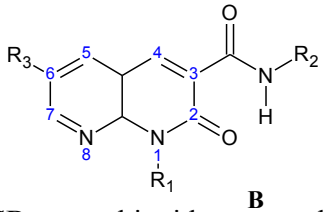
Current research indicates that the CB_2 cannabinoid receptor is a predominant target for the potential therapeutic benefit of cannabinoids in pathological processes such as cancer, neurodegenerative diseases, inflammatory states, stroke, atherosclerosis and pain (Sánchez et al., Clin. Immunol., 142 (2012) 57–67; Miller et al., Pharmacol Rev. 63 (2011) 461-70). In fact, the selective activation of CB_2 receptor could prevent the undesired central effects mediated by activation of the CB_1 receptor (Atwood et al., B. J. Pharmacol. 160 (2010) 467-79). It is, therefore, necessary to develop compounds with high affinity for the CB_2 receptor and low affinity for the CB_1 receptor. Our previous study has demonstrated the synthesis of 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives with the general structure **A**. Binding results revealed that these compounds exhibited a higher affinity and selectivity for CB_2 compared to CB_1 receptors (Manera et al., J. Med. Chem. 52 (2009) 3644-3651).



A



B

In the present study, to improve naphthyridine–based CB_2 cannabinoid receptor ligands, we have designed and synthesized a series of derivatives of **B** characterized by various substituents in position 6 and 1 on the 1,8-naphthyridine core. The aliphatic carboxamide group in position 3 has been selected on the basis of our previous studies (Manera et al., J. Med. Chem. 52 (2009) 3644-3651). The new compounds were tested in competitive binding assays using human recombinant CB_1 or CB_2 cannabinoid receptors expressed in HEK293 cells and were demonstrated to be highly specific for the CB_2 receptor. To further validate the ability of the newly synthesized compounds to act at CB_2, we employed the previously characterized βarrestin-2 assay (Sharir et al., J Neuroimmune Pharmacol. (2012) PMID:22454039) using U2OS cells co-expressing CB_2 and β-arrestin-2/GFP. Upon, prolonged stimulation with agonist, GFP-labeled β-arrestin translocates to intracellular pits/vesicles. The high local concentrations of fluorescent arrestins that develop as a result of receptor activation enable visualization of the entire process. These are detected in the images as small fluorescent aggregates or spots. Importantly this assay can be used to evaluate the compounds both as agonists or antagonists. We now have identified both agonists and antagonists in this series.

Acknowledgements: This work was supported by NIDA (Abood)

IN VIVO METABOLIC EFFECTS OF PIMSR, A CANNABINOID CB1 RECEPTOR NEUTRAL ANTAGONIST

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The use of the cannabinoid CB1 receptor inverse agonist SR141716 initially showed great promise in the regulation of obesity and diabetes related issues until dysphoric psychological side effects prompted its withdrawal from use. The possibility of inverse agonism as the cause of the dysphoric effects, coupled with the discovery of the CB1 neutral antagonist PIMSR, lead to our exploration of the consequences of neutral antagonism. The encouraging result that PIMSR (the C-3 piperidinoiminomethyl analog of SR141716) was shown to be free of dysphoric effects in electrical brain stimulation reward studies, led to a study of its potential as a regulator of metabolic disease related effects. This study reports the effect of PIMSR in high-fat diet (HFD) mice on body weight, food intake, glycemic control, and lipid homeostasis. Tissue distribution and markers of liver condition and function are also reported.

Mice on a HFD for 14 weeks when treated with 10 mg/kg/d (i.p.) for 28 days showed a 27% weight loss (vs 7% for control) and a persistently lower food intake versus vehicle treated mice. The adiposity index dropped to 40% of the vehicle treated mice. These mice also showed improved glycemic control in measures of glucose resistance, insulin sensitivity, basal hyperglycemia and hyperinsulinemia versus vehicle. Further, improved lipid homeostasis was shown by reduced serum triglycerides and cholesterol levels while HDL/LDL improved.

The tissue distribution 1 hour after an acute ip administration of 10 mg/kg PIMSR as determined by lc/ms/ms was found as 5.24 ug/mL in plasma with brain as 24%, liver as 414% and fat as 345% (ug/gm) that of plasma. The brain levels of low uM would support significant brain CB1 receptor occupancy (re: Ki = 17 nM), thus potentially including both CNS and peripheral influences on the observed weight loss. The extremely high levels of PIMSR measured in liver might, in part, be responsible for the elevated liver weight, and ALT as markers of hepatocellular damage. This is likely not a consequence of CB1R occupancy and hence similarly not due to neutral antagonism, but rather is due to the chemical properties and pharmacokinetics of this particular analog. Overall, this study suggests that marked improvements in aspects of metabolic disease can be realized with CB1R neutral antagonists and hence warrants the exploration of further members of this class of cannabinoid ligands.

TOWARDS RATIONAL DEVELOPMENT OF PERIPHERALLY SELECTIVE ANTAGONISTS OF CANNABINOID RECEPTOR 1

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Antagonists of cannabinoid receptor 1 (CB1R) have the potential for treating several important diseases such as drug addiction, obesity, diabetes, and liver disease. Unfortunately, adverse central nervous system (CNS)-related side effects including depression and suicidal ideation were reported with rimonabant (SR141716A), the first clinically approved CB1R antagonist (inverse agonist), leading to its withdrawal and the development of other CB1R antagonists were halted as well. However, CB1R is functionally expressed in peripheral organs and regulation of CB1R in these tissues by peripherally selective ligands is a promising approach to treat conditions like obesity, hepatic steatosis, and diabetes without eliciting the types of adverse effects noted with non-tissue selective agents. We have previously reported on the successful development of peripherally selective CB1R antagonists based on rimonabant (Bioorg Med Chem Lett. 21:5711-4; J Med Chem. 55:2820-34). Here we describe our efforts at developing peripherally selective CB1R antagonists that have a purine core, similar to otenabant (CP945598). These compounds were designed to have high topological polar surface areas (TPSA) because compounds with high TPSAs do not normally cross the blood brain barrier (BBB). Compounds were analyzed to determine their functional activity at CB1R using a calcium mobilization assay. Binding affinities and receptor selectivity of selected ligands were determined by radioligand displacement at CB1R and CB2R. Compounds with high TPSAs (TPSA>90), excellent functional activity (Ke ranging between 0.3-30 nM at CB1R), and high selectivity (>100-fold selective for CB1R versus CB2R) were identified. In vitro ADME profiling and testing in the MDCK-mdr1 model of BBB penetration led to the identification of compounds that were advanced into in vivo models to assess their CNS-permeability. Promising compounds were identified and they are currently undergoing further refinement and evaluation.

Supported by 1R21AA019740-01 to R. Maitra.

CLOUD COMPUTING WEBSERVER FOR CANNABINOID RESEARCH

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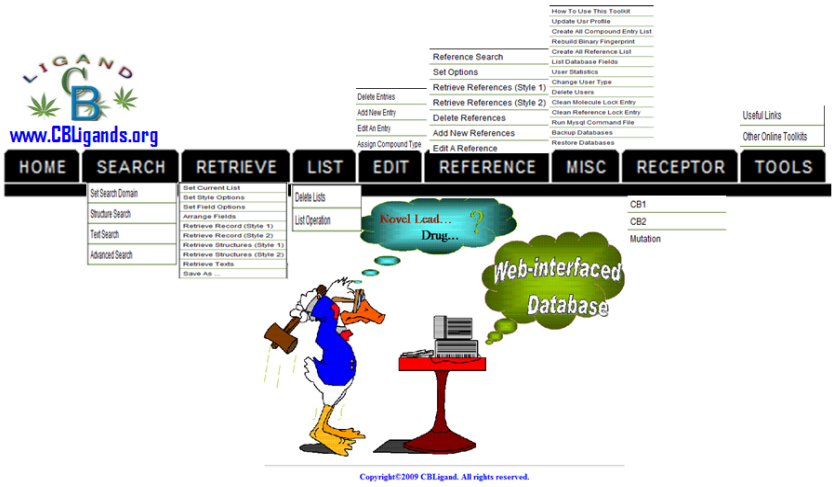
We have developed web-interfaced cannabinoid molecular information database (CBID) repository (*www.CBligand.org/cbid*), and implemented cheminformatics tools to facilitate the cannabinoid molecular information exchanges and assist new cannabinoid drug design. With cloud computing, *CBligand* or CBID provides user-friendly query interface with online data analysis tools and functions for molecular structure, substructure search and similarity calculations, as well as biological information related to cannabinoid ligands. The CBligand system was constructed using MySQL/php and our in-house cheminformatics data-mining algorithms. *CBligand* currently contains 2456 ligands that have been reported for cannabinoid receptor subtypes CB1 and CB2, and 4502 entries of affinity or function data from 457 journal papers and patents. *CBligand* provides 3D structures view/rotating in SDF format, and hyperlinks to access the corresponding PubMed references. *CBligand* also includes data-mining and modeling tools for CB receptor subtype specific pharmacophore generations, CB1 and CB2 ligand fragment libraries, and subtype binding/function predictions as well as other online cheminformatics tools, including TargetHunter, Off-TargetPredictor, and BBBPredictor, etc. Overall, *CBligand* and the developed cheminformatics tools will mediate the rapidly-growing chemical genomics studies of cannabinoid receptors for drug discovery and also facilitate the data analysis and information sharing/exchange and communications among the cannabinoid and related scientific communities (*Sean Xie, email: xix15@pitt.edu, www.CBLigand.org/CCGS).



PHARMACOKINETICS AND PHARMACODYNAMICS OF A POTENT, SELECTIVE AND ORALLY BIOAVAILABLE FAAH INHIBITOR, MM-433593 IN SPRAGUE-DAWLEY RATS, CYNOMOLGUS MONKEYS AND HUMAN

James D Wakefield, Kristie A Sykes, Nisha Perez, Stacy D Arnett, Vasu Sethuraman, Christopher M Graul, James P Pearson, G Todd Milne, Albert Profy, Robert W Busby and Kamlesh P Vyas

Ironwood Pharmaceuticals Inc., Cambridge Massachusetts, USA

MM-433593 is a potent and selective inhibitor of fatty acid amide hydrolase (FAAH), an enzyme responsible for the hydrolysis of bioactive fatty acid amides (FAA), including anandamide (AEA) oleoylethanolamide (OEA) and linoleoylethanolamide (LEA)]. LC-MS/MS methods were developed for the quantification of MM-433593 and three FAA biomarkers in plasma of the three species and for biomarkers only in rat brain. Initial studies in female rats, designed to investigate effects of MM-433593 on biomarker elevations as a function of dose (1-100 mg/kg, PO; 4 hr) and time (100 mg/kg, PO; 2-48 hr), demonstrated a dose-dependent increase in brain concentrations of all three biomarkers with maximum elevations of 6- to 8-fold relative to vehicle following a single 100 mg/kg dose. The pharmacodynamic effects on biomarker elevations were seen at doses as low as 1 mg/kg. At the highest dose level tested, brain and plasma concentrations of all three biomarkers increased with time before reaching maximum values at 6-8 hr post dosing, with significant elevations (2- to 11-fold) still seen at 48 hr. Concentrations of biomarkers in the brain were 45- to 80-fold higher than those in the circulation. Plasma concentrations of MM-433593 correlated well with changes in biomarker levels; such that there was a dose-dependent increase in drug concentrations at 4 hr post dosing as well as the maximum concentration of MM-433593 (3240 ng/mL) was observed at 6 hr post dosing. A more comprehensive PK/PD relationship of MM-433593 (5-600 mg/kg, PO) established in male monkeys using a simple cross-over design. Maximum plasma concentrations of MM-433593 were typically reached at 6 hr, and a less than dose-proportional increase in systemic exposure was observed over the dose range. Plasma concentrations of AEA, OEA and LEA were elevated above the vehicle control baseline at all dose levels, with maximal elevations (4-fold) observed at doses $\geq$ 30 mg/kg. In some instances a counterclockwise hysteresis was observed in the PK/PD relationship. The estimated MM-433593 plasma concentrations necessary to elevate circulating fatty acid amides to 50% of the theoretical maximum (EC_{50}) were 591, 299, and 378 ng/mL for AEA, OEA and LEA, respectively. Finally, in healthy human subjects, a single 25 mg oral dose of MM-433593 caused a modest, but significant elevation (2-fold above the placebo group) in plasma concentrations of two biomarkers (AEA and OEA) at 3-4 hr post dosing. MM-433593 was rapidly absorbed as the peak concentrations of 510 ng/mL were observed at 1 hr post treatment. In conclusion, oral treatment with the potent and selective FAAH inhibitor, MM-433593 results in significant increases in concentrations of FAA biomarkers in the rat, monkey and human.

A NOVEL PERIPHERAL CB1 ANTAGONIST REDUCES FOOD INTAKE AND EXHIBITS EFFICACY IN IMPROVING ADIPOSITY AND INSULIN RESISTANCE

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The endocannabinoid system regulates appetite and energy homeostasis. Cannabinoid receptor 1 (CB1), a component of the system, plays an important role in this metabolic regulation via both central and peripheral actions. To limit the psychological adverse effects resulting from blockade of central CB1, discovery of peripheral CB1 antagonists is actively pursued. Compound 1 (CB1 IC_{50} = 3.6 nM) is orally active and its B/P ratio at 2h after acute dosing (20 mg/kg) to C57BL6/J mice is less than 2%. In addition, it exhibited low brain occupany and did not reverse CP55940-induced hepothermia and analgesia. These results strongly support the peripheral selectivity of compound 1. Acute treatment of compound 1 at 10 mg/kg reduced food intake and weight gain effectively in Wistar rats. Chronic treatment of compound 1 in diet-induced obese mice substantially reduced adiposity and improved glucose intolerance at 10 mg/kg. More encouragingly, the amount of compound 1 detected in the brain was minimal. A preliminary study on *db/db* mice also demonstrated its efficacy in improving insulin resistance. In conclusion, compound 1 is a potent peripheral CB1 antagonist with limited brain penetration. Its *in vivo* efficacy supports the therapeutic potential of this second generation of CB1 antagonists in the treatment of metabolic disorders such as obesity and type 2 diabetes.

Acknowledgements: Funded by National Health Research Institues and National Science Council, Taiwan.

MYRTANIL SUBSTITUENT IN 3 POSITION OF PIRAZOLE RING DETERMINES CB_1 AGONISM OF NOVEL 4,5-DIHYDROBENZO-OXA-CYCLOHEPTAPYRAZOLE CANNABINOIDS

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We have previously described synthesis, structure-affinity relationships and pharmacological profile of three series of tricyclic compounds as 1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazoles (**1**), 4,5-dihydro-1*H*-benzo[*g*]indazoles (**2**) and 1,4-dihydroindeno[1,2-*c*]pyrazoles (**3**) (Figure 1), as potent cannabinoid receptor ligands.

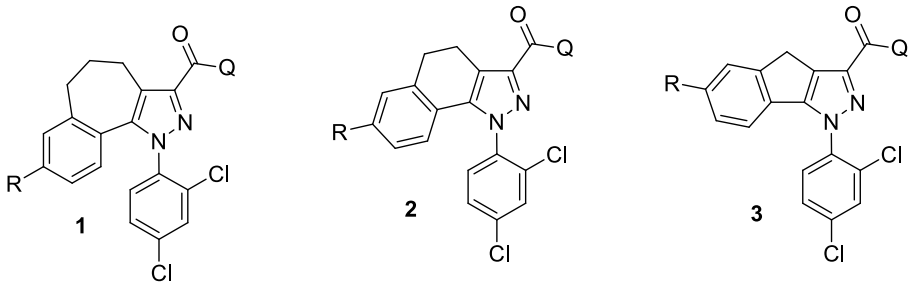

Figure 1. Tricyclic compounds acting on CB receptors.

In general it was highlighted a significant CB_1 affinity and selectivity for **1** compounds, good affinity towards both cannabinoid receptors for class **2**, CB_2 affinity and selectivity for derivatives encompassed by formula **3**. Moreover CB_2 agonist profile was determined for this last class, with very interesting activity of a lead compound towards neuropathic pain, while compounds **1** and **2** showed CB_1 antagonist activity.

As a continued effort to further characterize the structure-affinity relationships within the **1** class of compounds and with the aim to individuate new potential substitutes of rimonabant with neglect or reduced side effects, we decided to replace the methylene in position 6 of **1** with an oxygen atom. We report the preparation, the cannabinoid affinity evaluation and *in vitro*, *in ex-vivo* and *in vivo* assessment of novel 4,5-dihydrobenzo-1*H*-6-oxa-cyclohepta[1,2-*c*]pyrazoles **11-21** bearing different substituents in position 3 of the pyrazole ring.

All the synthesized compounds show affinity to CB_1 receptors. Amongst the new derivatives, those bearing (hetero)cyclic amines or a myrtanyl group in the C_3-carboxamide moiety bind with high affinities to CB_1 receptors as compared to aryl amides analogues. In particular compound **11** reveals the highest CB_1 affinity and additionally very high CB_1 selectivity, demonstrating as aliphatic carboxamides seems to be the optimal substituents for assure CB_1 receptor affinity.

By comparison of the novel compounds with the corresponding homologues encompassed by the formula **1**, no significant effect on cannabinoid receptor affinity was detected by the replacement of the methylene in position 6 of **1** with an oxygen atom.

Interestingly, while carboxamide derivatives with an aliphatic monocyclic ring **11-14** act as antagonists at the CB_1, the introduction in C_3-carboxamide position of a bicyclic bulky group as myrtanyl is responsible of CB_1 partial agonist activity of **15**, as confirmed by both *in vitro* studies and isolated organ assays.

DISCOVERY AND OPTIMIZATION OF 1-(4-(PYRIDIN-2-YL)BENZYL) IMIDAZOLIDINE-2,4-DIONE DERIVATIVES AS A NOVEL CLASS OF SELECTIVE CANNABINOID CB2 RECEPTOR AGONISTS

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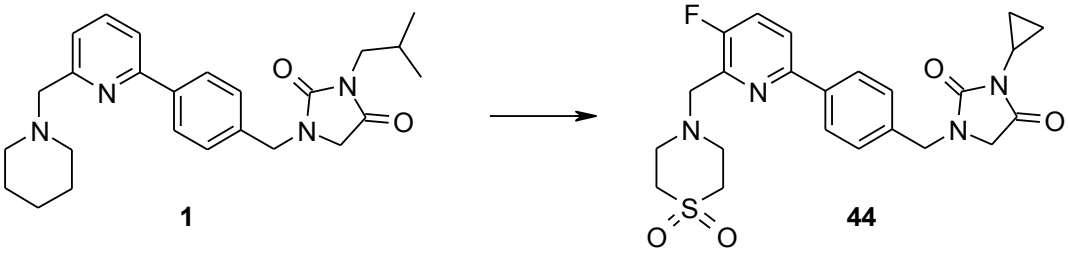
We will present the identification and optimization of 1-(4-(pyridin-2-yl)benzyl)imidazolidine-2,4-dione derivatives as a novel chemotype with selective cannabinoid CB2 receptor agonist activity. **1** is a potent and selective cannabinoid CB2 receptor agonist (hCB2 pEC50 = 8.6). The compound was found to be metabolically unstable, which resulted in low oral bioavailability in rat (F_{po} = 4%) and possessed off-target activity at the hERG ion channel (pKi = 5.5). Systematic modification of physico-chemical properties, such as lipophilicity and basicity, was used to optimize the pharmacokinetic profile and hERG affinity of this novel class of cannabinoid CB2 receptor agonists. This led to the identification of **44** as a potent, selective and orally bioavailable cannabinoid CB2 receptor agonist (hCB2 pEC50 = 8.0; hERG pKi < 4; F_{po} = 100%), which was active in a rat spinal nerve ligation model of neuropathic pain.



1

hCB2 pEC50 = 8.6
hERG pKi = 5.5
F (po) = 4 %

44

hCB2 pEC50 = 8.0
hERG pKi < 4
F (po) = 100 %

STIMULATORY EFFECTS OF PHOSPHOLIPIDS AND DIHYDROLIPOIC ACID ON *N*-ACYLETHANOLAMINE-HYDROLYZING ACID AMIDASE (NAAA)

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N-Acylethanolamine-hydrolyzing acid amidase (NAAA) is a lysosomal enzyme which catalyzes the hydrolysis of bioactive *N*-acylethanolamines to free fatty acids and ethanolamine only at acidic pH. Since the enzyme preferably hydrolyzes palmitoylethanolamide, an endogenous anti-inflammatory and analgesic substance, its specific inhibitors are expected as therapeutic drugs. Non-ionic detergent (Triton X-100 or Nonidet P-40) and the SH reagent dithiothreitol have been used as activators of NAAA. However, these compounds are artificials and naturally occurring NAAA stimulators remain poorly understood. In the present study, we examined stimulatory effects of endogenous phospholipids and thiol compounds on recombinant rat NAAA. Among various phospholipids, choline- or ethanolamine-containing phospholipids (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) were the most effective. Phosphatidylcholine at 3 μM–1 mM dose-dependently increased NAAA activity up to 6.6-fold. As to endogenous thiol compounds, dihydrolipoic acid (the reduced form of a-lipoic acid) was the most potent. The compound at 0.1–1 mM stimulated NAAA activity by 8.5–9.0-fold. Thus, endogenous phospholipids and dihydrolipoic acid may contribute to keeping NAAA active in lysosomes. Interestingly, the preference of palmitoylethanolamide by NAAA was not altered in the presence of phosphatidylcholine and dihydrolipoic acid. Fatty acid amide hydrolase (FAAH) is well known as the principal *N*-acylethanolamine-hydrolyzing enzyme. We examined a possible compensatory induction of NAAA in FAAH-deficient mice. However, in all the tissues examined, we could not detect significant changes in NAAA mRNA levels between FAAH-deficient and wild-type mice.

NOVEL OXADIAZOLONE ANALOGUES AS FAAH AND/OR MGL INHIBITORS

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Fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGL) inhibitors cause increase in the levels of endocannabinoids, arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG). In the past few years, various FAAH and/or MGL inhibitors have been developed based on known serine hydrolase inhibitors and substrate analogues. These include various scaffolds like ureas, α-keto heterocycles, carbamates, boronic acids, isoxazolones and oxadiazolones as FAAH and/or MGL inhibitors.

Earlier our colleagues have reported oxadiazolone analogues[1] (general structure **1**, Figure 1) as potent inhibitors of FAAH and/or MGL. In continuation of our efforts for the development of potent and selective FAAH and/or MGL inhibitors[1,2], herein, we report convenient synthesis of novel oxadiazolone analogues as FAAH and/or MGL inhibitors (Fig.1). The FAAH and MGL inhibitory activity of these compounds were measured by the procedures described previously.[3,4]

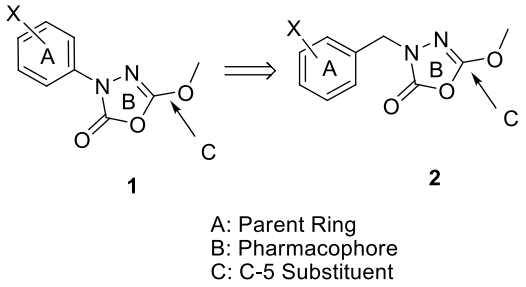

Fig. 1. Oxadiazolone derivatives

The modified oxadiazolones still maintain its utility as a useful template for the development of FAAH and/or MGL inhibitors through *in vitro* study. Further structural modification has been suggested in order to achieve higher potency and better selectivity.

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[2] Manuscript submitted to *Bio. Med. Chem.*
[3] Saario et al. *J. Med. Chem.* **2006**, *49*, 4650
[4] Saario et al. *Biochem. Pharmacol.* **2004**, *67*, 1381.

NOVEL INHIBITORS OF MONOACYLGLYCEROL LIPASE

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Introduction: Monoacylglycerol lipase (MGL) is a key enzyme involved in the metabolism of the endocannabinoid 2-arachidonoylglycerol and a potential target for drug development. However, irreversible inhibition of the enzyme leads to tolerance (Schlosburg *et al., Nat Neurosci* 13 [2010] 1113–9). There is a need for identification of novel reversible inhibitors of the enzyme. In consequence, we conducted a high-throughput screen of a chemical library and followed up potential hits using a standard structure-activity relationship approach.

Method: Initial screening was undertaken at Laboratories for Chemical Biology Umeå using a library of commercial compounds from Chembridge (www.chem.umu.se/english/lcbu), a 384 well format, and a fluorimetric assay measuring the hydrolysis of umbelliferyl arachidonate by recombinant human MGL. Follow-up assays used rat brain preparations with 2-oleoylglycerol (2-OG, cytosolic fraction) and anandamide (AEA, membrane fraction) as substrates for MGL and FAAH, respectively..

Results: A total of 17500 compounds were screened in the initial phase. Of these, 95 compounds produced >25% MGL inhibition at a test concentration of 10 μM. Based on considerations such as "drugability" (Lipinski's rule of five) and upon the outcome of preliminary concentration-response curves, 11 compounds were selected for secondary screening, where the criteria were selectivity *vs*. FAAH and a lack of time-dependent inhibition (such as would be seen with an irreversible compound). One compound, **I** [*N*-(3,4-dichlorophenyl)-3-[3-2-ethoxyphenyl)-1,2,4-oxadiazol-5-yl]propanamide], inhibited rat brain 2-OG hydrolysis with IC_{50} values of 2.3 and 2.7 μM following preincubation for 0 and 60 min, respectively, whilst AEA hydrolysis was inhibited with an IC_{50} value of 8.6 μM. A further 24 compounds from ChemBridge structurally related to **I** were investigated. Of these, two compounds were MGL-selective. **II** (*N*-(2-ethoxyphenyl)-3-[3-(2-ethoxyphenyl)-1,2,4-oxadiazol-5-yl]propanamide) inhibited rat brain 2-OG hydrolysis (no preincubation) with an IC_{50} value of 5.9 μM, and produced no inhibition of AEA hydrolysis at the highest concentration tested (30 μM). **III** (3-[3-(2-chlorophenyl)-1,2,4-oxadiazol-5-yl]-*N*-(3,4-dichlorophenyl) propanamide) was more potent, inhibiting 2-OG hydrolysis (no preincubation) with an IC_{50} value of 1.6 μM, and with a ten-fold selectivity vs. AEA hydrolysis (IC_{50} 17 μM). Neither compound affected the binding of [^{3}H]CP55,940 to rat brain CB_1 receptors over the concentration range tested (1-30 μM).

Conclusion: We have identified a novel class of MGL inhibitors with selectivity vs. FAAH and *vs*. the CB_1 receptor. Hopefully, these compounds can be used as a template for the design of potent MGL inhibitors with which to explore further the potential of this enzyme as a target for drug development.

Supported by grants from the Swedish Science Research Council, the Kurt & Alice Wallenburg Foundation, and the Kempe Foundation.

HIDDEN DIFFERENCES BETWEEN RAT AND HUMAN FATTY ACID AMIDE HYDROLASES

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Fatty acid amide hydrolase (FAAH) is a membrane protein that plays a relevant role in the metabolism of fatty acid amides and esters. It degrades important neurotransmitters such as oleamide and anandamide, and it has been involved in a number of human pathological conditions, representing therefore a valuable target for biochemical and pharmacological research. In this study, we have investigated *in vitro* the structure-function relationship of rat and human FAAHs. In particular circular dichroism, fluorescence spectroscopy and light scattering measurements have been performed, in order to characterize the structural features of the two proteins, both in the presence and absence of the irreversible inhibitor methoxyarachidonyl-fluorophosphonate. The results demonstrate that the structural properties of the two FAAHs are different, despite their high sequence homology and overall similarity in temperature-dependence. Additionally, membrane binding and kinetic assays of both FAAHs indicate that also the functional properties of the two enzymes are different in their interaction with lipid bilayers and with exogenous inhibitors.

Conclusions. These findings suggest that pre-clinical studies of FAAH-dependent human diseases based only on animal models should be interpreted with caution, and that the efficacy of new drugs targeted to FAAH should be tested *in vitro*, on both rat and human enzymes.

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DETERMINANTS OF FATTY ACID ACTIVATION OF THE PRONOCICEPTIVE ION CHANNEL TRPA1

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The transient receptor potential ankyrin 1 (TRPA1) channel expressed on primary afferent nociceptors detects potentially damaging environmental stimuli such as noxious cold, changes in pH, noxious chemicals and endogenous products of inflammation. Many irritant chemicals activate TRPA1 via covalent modification of intracellular N-terminal cysteines but this mechanism cannot explain the activation of this receptor by unreactive compounds, such as Delta-9-tetrahydrocannabinol (THC). In this study, we have investigated the activation of human TRPA1 by chemically unreactive fatty acids and their derivatives; including *N*-acyl neurotransmitter/amino acid conjugates and endocannabinoids like anandamide. HEK293 cells expressing human TRPA1 under the control of a tetracycline-sensitive repressor were grown in 96 well microplates. Intracellular calcium was measured using the calcium 5 kit from Molecular Devices using a FLEX Station Microplate Reader at 37^{o}C. Mutant hTRPA1 lacking 3 key reactive cysteines were also studied. Results are mean±sem of at least 3-5 determinations.

The covalent agonist cynnamaldehyde activated TRPA1 with an EC_{50} of 20±4μM, producing a maximum change of fluorescence of 500±30%. THC (30μM) only modestly activated the receptor (207±10%). Arachidonic acid (AA, 100μM) increased fluorescence by 430±140% with a notional EC_{50} of 10±2μM. Anandamide (180±40%), NA-tyrosine (120±20%), NA-glycine (70±35%) and NA-taurine (93±4%) all activated the channel at 30μM. Sub-threshold doses of AA (100 nM-1 μM) did not increase the maximum response of TRPA1 to cynnamaldehyde but cross-desensitization was observed between high concentrations of the two. In mutant TRPA1 lacking the three main reactive cysteines, the effect of AA (100 μM) was reduced by 43% while TRPA1 activation by cinnamaldehyde (300 μM) was reduced by 83%. Our data show that AA is a more potent activator of TRPA1 than its ethanolamide or amino acid/neurotransmitter derivatives. Our findings also suggest that AA and derivatives might activate TRPA1 via a mechanism distinct from that of cynnamaldehyde.

CHANGES IN CIRCULATING ENDOCANNABINOIDS AND N-ACYLETHANOLAMINES DURING OVERWINTERING AT CONCORIA RESEARCH STATION IN CONTINENTAL ANTARCTICA

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Background and Methods. Concordia station (Dome C, http://www.concordiabase.eu/) is located in one of the coldest, driest, and most inhospitable regions on the planet. The mean temperature at Dome C is -30°C during the summer and -60°C to -80°C during the winter. The summer season lasts three months with constant sunlight and the winter season from mid-February to mid-November with three months of unrelenting and total darkness. Overwintering at Dome C in Antarctica is characterized by an intense photoperiod during summer with high physiologic (outdoor) research activity by the crew followed by a pronounced drop in outside temperature and light and relatively low (mainly indoor) activity with longer periods of monotony and physiologic inactivity. During the nine month winter season there is no possibility of evacuation or delivering supplies to the station and communications with the outside world are very restricted. Thus, the crew living in this harsh environment is exposed to confinement and extreme isolation and has to be totally self-reliant[1].

The endocannabinoid system is known to be a key regulator of habituation and adaptation to stressful situations and environments[2]. In order to test the hypothesis that the extreme changes in environmental conditions experienced by the crew at Dome C influences circulating ECs, we profiled plasma concentrations of anandamide (ANA), 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and stearoylethanolamine (SEA) in 14 healthy male subjects during two winter-over periods lasting each one year.

Results. During the transition phase from the period of constant sunlight in summer to unremitting darkness at the peak of the Antarctic winter (April to September), plasma concentrations of ANA and OEA declined continously and significantly (ANA: 0.39±0.10 to 0.34±0.13 ng/ml, $p=0.03$; OEA: 2.83±0.89 to 2.31±0.70 ng/ml, $p<0.01$). PEA, SEA and 2-AG levels also declined but these changes were not statistically significant. At the end of the Antarctic winter (October/November), EC levels returned to baseline (February) values.

Conclusion. The dramatic environmental changes and their behavioral consequences during overwintering at Dome C are reflected by variations in EC signalling. Changes in EC plasma levels have also been observed in hibernating animals[3] which suggests biologic similarities between torpor states in animals and humans exposed to hibernation inducing conditions. This association should be studied in more detail as it may have consequences for a number of human conditions ranging from long-term exploratory space missions to organ protection during critical illness and the regulation of food intake and obesity.

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3. Vaughn LK et al. British journal of pharmacology 2010; 160: 530-43

GENERAL ANESTHESIA WITH PROPOFOL INFLUENCES CIRCULATING ENDOCANNABINOIDS AND N-ACYLETHANOLAMINES: A STUDY IN VOLUNTEERS

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Background. N-acylethanolamines (NAEs) and 2-AG are degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (2-AG), respectively. FAAH inhibition is known to increase endogenous levels of NAEs such as anandamide (AEA) and oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and stearoylethanolamide (SEA). Propofol is a known inhibitor of FAAH and influences NAE signaling in addition to its known potentiating effect on $GABA_A$ receptor activity. In the present study we profiled NAE and 2-AG plasma levels by HPLC-tandem-MS in volunteers undergoing anesthesia with propofol in order to characterize the relationship between NAEs and 2-AG, propofol plasma concentrations and depth of anesthesia which was quantified using an EEG derived parameter, the bispectral index (BIS).

Methods. Twenty volunteers (ASA I, age 29.3±8.0 yrs., 11 female, BMI 25.4±4.3) were administered propofol as an intravenous infusion (0.4 mg/kg/min) for 10 min followed by a 20 min recovery period. Propofol administration was then resumed with four escalating infusion rates (for 15 min each) to achieve targeted plasma concentrations of 2, 3, 4 and 5 mg/ml, followed by termination of the infusion and continued data collection for 270 min.

Results. NAE and 2-AG plasma concentrations increased after induction of anesthesia reaching maximal levels at 90 min when plasma concentrations of propofol were also maximal (6.50±1.92 mg/ml) and BIS values reached a nadir (27.6±8.0) indicating a deep state of anesthesia. As compared to baseline concentrations, the increases of OEA (from 2.68±1.31 to 3.72±1.85 ng/ml) and 2-AG (from 1.72±0.75 to 3.00±1.60 ng/ml) were statistically significant ($p<0.01$) and correlated with plasma propofol concentrations at 90 min after induction of anesthesia (2-AG: $r=0.54$, $p=0.01$; OEA: $r=0.54$, $p=0.03$). At 180 min after termination of the propofol infusion with the volunteers fully awake, plasma levels of OEA, AEA, PEA were significantly lower and 2-AG concentrations significantly higher (2.28±1.10 vs. 1.72±0.75 ng/ml, $p=0.03$) than baseline levels before induction of anesthesia.

Conclusions. Propofol increases plasma concentrations of endocannabinoids which may mirror central changes in endocannabinoid (EC) signaling. This finding may help to explain specific properties of propofol such as a lower risk for postoperative nausea and vomiting, an increased incidence of traumatic memories after intraoperative awareness compared to volatile anesthetics or mood enhancement and dreams. Pharmacologic manipulation of EC signaling could help to improve safety and outcome of general anesthesia.

LIPOPHILIC AMINES AS INHIBITORS OF *N*-ACYLETHANOLAMINE-HYDROLYZING ACID AMIDASE (NAAA)

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Bioactive *N*-acylethanolamines including anandamide (an endocannabinoid) and palmitoylethanolamide (analgesic and anti-inflammatory substance) are hydrolyzed to free fatty acids and ethanolamine by fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA). NAAA is a lysosomal enzyme acting only at acidic pH, which preferably hydrolyzes palmitoylethanolamide among various *N*-acylethanolamines. Thus, specific inhibitors of NAAA are expected as therapeutic drugs for pain and inflammation.

We screened simple long-chain alkylamines and long-chain amines having an ester or an ether moiety in the amine alkyl chains for inhibition of rat lung NAAA. Pentadecylamine and tridecyl 2-aminoacetate (tridecyl ester of glycine) were found to exhibit relatively potent inhibitory activities (IC_{50} = 5.7 μM and 11.8 μM, respectively), with much weaker effects on FAAH. As examined by Lineweaver-Burk plot, the inhibitions by both compounds were of competitive type. The alkylamines and alkylesters of glycine with shorter or longer carbon chains were less inhibitory for NAAA. These simple structures would provide a scaffold for further improvement in NAAA inhibitory activity.

BIOSYNTHETIC PATHWAYS OF *N*-ACYLETHANOLAMINES FROM *N*-ACYLATED PLASMALOGEN PHOSPHOLIPID

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In the brain, bioactive *N*-acylethanolamines, including the endocannabinoid anandamide, are formed from *N*-acylphosphatidylethanolamines (NAPEs) either by direct hydrolysis catalyzed by a specific phospholipase D (NAPE-PLD), or through multi-step pathways involving *O*-deacylation of NAPEs. In addition to phosphatidylethanolamine, which possesses two acyl chains at both *sn*-1 and *sn*-2 positions of the glycerol backbone, the brain abundantly contains plasmalogen-type ethanolamine phospholipid (plasmenylethanolamine, PlsEt) which has a vinyl ether bond at *sn*-1 position. *N*-Acyl-PlsEt may thus exist in the brain and serve as another precursor of *N*-acylethanolamine. Here, we examined this possibility with the brain of wild-type and NAPE-PLD-deficient (NAPE-PLD$^{-/-}$) mice. As analyzed by LC-MS/MS, NAPE-PLD$^{-/-}$ mice exhibited a decrease in *N*-acylethanolamines, including anandamide, and remarkable accumulations of NAPEs as well as lyso-NAPEs. Moreover, remarkable increases in *N*-acyl-PlsEts and *N*-acyl-lyso-PlsEts were also observed in NAPE-PLD$^{-/-}$ mice. The brain homogenate of NAPE-PLD$^{-/-}$ mice generated *N*-acylethanolamine from both of NAPE and *N*-acyl-PlsEt. The homogenate also formed various *N*-acylethanolamines from their corresponding *N*-acyl-lyso-PlsEts by a Mg^{2+}-dependent "lysophospholipase D". On the other hand, recombinant NAPE-PLD directly released *N*-acylethanolamine from *N*-acyl-PlsEt. These results strongly suggest that in brain tissue *N*-acylethanolamine is formed from both NAPE and *N*-acyl-PlsEt through the NAPE-PLD-independent pathway via *N*-acylated lysophospholipids as well as by its direct release by NAPE-PLD.

FREE AND ESTERIFIED N-ACYL ETHANOLAMINES IN PLASMA AND BLOOD CELLS: EVIDENCE FOR PREVIOUSLY IGNORED POOLS OF ESTERIFIED NAEs

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The origin of plasma *N*-acyl ethanolamines (NAEs) is not exactly known, and it is assumed that plasma levels are a reflection of system levels as plasma may act as a 'spill-over' sink. However, plasma contains numerous classes of lipids which could contain esterified NAEs, and blood cells might also synthesize these compounds. In the present study, we i) compared free to esterified plasma NAE levels in mice fed diets with different amount of n-3 fatty acids, and ii) investigated the presence of NAE in blood cells. For this purpose a UPLC-MS/MS method was developed and validated for the quantification of AEA, 2-AG, DHEA, DLE, EPEA, OEA, PEA and SEA in 100 µL plasma using a simple acetonitrile extraction step. This method was used to determine levels NAEs in plasma without and after an esterification step from animals that were on diets containing different mounts of n-3 fatty acids.

Plasma extracts contained 20-60 fold higher levels of esterified NAEs than free NAEs. Moreover, the effect of dietary n-3 fatty acids on free plasma NAE profiles was similar for esterified NAEs. Finally, esterified NAEs were also present in blood cells, and their pattern followed the same diet effect as observed for free and esterified plasma NAEs. Together, these data point to the presence of previously ignored pools of esterified NAEs in plasma and blood cells, which correlated with free plasma NAE levels.

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CHARACTERIZATION OF THE ENDOCANNABINOID SYSTEM DURING ZEBRAFISH (*DANIO RERIO*) EMBRYOGENESIS AND POSSIBLE DRUG SCREENING APPLICATIONS.

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The zebrafish (*Danio rerio*) has become a widely used vertebrate model organism in the last two decades. A combination of features like high fecundity, short development time and a well-know genome make this animal an important model for many research fields such as physiology, toxicology and pharmacology. Very little is known about the 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. However, these studies were based mainly on *in silico* methods that often fail to provide an understanding of the biological role for conserved genes. Therefore, we investigated the presence and the modulation of 14 orthologous genes of the endocannabinoid system during embryonic and larval zebrafish stages. In particular we quantified the mRNA expression of receptors and synthetic or catabolic enzymes for endocannabinoids. Our data reveal a time-dependent increased expression from 12 hours post fertilization to 7 days post fertilization of all the transcripts studied except for the 2-AG catabolic enzymes which are strongly down-regulated during hatching (from 48 to 72 hpf). Consistently, the quantification of endocannabinoid levels confirmed a significant increase of 2-AG with no appreciable changes in anandamide during hatching stages. These results reveal for the first time the presence of a complete endocannabinoid system in zebrafish and emphasize the conserved role of 2-arachidonyl glycerol during embryo development. In addition, given that zebrafish embryos appear to contain a fully fuctional endocannabinoid system, we propose this model is a suitable candidate for cannabinoid drug screenings.

Acronyms: N-arachidonylethanolamine, 2-AG: 2-arachidonyl glycerol.

BRAIN REGIONAL CANNABINOID CB_1 RECEPTOR SIGNALLING AND ALTERNATIVE ENZYMATIC PATHWAYS FOR 2-ARACHIDONOYLGLYCEROL GENERATION IN BRAIN SECTIONS OF DAGL-ALPHA AND DAGL-BETA DEFICIENT MICE

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In the central nervous system, endocannabinoids are produced and released locally by postsynaptic neurons. Then, endocannabinoids activate presynaptic CB_1 receptors in a retrograde manner to modulate the strength at various central synapses. The principal endocannabinoid in the brain, 2-arachidonoylglycerol (2-AG), is enzymatically produced from phospholipid precursors. The primary pathway for 2-AG generation is believed to be enzymatic conversion from the diacylglycerols (DAGs) by the two isoforms of *sn*-1-specific lipases, DAGLα and DAGLβ. Previous studies with DAGL-deficient mice have indicated that DAGLα is the major enzyme involved in the biosynthesis of 2-AG needed for retrograde synaptic signalling. In the current study, we investigated whether there are region-specific alterations in cannabinoid CB_1 receptor-dependent $G_{i/o}$ protein activity in brain cryosections of DAGL-deficient mice when compared to wild-type mice. We also wished to clarify whether *sn*-1-specific DAGLs are responsible for the 2-AG-dependent CB_1 receptor activity previously observed in brain cryosections after a comprehensive pharmacological blockade of 2-AG hydrolysis.

Functional autoradiography indicated that CB_1 receptor-dependent $G_{i/o}$-activity remains unaltered in all the studied brain regions of DAGLα-knockout and DAGLβ-knockout mice when compared to wild-type mice. When the degradation of endogenous 2-AG is pharmacologically blocked with an irreversibly acting inhibitor methylarachidonoylfluorophosphonate (MAFP), brain cryosections of all the genotypes are able to generate equal amounts of 2-AG, sufficient to activate CB_1 receptors throughout the brain regions endowed with these receptors. As demonstrated by LC/MS/MS, the CB_1 receptor-activating pool of 2-AG is generated via a tetrahydrolipstatin (THL) -sensitive enzymatic pathway but evidently not via DAGLα or DAGLβ. Therefore, this pool of 2-AG must be distinct from the DAGLα-dependent pool that is used for retrograde 2-AG signalling in living brain. We conclude that in addition to the *sn*-1-specific DAGLs, there are additional enzymatic pathways capable of generating 2-AG in brain tissue.

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TLR-3 MEDIATED CYTOKINE EXPRESSION IN THE RAT HIPPOCAMPUS IS ALTERED FOLLOWING PHARMACOLOGICAL INHIBITION OF FAAH

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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 data investigating their effects on inflammation associated with the activation of other TLRs. The present study examined the effects of URB597, a selective inhibitor of fatty acid amide hydrolase (FAAH), on endocannabinoid levels and cytokine expression in the hippocampus following systemic administration of the TLR-3 agonist polyinosinic:polycytidylic acid (poly I:C). Male Sprague Dawley rats received URB597 (1mg/kg, i.p.) or vehicle 30 minutes prior to systemic administration of poly I:C (3mg/kg, i.p.) or sterile saline. Animals were sacrificed at 2, 4, 8 and 24 hours post poly I:C challenge, the hippocampus dissected, snap-frozen and stored at -80°C. The expression of interferon (IFN)α, IFNβ, the chemokine IP-10, TNFα, IL-1β and IL-6 were determined using quantitative RT-PCR. Concentrations of the endocannabinoids, anandamide and 2-AG, and the related fatty acid ethanolamines, N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA), were determined using LC-MS-MS. Data were analysed using ANOVA followed by Fisher's LSD *post-hoc* test. $P < 0.05$ was deemed significant.

Systemic poly I:C administration significantly increased the expression of hippocampal IFNα at 24 hours, IL-1β at 4hr, TNFα and IP-10 at 4 and 8 hours when compared to saline-treated controls. In the presence of URB597, IFNα expression was increased in the hippocampus at 4 hours, IL-6 and IP-10 at 4 and 8 hours and TNFα at 8 hrs following poly I:C administration. Furthermore, URB597 attenuated the poly I:C-induced increase in TNFα and IL-1β mRNA expression, 4 hours post administration. URB597 did not alter anandamide or 2-AG levels in the hippocampus at any of the time points examined, but significantly increased OEA and PEA concentrations at 2, 4 and 8 hrs post poly I:C administration. In conclusion, the present study demonstrates that enhanced levels of OEA and PEA in the hippocampus following inhibition of FAAH are associated with an altered profile of expression of inflammatory mediators following TLR-3 activation. Improved understanding of FAAH-mediated regulation of neuroimmune functions will aid in the identification of new therapeutic targets for various neuroinflammatory disorders.

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IDENTIFICATION OF MRNA FOR ENDOCANNABINOID BIOSYNTHETIC ENZYMES WITHIN HIPPOCAMPAL PYRAMIDAL CELLS AND CA1 STRATUM RADIATUM INTERNEURON SUBTYPES USING QUANTITATIVE REAL-TIME PCR

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The hippocampus is required for short-term memory and contains both excitatory pyramidal cells and inhibitory interneurons. Both cell types exhibit various forms of synaptic plasticity, the mechanism underlying learning and memory. Recently, endocannabinoids (eCBs) have been identified to be involved in many types of plasticity including a novel long-term depression between CA3 pyramidal cells and CA1 stratum radiatum interneurons where the interneuron itself was suggested to produce the eCB involved. However, the expression pattern of eCB biosynthetic enzymes within stratum radiatum interneurons has been unclear, and their capacity to produce eCBs debated. Therefore, our goal was to describe the distribution of eCB biosynthetic enzymes within CA1 stratum radiatum interneurons and CA3/CA1 pyramidal cells using real-time quantitative PCR (RT-qPCR). We extracted RNA from single cells using patch electrodes and after reverse transcription, used probe based RT-qPCR to detect the presence of 12-lipoxygenase, N-acyl-phosphatidylethanolamine-specific phospholipase D, diacylglycerol lipase α, as well as type I metabotropic glutamate receptors, which are known to be involved in eCB production and plasticity. Interneuron markers such as parvalbumin, calbindin, calretinin, and cholecystokinin were used to correlate eCB biosynthetic enzyme expression to interneuron subtype. Spiking patterns recorded from interneurons, while varied, support categorization of interneuron subtypes based on these markers. Results identified that eCB biosynthetic enzyme mRNA is expressed within interneurons and is indeed coexpressed with type I metabotropic glutamate receptors, suggesting that stratum radiatum interneurons could putatively produce eCBs necessary for synaptic plasticity. This expression is also subtype-specific. For example, mGluR5 was coexpressed with diacylglycerol lipase α and N-acylphosphatidylethanolamine-specific phospholipase D, but not 12-lipoxygenase; while mGluR1 was coexpressed with all tested eCB biosynthetic enzymes, mainly in cholecystokinin-calbindin cells. In addition, we identified that CA3 and CA1 pyramidal cells express eCB biosynthetic enzyme mRNA. Finally, quantitative levels of expression illustrate very similar amounts of type I metabotropic glutamate receptors in interneurons versus pyramidal cells, with some differences in eCB biosynthetic enzyme mRNA between them. Collectively, our data provide the first RT-qPCR evidence for putative eCB production in interneurons, suggesting their potential ability to regulate eCB-mediated processes such as synaptic plasticity.

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CNR2 AND *ABHD4* GENE EXPRESSION IS UP-REGULATED IN ASTHMA PATIENTS

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Asthma is defined as a chronic inflammatory disorder of the airways. The complex immune mechanisms that are associated with and probably causal to, persistent airways inflammation and asthma exacerbation indicate that an imbalanced immune system is the primary driving force underlying asthma. It is generally believed that endocannabinoids act as native modulators of immune system, probably through cannabinoid receptors activation. Thus, the aim of this study was to determine if gene expression levels of CB2 (*CNR2*), α,β-hydrolase 4 (*ABHD4*) and fatty acid amide hydrolase (*FAAH*) in untreated atopic and non-atopic asthma patients and if there was a correlation with clinical parameters.We analyzed a case-control cohort composed of 202 children with newly detected mild or moderate persistent asthma, aged 5-18 years and 184 healthy unrelated age and sex matched controls. Several important clinical and laboratory parameters were measured in asthmatics and handled as quantitative variables. RNA was extracted from total blood leukocytes and gene expression levels were measured by qPCR.

Median gene expression levels of *CNR2* and *ABHD4* were significantly higher in all asthma patients (*CNR2*, p = 1.0E-03; *ABHD4*, p = 2.0E-04), atopic (*CNR2*, p = 0.02; *ABHD4*, p = 4.0E-04) and non-atopic (*CNR2*, p = 0.02; *ABHD4*, p = 0.03), while for *FAAH* no significant difference was found when compared with control. Spearmen correlation analysis of gene expression levels with clinical data revealed a positive correlation between: forced expiratory volume in 1 second (FEV1) and *ABHD4* in all asthmatics (p = 0.03); fraction of nitric oxide in exhaled air (F_ENO) with *ABHD4* (p = 7.2E-03) and *FAAH* (p = 0.02) in non-atopic asthmatics; eosinophilia and *CNR2* in all asthmatics (p= 0.05) and *FAAH* in atopic asthmatics (p=0.04); and, immunoglobulin E and *FAAH* in non-atopic asthmatics (p = 0.02). A negative correlation between $logPC_{20}$ (provocative concentration of methacholine causing a drop in FEV1 of 20%) of *ABHD4* (p = 7.5E-03), *FAAH* (p = 0.05) was found only in atopic asthmatics and *CNR2* (p = 0.03) is all asthmatics.

In conclusion, our results suggest that the endocannabinoid system is up-regulated in asthma patients but is correlated with bronchial hyperreactivity, markers of atopy and eosinophilic inflammation differently in atopic and non-atopic asthmatics. Future studies will focus on the effect of anti-asthmatic treatment on the expression levels of these genes and how these relate to plasma endocannabinoid levels.

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DISTRIBUTION OF ABHD12 PROTEIN IN MURINE BRAIN

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Cannabinoids are part of an endogenous signaling system consisting of cannabinoid receptors and endogenous cannabinoids (eCBs) as well as the enzymatic machinery to produce and break down these eCBs. Monoacylglycerol lipase (MGL) appears to serve as the chief enzyme responsible for metabolism of the eCB 2-arachidonoyl glycerol (2-AG). However evidence has surfaced that other enzymes are also capable of breaking down 2-AG, including ABHD6 and ABHD12 as well as COX2 and FAAH. Of these the least well characterized is ABHD12. Although ABHD12 accounts for a relatively minor portion (~10%) of 2-AG metabolism in brain homogenates, ABHD12 may play a central role in sub-regions of the CNS. Supporting this notion is a recently published account identifying deleterious mutations in ABHD12 as the cause of PHARC syndrome, a heritable disease with symptoms that include polyneuropathy and several ocular abnormalities.

It has remained difficult to study the function of ABHD12, partly due to a shortage of tools to determine the distribution of ABHD12 protein in the CNS. To address this shortcoming, we have developed an antibody against ABHD12, verified its specificity using ABHD12-/- mice, and have used this tool to study ABHD12 protein in the murine brain. We have found, consistent with available data regarding ABHD12 mRNA, that ABHD12 is widely distributed in the brain. For instance, staining is abundant in all major regions of the hippocampal formation, including the dentate gyrus, CA3 and CA1 regions. In the CA1 ABDH12 is found throughout the synaptic layers including stratum oriens and radiatum. Expression is characterized as being largely diffuse and punctate in nature. ABHD12 immunoreactivity colocalized with some neurons, including interneurons.

In summary, we have found that ABHD12 protein expression is abundant in the murine brain. As a consequence, ABHD12 is well-placed to play a role in eCB metabolism in many important physiological functions of the CNS. Given the profound consequences of deleterious mutations of ABHD12 as observed in patients with PHARC syndrome, this suggests that an investigation of the function of ABHD12 in CNS will be important to understanding the signaling of cannabinoids and other lipids and their role(s) in health, disease and neuronal function.

REGULATION OF ENDOCANNABINOID LEVELS BY STEROL CARRIER PROTEIN 2

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Considerable data indicate that prolonged hypothalamic-pituitary-adrenal (HPA) axis activation has detrimental physiological and psychological effects and is likely causative for depression, anxiety and post-traumatic stress disorders. The HPA axis is normally inhibited by several negative feedback loops. Corticosteroids activate glucocorticoid receptors (GR) in the hypothalamus, hippocampus and prefrontal cortex which activate circuits that inhibit CRF-releasing cells in the paraventricular region of the hypothalamus. The endocannabinoid system is a critical part of all of these negative feedback loops and loss of endocannabinoid signaling leads to excessive and prolonged activation of the HPA axis in rodent models. Furthermore, the amygdala functions as a part of the limbic system to link cortical regions including the prefrontal cortex which process sensory information with effector regions of the hypothalamus and brainstem and has long been associated with emotional control. Taken together, there are numerous areas in which eCBs function to maintain psychological health. Based on numerous previous studies, synthesis and mobilization of endocannabinoids, is central to regulation of mood by eCBs through regulation of neurotransmitter release. Occuring on the magnitude of minutes, synthesis and release of eCBs must occur in a rapid fashion. Since the endocannabinoids, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), are only marginally soluble in water, we hypothesize that they bind to lipid binding proteins to facilitate aqueous distribution and solubility. We hypothesize further that the binding of endocannabinoids, particularly AEA, to lipid binding proteins is a mechanism for intracellular sequestration and may also serve as a mechanism for regulated release. We have identified sterol carrier protein 2 (SCP-2) as a potential candidate for eCB trafficking. SCP-2 has been extensively studied in the transport of cholesterol and other fatty acids mainly in the liver, however, its ability to shuttle a variety of lipid molecules including arachidonic acid led us to determine if it plays a role in eCB mobilization. Indeed, in *in vitro* overexpression systems, SCP-2 allows for the uptake of AEA and concentration within cells in a temperature dependent fashion. Due to these promising results, we chose to examine n-acetylethanolamide and monoacylglycerol levels within the brains of mice lacking SCP-2. Interestingly, we have found alterations in both n-acetylethanolamide and monoacylglycerol levels within some regions of the brain. In particular, 2-AG and 2-palmitoylglycerol (2-PG) is significantly diminished in the amygdala of mice that lack the lipid carrier protein, sterol carrier protein 2 (SCP-2) compared to wild type counterparts. Furthermore, the prefrontal cortex shows trend of decreased 2-AG levels in SCP-2 knockout mice. Also, in these same brain regions, AEA also shows a decreasing trend. In other brain regions including the hippocampus and hypothalamus, alterations in eCBs were not observed as significantly different, potentially due to the differential concentration of SCP-2 within the various brain regions. Taken together, we believe that SCP-2 functions to store and transport endocannabinoids making them readily available for use in times of need.

SOCIAL PAIN AND THE ENDOCANNABINOID SYSTEM: ADOLESCENT PEER-REJECTION PERSISTENTLY ALTERS PAIN PERCEPTION AND CB1 RECEPTOR EXPRESSION IN FEMALE RATS

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Endocannabinoids and CB1 receptors are present in the major pain pathways and strongly modulate pain processing through central and peripheral mechanisms. A link between the neuronal mechanism responsible for the modulation of physical pain and the experience of social pain, such as social loss oder social rejection has been demonstrated in human imaging studies. However, no such link has been provided in animal research so far. Peer-interactions become particularly important during adolescence and hence, human teenagers display enhanced sensitivity towards social rejection which might even contribute to the development of neuropsychiatric disorders. Here we studied potential adverse consequences of inadequate social encounters in adolescent rats, which we propose as an operational model for adolescent peer-rejection. Female adolescent Wistar rats were either reared with another Wistar rat (adequate social rearing; WIS_{ASR}), or with an age-matched Fischer344 rat (inadequate social rearing; WIS_{ISR}). From day 50 on, all Wistar rats were group housed with same strain partners and tested for social behavior, emotional and pain-reactivity, as well as for neurobiological/endocrine differences. We detected profound differences in social play between Fischer344 and Wistar rats. Pairing of both strains decreased playful peer-interactions for WIS_{ISR} animals. Consequently, adult WIS_{ISR} rats showed increased emotional-reactivity and decreased pain-sensitivity than WIS_{ASR} animals. Both groups also differed in their endocrine stress-response and in expression-levels of thalamic CB1 receptors. Adolescent inadequate social rearing results in distinct behavioral and neurobiological/endocrine alterations, with similarities to consequences of social rejection reported in humans. The present animal model represents a novel and valid approach for assessing long-term consequences of peer-rejection and our findings provide further evidence for a common neuronal regulation of social and physical pain, which seems to involve the endocannabinoid system. Additionally, our model offers potential important insights for clinical conditions related to social rejection such as borderline personality disorder and posttraumatic stress disorder.

ENDOGENOUS CANNABINOIDS AND RELATED *N*-ACYL AMIDES ARE UP AND DOWN-REGULATED IN A SPINAL CORD INJURY MODEL OF NEUROPATHIC PAIN

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Neuropathic pain, which is described as any pain resulting from dysfunction or lesion to the nervous system, in its chronic form, remains one of the most prominent ailments of multiple diseases including spinal cord injury. Many of the signalling pathways involved in both development and treatment remain unknown, and much of the current treatments fail to alleviate the pain on a long term scale. Signalling molecules, such as prostaglandins (PGs), reactive oxidative species, and cytokines, have been found in the maintenance of chronic pain states in the CNS upon their release from microglia. Preventing this release lessens the presence of pain behaviour displayed by the animal. Knocking out Mammalian Transient Receptor Potential (TRP) channels (TRPV 1-4, TRPM8, and TRPA1) in the dorsal root ganglia (DRG) of rats, was also found to reduce pain behaviour. Endogenous cannabinoids (eCB) and *N*-acyl amides are signalling molecules that share structural homology to endogenous vanilloids and have been found to activate many TRP channels. Here, we test the hypothesis that PGs, eCBs and related *N*-acyl amides are up or down regulated in a spinal cord injury model of neuropathic pain.

A chronic pain state was induced using the Wieseler et al (2010) model for a T13/L1 avulsion[1]. After the actual or sham surgery, rats were sacrificed at 24 hours, 2 weeks, and 6 weeks. Spinal cord tissue was harvested from the site of the injury (T13) and the site associated with pain (L1). Partial purification of lipids was performed using methanolic extracts on C18 solid-phase extraction columns as previously described (Bradshaw et al, 2006)[2]. HPLC/MS/MS was then used to identify and quantify 15 lipids (3 eCBs-2-AG, AEA, and NAGly; 2 PGs-PGE2 and PGF2 alpha, 5 *N*-acyl ethanolamines, and 5 *N*-acyl serines). Each lipid was quantified using a standard curve made by synthetic lipids either purchased from Cayman chemical, Tocris Bioscience, or made in house. Comparisons were made to levels of the sham operated animal at the same level of the spinal cord at the same time point.

All 15 compounds were detected in each spinal level. While there were changes from baseline, neither PGs measured were significantly different in the avulsion or sham surgery at any time point. At 24 hours post surgery, no significant changes in eCB or *N*-acyl amides were seen in L1 tissue; however, levels of 3 *N*-acyl serines (palmitoyl, oleoyl, and docosahexaenoyl conjugates) at T13-the level of injury- were significantly increased. *N*-docosahexaenoyl ethanolamine (DHEA), decreased significantly after two weeks in L1 tissue, whereas, at the 6 week time point (when the level of pain behaviours has reached a maximum) there were significant decreases in 4 *N*-acyl ethanolamides (including AEA and DHEA) in both T13 and L1, whereas, levels of 2- AG significantly increased only in T13. These data provide evidence that eCBs and related *N*-acyl amides play an important role in the spinal mechanisms of neuropathic pain.

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LEVELS OF ENDOCANNABINOIDS AND RELATED N-ACYL AMIDES CHANGE IN THE CEREBELLUM, MIDBRAIN, BRAINSTEM AND THALAMUS IN A MODEL OF ACUTE PERIPHERAL INFLAMMATION

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Previous studies have demonstrated the importance of the endogenous cannabinoid system in a variety of inflammatory and pain conditions. Notably, the non-opioid component of acute stress-induced analgesia was shown to be a combination of differential signaling of 2-arachidonoyl glycerol (2-AG) and Anandamide (*N*-arachidonoyl ethanolamine; AEA) in the midbrain periaqueductal grey (Hohmann et al. 2005). In order to test the hypothesis that these findings extend to additional areas of the brain and for additional lipids, we use a model of acute peripheral inflammation that is associated with an increase in thermal and mechanical hyperalgesia and performed lipidomics screens of the midbrain, brainstem, thalamus and cerebellum for 2-AG, AEA and 79 structurally similar lipids (*N*-acyl amides and prostaglandins).

Using HPLC/MS/MS we performed scans for 81 lipids from methanolic extracts of MB, BS, TH, and CER in a rat model using either 3% λ-carrageenan to induce inflammation in the hindpaw or a vehicle injection (saline) as a control. Animals were sacrificed either 1 or 3 hours after injection. Lipids were partially purified from each dissected brain region on C18 solid-phase extraction columns and multiple fractions analyzed using HPLC/MS/MS. Standards were purchased from Caymen Chemical or made in house. Data were compared by time point and between vehicle control (VH) and carrageenan (CG) group.

Several patterns were observed in lipid production among the 4 brain areas using this inflammatory model. Few changes in lipid production were measured between the VH-1hour and CG-1 hour in any of the brain areas, however, both CER and BS had a significant decrease in *N*-arachidonoyl GABA (a TRPV1 activator) and in addition, CER and MB had significant increases in 2-AG. At 3 hours post-injection, among many other changes in lipid production, all 4 brain areas had an increase in 3 *N*-acyl ethanolamines (OEA, LEA, AEA), whereas, CER and TH also showed significant increases in DHEA. No differences were measured in levels of 2-AG at 3 hours post injection in any brain area. Of the four brain areas examined here, CER had the most dynamic changes in lipid profiles with over 20 lipids changing by 3 hours post injection. Notably, while CER levels of 2-AG were significantly increased at 1 hour post injection and back to baseline at 3 hours, CER levels of the prostaglandins PGE2 and PGF2alpha were increased at 3 hours post injection, supporting recent evidence that there is a potential biosynthetic link between 2-AG and PG production (Nomura et al., 2001). These data extend and support the hypothesis that peripheral inflammation associated with pain drive changes in eCBs and related lipid mediators in the MB and other areas of the CNS.

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PERIPHERAL INFLAMMATION DRIVES CHANGES IN *N*-ACYL AMIDES IN THE STRIATUM AND HIPPOCAMPUS

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N-acyl amides form a large and expanding family of endogenous lipids consisting of over 60 identified compounds. The most extensively characterized *N*-acyl amide is *N*-arachidonoylethanolamine (anandamide), an endogenous cannabinoid which has activity at CB_1, CB_2, and TRPV1 receptors. While several other *N*-acyl amides produce cannabimimetic effects and share synthetic and metabolic pathways with anandamide, they have low or no affinity at known cannabinoid receptors, and the physiological relevance of the majority of these molecules remains unknown. Some *N*-acyl amides have activity at G protein-coupled receptors and TRP receptors, and data suggest that these compounds play a role in inflammation and pain. This study seeks to compile and identify the effects of inflammation on levels of *N*-acyl amides in the brain, specifically in the striatum and hippocampus, which have been shown to be CNS regions involved in pain.

We examined levels of these putative signaling molecules in a rat model using either 3% λ-carrageenan to induce inflammation in the hindpaw or a vehicle injection (saline) as a control. Animals were allowed to recover for either 1 or 3 hours. Lipids were extracted from bilateral striata and hippocampi then partially purified on C18 solid-phase extraction columns and analyzed using high-performance liquid chromatography in conjunction with tandem mass spectrometry. *N*-acyl amide standards were purchased from Cayman Chemical or synthesized in house.

HPLC/MS/MS methods were analyzed for 81 compounds. 57 and 48 analytes were detected in striatal and hippocampal samples respectively. There were very few significant changes in levels of *N*-acyl amides in a comparison of 1 hour post-vehicle and 1 hour post-carrageenan conditions. However, there were significant increases in several *N*-acyl amides, particularly those in the *N*-acyl ethanolamine and *N*-acyl glycine families, in both brain areas following 3 hours of inflammation causing a significant increase in 14% of *N*-acyl amides detected in the striatum and a significant increase in levels of 27% of *N*-acyl amides in the hippocampus. Overall, the results of this study suggest that *N*-acyl amides perform a role in mediating the response to peripheral inflammation as well as a potential role in pain and stress.

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CHARACTERIZATION OF A MODEL OF OCULAR PAIN/DISCOMFORT WITH RESPECT TO CYCLO-OXYGENASE AND PROSTANOID RECEPTOR INVOLVEMENT

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Cyclo-oxygenase inhibitors are frequently used to alleviate the ocular surface pain associated with surgical procedures, which implies a role for prostanoids as hyperalgesic agents. These studies were intended to extend a previously reported animal model of ocular surface pain by elucidating the pharmacological basis of the prostanoid mediated component of the response to locally applied capsacian .The capsacian induced ocular pain model in rats was employed. All drugs and vehicle were applied to the eye in a 10μL volume. Prostanoid receptor antagonists, or vehicle, were applied as a 30 min pretreatment. Capsacian was applied at a 10mg/mL concentration and the behavioral response recorded was a 10 min period. Wiping of the eye with the hind paw was monitored, eye closure was ignored since it often occurred contemporaneously. After an initial set-up protocol of 30 min to monitor the behavioral response to ocular capsacian administration, it was clear that the response was of brief duration and the behavioral monitoring phase was reduced to 10 min. Cyclo-oxygenase inhibitors diclofenac and ketorolac, at the clinically employed dose, inhibited capsacian induced ocular surface pain/discomfort by about 50%. The activity of a series of potent and selective prostanoid receptor antagonists was examined at 0.01%, and 0.1%, and 1% concentration. Inhibition of the capsacian induced ocular pain/discomfort response was afforded by EP_1, EP_4, and FP receptor antagonism by SC-51322, GW 627368, and AS-604872, respectively. Antagonists at DP_1 (BW A868C) DP_2/TP (TM-30089), EP_2 (PFE-04418948), EP_3 (L-826266) and TP (SQ 29548) receptors were inactive. Two IP antagonists were used, RO-1138452 and RO-3244019 but only RO-324019 was active. Combination of active antagonists produced no meaningful additivity. COX-2 inhibitors were inactive. The prostamide$F_{2\alpha}$ antagonist AGN 211336 also significantly reduced capsacian induced ocular surface pain .

Conclusions: Capsacian induced ocular pain/discomfort exhibits a distinct prostanoid mediated pharmacological component. This accounts for about half the response. The involvement of EP_1, EP_4, and FP receptors was apparent: each operated in a non-additive, mutually exclusive manner. The prostamide antagonist AGN 211336 was also active and this, together with the FP antagonist data , casts doubt on the predictive utility of this model because FP and prostamide receptor agonists do not produce ocular surface pain in humans.

ACTIVATION OF PPARα IN DORSAL ROOT GANGLION NEURONS MODULATES NOCICEPTION AND REDUCES TUMOR PAIN IN MICE

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Anandamide (AEA) activation of cannabinoid type-1 receptors on nociceptors sets the threshold for the activation of the neurons in response to noxious stimuli. Previously we have shown that a decrease in this signaling pathway in tumor-bearing mice contributes to tumor-related mechanical hyperalgesia that is reduced by the elevation of the level of AEA in the area near the tumor (Khasabova et al., 2008). We provide evidence that a related lipid ethanolamide, palmitoylethanolamide (PEA), has the same effect on mechanical sensitivity, but its action is mediated by the peroxisome proliferator-activated receptor-alpha (PPARα). Intraplantar (i.pl.) injection of the PPARα antagonist GW6471 increased mechanical sensitivity locally in naive mice. Because PEA is the preferred substrate for *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), intraplantar injection of the NAAA inhibitor ARN077 was used to increase the local level of PEA. Co-administration of ARN077 with GW6471 blocked the effect of GW6471. ARN077 had no effect on mechanical sensitivity on its own, suggesting that PPARα is saturated under basal conditions. The pattern of drug activity was reversed in tumor-bearing mice. Mechanical hyperalgesia occurred in the tumor-bearing hind paw within 10 days of implantation of fibrosaroma cells in the calcaneous bone, and the hyperalgesia was accompanied by a decrease in PEA in DRGs innervating the tumor-bearing paw. Injection of ARN077 (i.pl) ipsilateral to the tumor reduced the mechanical hyperalgesia but had no effect on mechanical sensitivity in the contralateral paw. The effect of ARN077 was blocked by co-treatment with GW6471. The effect of each drug *in vivo* was mimicked *in vitro* in which the tumor condition was modeled by co-culture of DRG neurons with fibrosarcoma cells. In this model PEA as well as the NAAA inhibitor reduced the amplitude of the depolarization-evoked Ca^{2+} transient in isolated DRG neurons co-cultured with fibrosarcoma cells. Together these data support the conclusion that activation of PPARα by PEA and related substrates of NAAA modulate mechanical sensitivity of somatosensory neurons and may be a useful strategy in the management of cancer pain.

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NERVE INJURY-INDUCED MOLECULAR CHANGES: ROLE OF THE ENDOCANNABINOID AND ENDOVANILLOID SYSTEMS

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Neuropathic pain is a multifactorial condition caused by damage or dysfunction of the nervous system. There is considerable evidence supporting a role for endocannabinoids (ECs) in the modulation of pain. Anandamide (AEA), the best studies endocannabinoid activates cannabinoid receptors – CB1 and CB2 but is also the best characterized endogenous ligand of TRPV1and consequently AEA is also termed an endovanilloid. The potential involvement of TRPV1 in the analgesic effect of AEA has been raised. Recent animal studies indicate that AEA is effective against chronic pain of both neuropathic and inflammatory origin. AEA is synthesized on demand in three synthesis pathways: 1) direct conversion by NAPE-PLD; 2) PLC- and 3) PLA2-catalyzed. Due to efficient enzymatic degradation mainly by FAAH but also LOX-12/15 and COX-2, locally released ECs have a short half-life. Dysregulation of the EC system underlies several neurological disorders, thus therapeutic strategies manipulating the EC system are being developed to gain global picture of its components' regulation in the animal models of chronic pain.

Present research focuses on endocannabinoid-endovanilloid interaction in the development and maintenance of chronic constriction injury (CCI) model of neuropathic pain. We also studied other components potentially linked to ECs and TRPV1: bradykinin receptors (B1R and B2R), which amplify production of 12-LOX metabolites, activators of TRPV1 and secondary messengers like p38 (activated by TRPV1 elevation). As the TRPV1 is regulated by kinases and channel desensitization is calcium dependent we also investigated the role of PKA, PKC and CaMKII. Finally, since the anti-inflammatory effects of ECs are complex and may involve modulation of cytokines we also looked at proinflammatory interleukins production during the development of neuropathic pain.

Wistar rats underwent sciatic nerve ligation (CCI model). Three, 7 and 14 days after CCI lumbar spinal cord and L4-L6 DRGs were collected and stored at -20°C in RNAlater reagent. Isolated RNA has been rewritten in reverse transcription reaction. Quantitive PCR were performed using Assay-On-Demand Taqman probes. Genes expression levels were assessed against housekeeping gene.

CB1 and TRPV1 transcripts were present in the lumbar spinal cord (SC) of control rats and were slightly altered both in DRG and spinal cord of CCI rats in the first days after injury. mRNA levels of NAPE-PLD were not elevated neither in DRG nor in the SC tissue. The PTPN22/SHIP1 pathway was gradually elevated from day 3 to day 7 and reached back control values at day 14 in spinal cord, and still increased in DRG, suggesting an increase in PLC-depended AEA synthesis. The subsequent PLA-depended AEA anabolic pathway was significantly enhanced in DRG, but only a trend was observed in the SC of CCI rats. The main AEA-degradation enzyme, FAAH, was significantly enhanced both in DRG and SC of CCI rats. The 12-LOX pathways was slightly activated, whereas the 15-LOX remain unaffected in the DRG of CCI rats. Spinal levels of both 12- and 15-LOX were significantly altered in CCI rats. No changes in COX-2 expression levels occurred in DRG, while at the level of the spinal cord initial increase lasting up to day 7 was observed. Kinin B2, but not kinin B1 receptor (R) gene expression was increased in the sensory DRG after CCI, particularly 7 and 14 days after the injury. Spinal BK1R peaked at day 3, while no other changes were observed for day 7 and 14 neither for BK2R. p38 MAPK activation was observed both in DRG and SC of CCI rats exclusively at early time points of neuropathic pain development (day 3 and 7). CaMKII increased exclusively in the DRG of CCI rats. Finally the level of proinflammatory cytokines was significantly increased throughout the development of neuropathic pain.

The functional elements of ECs in the DRG and spinal cords of CCI rats were increased. The significant involvement of alternative AEA-anabolic pathway (both PLC- and PLA-catalyzed) may account for the recent finding that AEA tissue levels are unchanged in NAPE-PLD -/-. Our findings supported the hypothesis that CaMKII is required for the maintenance of neuropathic pain induced by peripheral nerve ligation and that p38 contributes to its development. Additionally data on the increased kinin B2 receptor corresponds to altered pain behavior after CCI.

Our data predict that both CB1 and TRPV1 system present in the DRG and spinal cord may be an important therapeutic target for the treatment of pain and ongoing inflammation associated with CCI in rats. They highlight the interaction between EC and endovanilloid systems and the complex activation of TRPV1 by AEA and products of its metabolism.

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PACLITAXEL-INDUCED NEUROTOXICITY: EFFICACY AND SAFETY OF CANNABIDIOL AS A NOVEL TREATMENT STRATEGY

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Paclitaxel (PAC) is associated with a chemotherapy-induced neuropathic pain (CINP) state that can lead to the cessation of treatment in late stage breast cancer patients, even in the absence of alternate therapies. Cannabinoid-based therapies are currently under investigation for the treatment of various neuropathic pain states as well as the direct targeting of tumor progression, but the psychoactivity of these agents may limit their clinical utility. We hypothesized that the non-psychoactive *Cannabis* constituent cannabidiol (CBD) would reverse or prevent the onset of PAC-induced CINP in female C57Bl/6 mice. We also hypothesized that CBD would not produce conditioned rewarding effects, disrupt cognitive performance, or attenuate the efficacy of PAC at inhibiting breast cancer cell activity. To test these hypotheses, we investigated the effect of CBD on both prevention and reversal of PAC-induced mechanical sensitivity using Von Frey filament testing, and on its ability to reverse a PAC-induced negative affective state using a modified place conditioning procedure. We also determined the effect of CBD alone on place conditioning and on a conditioned learning and memory task called autoshaping. In addition, we assessed the activity of CBD and PAC alone and in combination on breast cancer cell viability using the combination index (CI).

Treatment with PAC (4.0 and 8.0 mg/kg X 4 inj) produced significant mechanical sensitivity in female C57Bl/6 mice that could either be prevented or reversed by administration of CBD (2.5 – 10 mg/kg). Furthermore, the protective effect of CBD was reversed by co-administration of the 5-HT1A antagonist WAY 100635, but not the CB1 antagonist SR141716 or the CB2 antagonist SR144528. Mice treated with PAC also showed a significant place conditioning for CBD, while CBD produced no conditioned rewarding effects in naïve mice. CBD in this dose range also did not affect acquisition or retention in the autoshaping procedure. At optimal concentrations, CBD+PAC combinations produce additive to synergistic inhibition of breast cancer cell viability. Our data suggest that CBD is protective against PAC-induced neurotoxicity and that this effect is mediated by the 5-TH1A receptor system. Furthermore, CBD treatment was devoid of other nervous system effects such as conditioned reward or cognitive impairment. CBD also did not attenuate the efficacy of PAC in targeting breast cancers. Taken together, adjunct treatment with CBD during PAC chemotherapy treatment may be safe and effective in the prevention or attenuation of CINP.

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SELECTIVE ACTIVATION OF CANNABINOID CB_2 RECEPTORS SUPPRESSES CHEMOTHERAPY-INDUCED NEUROPATHY INDEPENDENT OF CB_1 OR CXCR4 RECEPTOR SIGNALING

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Cannabinoids suppress neuropathic pain produced by traumatic nerve injury, disease states and toxic insults. We previously showed that AM1710, a cannabilactone CB_2 agonist, produces antinociception without producing central nervous system side-effects typical of cannabinoid CB_1 receptor activation (Rahn et al. (2011) *Pharm, Biochem Behav* 98: 493-502). However, effects of AM1710 in models of pathological pain remain poorly characterized. Chemotherapeutic agents such as paclitaxel and cisplatin produce dose-limiting toxic neuropathy through mechanisms that remain poorly understood. CXCR4 (alpha-chemokine receptor) signaling contributes to the maintenance of neuropathic pain produced by traumatic nerve injury and HIV-associated neuropathy. However, whether CXCR4 signaling contributes to chemotherapy-induced neuropathy is unknown. We, therefore, examined the ability of the CB_2 agonist AM1710 to suppress mechanical and cold allodynia evoked by taxane (i.e. paclitaxel) and platinum (i.e. cisplatin) -based chemotherapeutic agents. We examined the contributions of CB_1, CB_2 and CXCR4 signaling to both chemotherapy-induced neuropathy and CB_2 agonist efficacy. Paclitaxel and cisplatin were used to produce neuropathy. The CB_2 agonist AM1710 suppressed mechanical and cold allodynia in paclitaxel and cisplatin models through a CB_2-specific mechanism without altering basal nociceptive thresholds. AM1710 suppressed paclitaxel-evoked mechanical and cold allodynia with similar efficacy in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. Thus, anti-allodynic effects of the CB_2 agonist were independent of CB_1 receptors. Pharmacological blockade of CXCR4 signaling with AMD3100 in rats failed to attenuate mechanical or cold allodynia in paclitaxel or cisplatin models. These findings suggest that the maintenance of chemotherapy-induced neuropathy is independent of CXCR4 signaling. Finally, blockade of CXCR4 signaling failed to enhance the anti-allodynic effects of AM1710, suggesting distinct mechanisms of action. In conclusion, the cannabilactone CB_2 agonist AM1710 suppressed the maintenance of mechanical and cold allodynia produced by both paclitaxel and cisplatin treatments through a CB_2-specific mechanism. By contrast, blockade of CXCR4 signaling with AMD3100 failed to reverse mechanical and cold allodynia in the same paradigms. Our studies suggest that CB_2 receptors represent a promising therapeutic target for the treatment of toxic neuropathies produced by diverse classes of chemotherapeutic agents.

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PHARMACOLOGICAL MODULATION OF THE ENDOCANNABINOID SYSTEM IN TWO RAT STRAINS DIFFERING IN NOCICEPTIVE RESPONSIVITY: BEHAVIOURAL EFFECTS AND RVM ZIF268 EXPRESSION

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Anxiety can enhance pain responding, a phenomenon referred to as anxiety-related hyperalgesia (ARH). The endocannabinoid system modulates both pain- and anxiety-related behaviour but its potential role in ARH has not been studied. Here, we investigated the effects of pharmacological modulation of the endocannabinoid system on nociceptive responding in Sprague-Dawley (SD) and Wistar-Kyoto (WKY) rats, two strains that differ in baseline anxiety-related behaviour. Associated alterations in expression of the immediate early gene and marker of neuronal activity, *zif268,* and *N*-acylethanolamine concentrations in the rostroventromedial medulla (RVM), were also investigated. Adult male SD and WKY rats (n=12 per group) received URB597, an inhibitor of the catabolism of *N*-acylethanolamines including anandamide, palmitoylethanolamide and oleoylethanolamide (0.1, 0.2 or 0.5mg/kg i.p.), AM251, a CB_1 receptor antagonist (1, 3 or 5mg/kg i.p.), or vehicle prior to assessment of nociceptive responding in the hot plate test. The effects of URB597 (0.5mg/kg i.p.), AM251 (3 mg/kg i.p.) or vehicle on nociceptive behaviour in the formalin test and associated alterations in *zif268* mRNA expression (qRT-PCR) and *N*-acylethanolamine concentrations (LC-MS/MS) in the RVM were also assessed. Data were analysed by ANOVA followed by Fisher's LSD post-hoc test ($p<0.05$ significant).

WKY rats exhibited enhanced nociceptive behaviour in the hot plate and formalin tests compared with SD counterparts, confirming the expression of ARH. Both AM251 and URB597 dose-dependently reduced hot plate nociceptive responding in WKY, but not SD, rats. URB597 attenuated formalin-evoked nociceptive behaviour in both strains, however, the magnitude of these effects was greater in WKY rats. AM251 enhanced formalin-evoked nociceptive behaviour in WKY rats only. URB597 increased levels of anandamide in the RVM of formalin-treated WKY, but not SD, rats, and increased levels of palmitoylethanolamide and oleoylethanolamide to a greater extent in WKY rats than in SD rats. The enhanced formalin-evoked nociceptive behaviour in WKY rats was associated with increased expression of *zif268* in the RVM, an effect not observed following administration of either AM251 or URB597. In conclusion, the data suggest that WKY rats exhibit ARH which may be associated with a deficit in endocannabinoid-CB_1 receptor signalling and that *zif268* expression in the RVM may represent a molecular correlate of ARH.

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CHARACTERISATION OF THE ENDOCANNABINOID SYSTEM WITHIN THE DESCENDING PAIN PATHWAY OF TWO RAT STRAINS DIFFERING IN NOCICEPTIVE RESPONSIVITY

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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. Given the well-established role of the endocannabinoid system in modulating both stress and peripheral and central pain processing, we hypothesised that differences in the expression and/or mobilisation of elements of the endocannabinoid system within the CNS may account for the differential nociceptive responsivity in WKY vs. SD rats. The aim of the present study was to complete a comparative molecular and neurochemical analysis of the endocannabinoid system within key components of the descending pain pathway of WKY and SD rats that had received intra-plantar injection of either saline or the noxious chemical formalin. Adult male WKY or SD rats (280-320g) received intra-plantar injection of either saline or formalin (2.5%), nociceptive behaviour assessed for 30 minutes, and then post-mortem tissue from the periaqueductal grey (PAG), rostroventromedial medulla (RVM) and dorsal horn of the spinal cord (DH) was harvested for analysis. Levels of the endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the related *N*-acylethanolamines, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), were quantified using LC-MS/MS. Quantitative RT-PCR was used to determine the expression of mRNA coding for various components of the endocannabinoid system including the cannabinoid$_1$ (CB_1) receptor, fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), n-arachidonoylphosphatidylethanolamine phospholipase D (NAPE-PLD), diacylglycerol lipase alpha (DAGLα), GPR55, PPARα and PPARγ.

WKY rats exhibited increased formalin-evoked nociceptive behaviour compared with SD counterparts, indicating a hyperalgesic phenotype. Levels of AEA, 2-AG, PEA and OEA in the ventrolateral PAG, and OEA and PEA in the RVM, were higher in saline-treated WKY rats, compared with SD counterparts. FAAH and MAGL mRNA expression levels were comparable in both SD and WKY saline-treated rats. CB_1 mRNA levels were significantly higher in the lateral PAG, and lower in the RVM of saline-treated SD rats compared with WKY counterparts. PPARγ expression was significantly higher in the lateral PAG of saline- and formalin-treated WKY rats compared with SD counterparts. Intraplantar formalin injection increased levels of AEA, OEA and PEA in the DH of both strains, and increased 2-AG, OEA and PEA levels in the RVM of SD, but not WKY, rats. In SD but not WKY rats, formalin injection was also associated with significant increases in NAPE-PLD and DAGLα mRNA in the dorsolateral PAG, and in the lateral PAG (DAGLα only). Both NAPE-PLD and DAGLα mRNA were also significantly increased in the RVM and DH of formalin-treated SD rats. A significant increase in CB_1 mRNA expression was observed in the DH of formalin-treated WKY, but not SD, rats. Formalin treatment was associated with significant decreases in FAAH mRNA in the ventrolateral PAG and DH of SD, but not WKY, rats. There was also a decrease in FAAH mRNA expression in the lateral PAG of formalin-treated WKY, but not SD, rats. MAGL mRNA expression was reduced in the lateral PAG of formalin-treated rats of both strains, but increased in the dorsolateral PAG of SD rats alone. Formalin injection was associated with increased expression of PPARγ mRNA in the dorsolateral and ventrolateral PAG of both rat strains. Formalin injection also produced an increase in PPARα mRNA expression in the dorsolateral PAG of WKY, but not SD, rats and an increase in PPARα mRNA in the ventrolateral PAG of SD, but not WKY, rats. Formalin treatment produced increases in GPR55 expression in the dorsolateral and ventrolateral PAG of both rat strains.

Overall, these data suggest a more active and efficient mobilisation of endocannabinoid system components in SD compared with WKY rats in response to intraplantar formalin injection as demonstrated by increases in endocannabinoid levels, increases in expression of genes coding for synthetic enzymes and a corresponding decrease in genes coding for catabolic enzymes. Further studies are needed to identify the extent to which these alterations underlie the differential response to nociceptive stimuli in the two rat strains.

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ASYMMETRIC SYNTHESIS OF NOVEL N-(1-PHENYL-2,3-DIHYDROXYPROPYL) ARACHIDONYLAMIDES AND EVALUATION OF THEIR ANTI-INFLAMMATORY ACTIVITY

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The original design and synthesis of novel N-(1-phenyl-2,3-dihydroxypropyl) arachidonylamides (C) and evaluation of their analgesic and anti-inflammatory potentials have been conducted. The resulting new compounds were found to have the capacity to increase production of 15-deoxy-Δ13, 14-PGJ2 (PGJ) and may increase the occurrence of programmed cell death (apoptosis) (Takenouchi et al., 2012). The murine macrophage cell line RAW 264.7 was used as a model for inflammatory responses in vitro (Burstein et al., 2012). It consists of cultured monolayers of PGJ are measured by ELISA following LPS (10 μg/ml) stimulation and by treatment with 0.1, 0.3, 1.0, 3.0 and 10 μM concentrations of the compounds. The data indicated that these agents are potential candidates for the therapy of conditions characterized by ongoing (chronic) inflammation and the associated pain.

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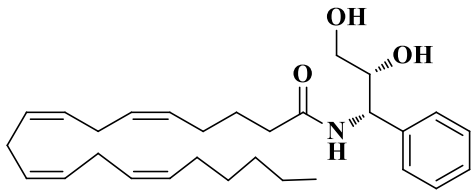

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THE ENDOCANNABINOID SYSTEM AND STRESS-MEDIATED ENHANCEMENT OF PAIN SUSCEPTIBILITY IN MICE

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Neuropsychiatric disorders, such as anxiety disorders, depression, have been reported to affect pain susceptibility in both humans and animal models. The aim of this study was to investigate how chronic stress exposure affects pain susceptibility in mice and whether the endocannabinoid (eCB) system plays a role in the regulation of this stress-mediated enhanced predisposition to pain. Male C57-BL/6J mice (8 week old) were exposed to chronic unpredictable stress (CUS) for 5-6 weeks.

Mice exposed to CUS showed reduced body weight and an anxiogenic behavior as evaluated by the elevated plus maze (EPM) test. On the other hand, EPM in association with food reward after overnight starvation combined with intense light exposure during the test revealed that CUS-exposed animals, as compared to the control group, showed an increased drive to enter the bright open arms and to reach the food reward, suggesting that their ability to recognize the danger (reward under intense light) was strongly impaired. Moreover, CUS-exposed mice showed a significantly lower pain threshold and thus, an enhanced sensitivity to pain as compared to the control group. This increased predisposition to pain was observed both in absence and presence of a noxious stimulus (nerve growth factor, NGF, injected into the muscle). Interestingly, chronic treatment with the fatty acid amide hydrolase (FAAH) inhibitor URB-597 (0.3 mg/kg/day during last week of CUS and during behavioral and pain tests) appeared to ameliorate the lower pain threshold only in those animals exposed to CUS together with a pre-existing increased susceptibility to pain (as induced by NGF injection into the muscle). Additionally, we found changes in the serum level of endocannabinoids and related lipids and a decrease in the [^{35}S]-GTPγS bound CB_1 receptor in the brain of CUS-exposed mice as compared to control animals. In conclusion, our findings demonstrate that chronic stress exposure increases pain susceptibility in mice and disclose, although preliminarily, a possible role of the eCB system in the regulation of the stress-induced pain perception in combination with NGF-induced pain.

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INTERACTION BETWEEN CANNABINOID AND METABOTROPIC GLUTAMATE RECEPTORS IN THE PERIAQUEDUCTAL GRAY-ROSTRAL VENTROMEDIAL MEDULLA PATHWAY IN NEUROPATHIC RAT

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Cannabinoids have shown effectiveness in neuropathic pain which still lacks an effective therapeutic treatment. Apart from cannabinoids, glutamate also has a key role in controlling pain responses and development. By this subject interactions between cannabinoid and metabotropic glutamate (mGlu) receptors, may have a critique relevance in controlling pain and may be exploited for treating neuropathic pain with particular regard to the periaquectal grey (PAG)- rostral ventromedial medulla (RVM) descending pathway, which represents an endogenous source whose role is to counteract pain. ON and OFF cells are two cell populations found within RVM which respond differentially to pain stimuli and analgesics and may be exploited for studying the potential of centrally acting analgesics. ON cells are excited by nociceptive stimulation and inhibited by analgesics whereas OFF cells are inhibited by painful stimuli and enhanced by analgesics. The following study has investigated the effect of intra-VL PAG administration of a CB receptor agonist on tail flick latency and on the activity of the RVM ON and OFF cells in neuropathic pain conditions. The effect of group I mGlu receptor antagonists has been also considered together with CB and mGlu receptor and endocannabinoid-related protein expression. Intra-VL PAG microinjection of WIN 55,212-2, (2-4-8 nmol), a CB receptor agonist, increased the tail flick latency and changed the ongoing activity of RVM OFF and the tail flick-related activity of the ON and OFF cells, accordingly. These effects were prevented by SR141716A and MPEP, selective CB1 and mGlu5 receptor antagonists, respectively, though not by CPCCOEt, a selective mGlu1 receptor antagonist. A higher dose up to 16 nmol of WIN 55,212-2 was necessary to increase tail flick latency and change ON and OFF cell activity in CCI rats. Consistently, CCI rats showed a decrease in the expression of CB1 receptors, NAPE-PLD, Gai3 and CRIP 1a proteins in the VL PAG. The expression of diacylglycerol lipase (DAGLA) proved to be increased while fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) did not change. MPEP and SR141716A blocked WIN 55,212-2-induced effects also in CCI rats. Altogether these data show a down regulation of the endocannabinoid system and a functional interaction between mGlu5 and CB1 receptor for cannabinoid-mediated effect in the PAG-RVM pain circuitry in neuropathic rats.

COMBINED INHIBITION OF MAGL AND FAAH SUPPRESSES EDEMA AND PRODUCES AUGMENTED ANTI-ALLODYNIC EFFECTS IN THE CARRAGEENAN MOUSE MODEL

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Direct-acting cannabinoid receptor agonists elicit anti-inflammatory and anti-nociceptive effects in a variety of laboratory animal models. However, their marijuana-like side effects diminish enthusiasm for their therapeutic development. In the present study, we evaluated the impact of inhibiting monoacylglycerol lipase (MAGL), the primary catabolic enzyme for 2-arachidonyl glycerol (2-AG), and fatty acid amide hydrolase (FAAH), the principle degradative enzyme for anandamide (AEA), in the mouse carrageenan model of inflammatory pain.

The present study tested whether combination of JZL184 and PF-3845, administered alone or in combination, would decrease paw edema and allodynia (a nociceptive response to normally non-noxious stimuli).We first evaluated the efficacy of JZL184 (1.6, 4, 16, or 40 mg/kg) or PF-3845 (1, 3, or 10 mg/kg) given alone. Additionally, we determined whether JZL184 would retain its anti-edematous and anti-allodynic effects after repeated administration. JZL184 and PF-3845 partially attenuated carrageenan-induced edema and allodynia. Repeated administration of high doses of JZL184 (16 and 40 mg/kg) underwent tolerance to its anti-edematous and anti-allodynic effects; however, repeated administration of low dose JZL184 (4 mg/kg) retained efficacy. Based on these results, we tested whether combined administration of JZL184 and PF-3845 would produce enhanced efficacy in the carrageenan model. Partial blockade of MAGL, with a low dose of JZL184 (4 mg/kg), and full blockade of FAAH, with a high dose of PF-3845 (10 mg/kg), enhanced the anti-allodynic effects, but no further increases in the anti-edematous effects were found. Brain AEA and 2-AG levels were elevated approximately 8-10-fold and 3-4 fold, respectively. Additionally, repeated administration of this combination did not result in tolerance. In the final experiment, we tested a novel FAAH-MAGL dual inhibitor, SA-57, which is far more potent in inhibiting FAAH than MAGL. SA-57 elevated brain AEA levels ~10-fold at all doses (0.125, 1.25, 2.5, 5, and 12.5 mg/kg). SA-57 did not increase 2-AG levels at 0.125 or 1.25 mg/kg, but 2-AG levels were increased 3-fold, 8-fold, and more than 10-fold at 2.5, 5, and 12.5 mg/kg, respectively. SA-57 reversed allodynia at doses of 5 and 12.5 mg/kg and also decreased carrageenan induced edema at doses of 2.5, 5, and 12.5 mg/kg. Taken together, these findings suggest that dual MAGL and FAAH inhibition represents a promising avenue for the treatment of inflammatory disease states. More specifically, partial MAGL inhibition combined with full FAAH inhibition produces augmented antinociceptive effects with no observed cannabimimetic side effects.

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