# 19TH ANNUAL SYMPOSIUM OF THE INTERNATIONAL CANNABINOID RESEARCH SOCIETY

PHEASANT RUN

ST. CHARLES, ILLINOIS USA

JULY 7 - 11, 2009

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# INTERNATIONAL CANNABINOID RESEARCH SOCIETY

## Pheasant Run

St. Charles, Illinois, USA, July 7 -11, 2009

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# Registration: July 7, 2009 (12.00 – 22.00)

# Welcome Reception [atrium]: 19.00 – 21.00

# Day 1
# Wednesday, July 8th

| | | | |
|---|---|---|---|
| 7.00 | Breakfast [Atrium] | | |
| **Oral Session 1.** Inhibitor / Ligand Development<br>**Moderators: John Huffman and Patti Reggio** | | | ABSTRACT # |
| 8.45 | **Welcome and Opening Remarks** | | |
| 9.00 | Didier M. Lambert, Coco N. Kapanda, Giulio G. Muccioli, Geoffray Labar and Jacques H. Poupaert | BIS[(DIALKYLTHIOCARBAMOYL)]DISULFIDE DERIVATIVES AS POTENT AND SELECTIVE MONOGLYCERIDE LIPASE INHIBITORS | 1 |
| 9.15 | John W. Huffman, Valerie J. Smith, Jianhong Chen, Jenny L. Wiley and Billy R. Martin | STRUCTURE-ACTIVITY RELATIONSHIPS AT THE CB1 AND CB2 RECEPTORS FOR 1-ALKYL-3-(1-NAPHTHOYL-4 AND 8-HALOGEN SUBSTITUTED) INDOLES | 2 |
| 9.30 | Xiang-Qun (Sean) Xie, Yuxun Zhang, Lirong Wang, Jianzhong Chen, Juerg Gertsch and Dana E. Selley | DISCOVERY OF NEW CB2 LIGANDS WITH NOVEL CHEMICAL SCAFFOLD | 3 |
| 9.45 | Michael Meyer, Betty Yao, Anthony Daza, Yihong Fan, Lanlan Li, Loan Miller, Odile El Kouhen, Gin Hsieh, Erica Wensink, Anita Salyers, Prasant Chandran, Michael Dart, Tongmei Li and William Carroll | A-1036654, A NOVEL ORALLY BIOAVAILABLE CB2-SELECTIVE AGONIST EXHIBITING ANALGESIC ACTIVITY IN RODENT MODELS OF OSTEOARTHRITIC AND NEUROPATHIC PAIN | 4 |

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|---|---|---|---|
| 10.00 | Aron H. Lichtman, Anu Mahadevan, Raj K. Razdan, Sreenivasulu P. Naidu, Steven G. Kinsey, Bingjun Zhao, Hang Sun, Dana E. Selley and M. Imad Damaj | COMPARISON BETWEEN THE CB2 RECEPTOR AGONIST O-3223 AND THE POTENT, MIXED CB1/CB2 RECEPTOR AGONIST CP55,940 IN A BATTERY OF NOCICEPTIVE ASSAYS | 5 |
| 10.15 | Jenny L. Wiley, Pinglang Wang, Anu Mahadevan, Raj K. Razdan and Billy R. Martin | PYRAZOLE CANNABINOIDS WITH NON-CB1, NON-CB2 ACTIVITY | 6 |
| 10.30 – 11.00 | COFFEE BREAK [POSTER AREA] | | |
| **ORAL SESSION 2.** CB2 RECEPTORS: FUNCTION / SIGNALING<br>**MODERATORS: NANCY BUCKLEY AND ZHAO-HUI SONG** | | | ABSTRACT # |
| 11.00 | Zhuanhong Qiao, Jian Cai, William M. Pierce Jr. and Zhao-Hui Song | DETERMINATION OF AN INTERNAL DISULFIDE BOND IN THE SECOND EXTRACELLULAR LOOP OF CANNABINOID CB2 RECEPTOR BY MASS SPECTROMETRY | 7 |
| 11.15 | Mitzi Nagarkatti, Rupal Pandey and Prakash Nagarkatti | TARGETING CANNABINOID RECEPTORS AS A NOVEL APPROACH TO PREVENT GRAFT-VERSUS-HOST DISEASE | 8 |
| 11.30 | Orr Ofek, Malka Attar-Namdar, Esther Shohami, Raphael Mechoulam and Itai Bab | OSTEOBLASTIC CB2 CANNABINOID RECEPTOR ACTIVATES GI PROTEIN-ERK1/2-MAPKAPK2-CREB SIGNALING PATHWAY | 9 |
| 11.45 | Nancy E. Buckley, Beverly McDowell, Ehsan Taqavi and Gideon Blumstein | DELTA-9-TETRAHYDROCANNABINOL, CB2R AND IMMUNITY TO YEAST INFECTION | 10 |

| | | | |
|---|---|---|---|
| 12.00 | Betty B Yao, Odile El Kouhen, Gin Hsieh, Yihong Fan, Bradley A Hooker, Loan Miller, David G Witte, Lanlan Li, Steve McGaraughty, Kathleen Chu, Madhavi Pai, Erica J Wensink, Anita K Salyers, Chang Z Zhu, William A Carroll, Michael J Dart and Michael D Meyer | $CB_2$ RECEPTOR-MEDIATED ANTI-ALLODYNIC EFFECTS IN NEUROPATHIC PAIN MODELS IS LIKELY MEDIATED THROUGH NON-NEURONAL CELLS | 11 |
| 12.15 – 14.00 | LUNCH [ATRIUM] | | |
| **ORAL SESSION 3.** CB1 RECEPTOR SIGNALING<br>**MODERATORS: SACHIN PATEL AND MATTHEW HILL** | | | ABSTRACT # |
| 14.00 | Kofi-Kermit Horton, Sarah Bough, Anushka Goonawardena, John Sesay, Allyn Howlett and Robert E. Hampson | CHRONIC RIMONABANT ALTERS BEHAVIOR AND GENE EXPRESSION IN HIPPOCAMPUS DURING LEARNING | 12 |
| 14.15 | Peter Nguyen, Cullen L. Schmid, Kirsten M. Raehal, Dana E. Selley, Laura M. Bohn and Laura J. Sim-Selley | BETA-ARRESTIN 2 REGULATES CB1 RECEPTOR DESENSITIZATION AND THC-MEDIATED ANTINOCICEPTIVE TOLERANCE | 13 |
| 14.30 | Alex Straiker and Ken Mackie | DIFFERENTIAL REGULATION OF DSI TIME COURSE IN IDENTIFIED POPULATIONS OF AUTAPTIC HIPPOCAMPAL NEURONS | 14 |
| 14.45 | Val J. Watts and Julie A. Przybyla | EXPRESSION, LOCALIZATION, AND DRUG MODULATION OF CANNABINOID CB1 AND DOPAMINE D2 RECEPTOR HETERODIMERS IN CATH.A DIFFERENTIATED NEURONAL CELLS | 15 |

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| 15.00 | Joel E. Schlosburg, James J. Burston, Steven G. Kinsey, Lamont Booker, Rehab A. Abdullah, Jonathan Z. Long, Dana E. Selley, Benjamin F. Cravatt and Aron H. Lichtman | BEHAVIORAL AND FUNCTIONAL ADAPTATION OF THE ENDOCANNABINOID SYSTEM FOLLOWING REPEATED MONOACYLGLYCEROL LIPASE (MAGL) INHIBITION | 16 |
| 15.15 | Xia Zhang | LONG-TERM DEPRESSION IN VENTRAL TEGMENTAL DOPAMINE NEURONS MEDIATES LEARNING TO ASSOCIATE CANNABINOID EXPOSURE WITH ENVIRONMENTAL CUES | 17 |
| 15.30 | Sachin Patel, Philip J. Kingsley, Ken Mackie, Lawrence J. Marnett and Danny G. Winder | REPEATED HOMOTYPIC STRESS ELEVATES 2-AG AND ENHANCES DEPOLARIZATION-INDUCED SUPRESSION OF INHIBITION IN BASOLATERAL AMYGDALA | 18 |
| 15.45 | Tiziana Rubino, Natalia Realini, Daniela Vigano', Guidali Cinzia and Daniela Parolaro | URB597 RECOVERS SOME OF THE SIGNS PRESENT IN THE DEPRESSIVE PHENOTYPE INDUCED BY ADOLESCENT EXPOSURE TO THC | 19 |
| 16.00 – 17.00 | **KANG TSOU MEMORIAL LECTURE**<br>"THE GLUTAMATE HOMEOSTASIS HYPOTHESIS OF ADDICTION"<br>**PETER KALIVAS, PH.D.**<br>Medical University of South Carolina | | PS1 |
| 17.00 – 19.00 | **POSTER SESSION 1**<br>SNACKS | | |
| 19.00 | DINNER [ATRIUM] | | |

# Day 2
# Thursday, July 9th

| | | | |
|---|---|---|---|
| 7.00 | Breakfast [Atrium] | | |
| **Oral Session 4.** GPR55 Pharmacology / Signaling<br>**Moderators: Mary Abood and Takayuki Sugiura** | | | ABSTRACT # |
| 8.30 | Chris Henstridge, Simon Arthur and Andrew Irving | LACK OF SPECIFICITY FOR CANNABINOID CB1 RECEPTOR ANTAGONISTS: INTERACTIONS WITH GPR55 | 20 |
| 8.45 | Khalil Eldeeb, Stephen Alexander, David Pritchard and David Kendall | LPI-EVOKED INCREASES IN INTRACELLULAR CALCIUM INCREASES IN MICROGLIAL CELLS IN CULTURE | 21 |
| 9.00 | Ankur Kapur, Pingwei Zhao, Haleli Sharir, Yushi Bai, Marc G. Caron, Larry S. Barak and Mary E. Abood | GPR55: TO BE OR NOT TO BE A CANNABINOID RECEPTOR | 22 |
| 9.15 | Nariman Balenga, Ralf Schröder, Wolfgang Platzer, Julia Kargl, Christopher M Henstridge, Andrew J Irving, Evi Kostenis and Maria Waldhoer | FUNCTIONAL SELECTIVITY OF CANNABINOIDS ON THE NOVEL CANNABINOID RECEPTOR GPR55 | 23 |
| 9.30 | Takayuki Sugiura, Atsushi Yamashita and Saori Oka | LYSOPHOSPHATIDYLINOSITOL INDUCES RAPID CYTOSKELETAL REARRANGEMENTS AND MORPHOLOGICAL CHANGES IN HEK293 CELLS EXPRESSING GPR55: FURTHER EVIDENCE THAT LYSOPHOSPHATIDYL-INOSITOL IS THE NATURAL LIGAND FOR GPR55 | 24 |

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|---|---|---|---|
| 9.45 – 10.15 | COFFEE BREAK [POSTER AREA] | | |
| **ORAL SESSION 5.** PHYTOCANNABINOIDS<br>**MODERATORS: ETHAN RUSSO AND ROGER PERTWEE** | | | ABSTRACT # |
| 10.15 | Lisa A.Gauson, Maria-Grazia Cascio, Ruth A. Ross and Roger G. Pertwee | CANNABIGEROL DISPLAYS SIGNIFICANT POTENCY AS A 5-HT1A RECEPTOR ANTAGONIST | 25 |
| 10.30 | Sean D. McAllister, Darryl Lau, Rigel T. Christian, Arash E. Pakdel, Anne J. Zielinski, Jasmine Lee, Dan H. Moore and Pierre-Yves Desprez | CANNABIDIOL AS A NOVEL INHIBITOR OF ID-1 GENE EXPRESSION IN AGGRESSIVE BREAST CANCER CELLS | 26 |
| 10.45 | Nicholas A. Jones, Andrew J. Hill, Gary J. Stephens, Claire M. Williams and Benjamin J. Whalley | ANTICONVULSANT EFFECTS OF CANNABIDIOL UPON SPONTANEOUS EPILEPTIFORM ACTIVITY IN ACUTE HIPPOCAMPAL BRAIN SLICES | 27 |
| 11.00 | Tamás Bíró, Attila Oláh, Balázs I. Tóth, Gabriella Czifra, Christos C. Zouboulis and Ralf Paus | CANNABIDIOL AS A NOVEL ANTI-ACNE AGENT? CANNABIDIOL INHIBITS LIPID SYNTHESIS AND INDUCES CELL DEATH IN HUMAN SEBACEOUS GLAND-DERIVED SEBOCYTES | 28 |
| 11.15 | Erin M. Rock, Cheryl L. Limebeer, Raphael Mechoulam and Linda A. Parker | CANNABIDIOL (THE NON-PSYCHOACTIVE COMPONENT OF CANNABIS) MAY ACT AS A 5-HT1A AUTO-RECEPTOR AGONIST TO REDUCE TOXIN-INDUCED NAUSEA AND VOMITING | 29 |
| 11.30 | Daniele De Filippis, Giuseppe Esposito, Mariateresa Ciprian, Caterina Scuderi, Joris De Man and Teresa Iuvone | CANNABIDIOL CONTROLS INTESTINAL INFLAMMATION THROUGH THE MODULATION OF ENTERIC GLIAL CELLS | 30 |

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|---|---|---|---|
| 11.45 – 17.00 | LUNCH – On your own<br>Free Time | | |
| 17.00 – 19.00 | **Poster Session 2**<br>Pizza Buffet | | |
| 19.00 – 20.00 | **Plenary Lecture**<br>Individual Differences in Responses to Drugs in Humans<br>**Harriet de Wit, Ph.D.**<br>University of Chicago | | PS2 |
| **Oral Session 6.** Human Studies / Drug Abuse<br>**Moderators: Eliot Gardner and Jenny Wiley** | | | ABSTRACT # |
| 20.00 | Joshua A. Lile, Thomas H. Kelly and Lon R. Hays | $GABA_A$ MODULATION OF THE DISCRIMINATIVE STIMULUS EFFECTS OF $\Delta^9$-THC IN HUMANS | 31 |
| 20.15 | Amanda Reiman | CANNABIS AS A SUBSTITUTE FOR ALCOHOL AND OTHER DRUGS | 32 |
| 20.30 | Andrea M Dlugos, Ajna Hamidovic, Colin A Hodgkinson, David Goldman, Abraham A Palmer and Harriet de Wit | MORE AROUSED, LESS FATIGUED: FAAH GENE POLYMORPHISMS INFLUENCE ACUTE RESPONSE TO AMPHETAMINE | 33 |

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| 20.45 | José Alexandre S. Crippa, Guilherme Nogueira Derenusson, Thiago Borduqui Ferrari, Lauro Wichert-Ana, Fábio Duran, Rocio Martin-Santos, Marcus Vinícius Simões, Sagnik Bhattacharyya, Paolo Fusar-Poli, Alaor Santos Filho, Maria Cecília Freitas-Ferrari, Philip K. McGuire, Antonio Waldo Zuardi, Geraldo Busatto Filho, Jaime Eduardo Cecílio Hallak and Richard E. Musty | NEURAL BASIS OF ANXIOLYTIC EFFECTS OF CANNABIDIOL (CBD) IN GENERALIZED SOCIAL ANXIETY DISORDER | 34 |
| 21.00 | Sara Jane Ward and Ellen A. Walker | SEX AND CANNABINOID CB1 GENOTYPE DIFFERENTIATE PALATABLE FOOD AND COCAINE SELF-ADMINISTRATION IN MICE | 35 |
| 21.15 | Daniel Thomas Malone, Dennis Jongegan and David Alan Taylor | THE EFFECT OF CANNABIDIOL AND $\Delta^9$-TETRAHYDROCANNABINOL ON SOCIAL INTERACTION OF RATS | 36 |
| 21.30 | Emmanuel Shan Onaivi | GENETIC BASIS OF MARIJUANA USE | 37 |
| 21.45 | Mark Ware, Tongtong Wang, Stan Shapiro and Jean-Paul Collet | CANNABIS FOR THE MANAGEMENT OF PAIN: ASSESSMENT OF SAFETY STUDY (COMPASS) | 38 |
| 22.00 | DESSERT [POSTER AREA] | | |

*Notes:*

# Day 3
# Friday, July 10th

| | | | |
|---|---|---|---|
| 7.00 | Breakfast [Atrium] | | |
| **Oral Session 7.** Neuroprotection<br>**Moderators: Julian Romero and Ronald Tuma** | | | ABSTRACT # |
| 9.00 | Lourdes Ruiz-Valdepeñas, José Antonio Martínez Orgado, Cristina Otalora, Marta Moreno, África Millán, Rosa María Tolón and Julián Romero | CANNABIDIOL REDUCES LIPOPOLYSACCHARIDE-INDUCED VASCULAR DYSFUNCTION IN THE MOUSE BRAIN: AN INTRAVITAL MICROSCOPY STUDY | 39 |
| 9.15 | Stefania Petrosino, Marcello Naccarato, Daniela Pizzuti, Marco Simonetto, Fabio Chiodo Grandi, Gilberto Pizzolato and Vincenzo Di Marzo | BLOOD ENDOCANNABINOID AND PALMITOYLETHANOLAMIDE LEVELS IN HUMAN ISCHEMIC STROKE | 40 |
| 9.30 | Ming Zhang, Martin W. Adler, Mary Abood, Doina Ganea and Ronald F. Tuma | THE COMBINATION TREATMENT WITH A CB2 AGONIST AND CB1 ANTAGONIST ATTENUATED CEREBRAL ISCHEMIC INJURY THROUGH DIFFERENT MECHANISMS | 41 |
| 9.45 | Yosefa Avraham, Nicholaos C Grigoriadis, Iddo Magena, Theofilos Poutahidis, Lia Vorobiava, Yaron Ilan, Raphael Mechoulam and Elliot M Berry | CAPSAICIN AFFECTS BRAIN FUNCTION IN A MODEL OF HEPATIC ENCEPHALOPATHY ASSOCIATED WITH FULMINANT HEPATIC FAILURE IN MICE | 42 |
| 10.00 | Somnath Mukhopadhyay, Ju-Ahng Lee and Shailendra Devkota | ENDOCANNABINOID AND CB1 RECEPTOR-MEDIATED REGULATION OF NEUROGENESIS IN ZEBRAFISH | 43 |
| 10.15 – 10.45 | Coffee Break [Poster area] | | |

## Oral Session 8. Endocannabinoids

**Moderators: Vincenzo Di Marzo and Dale Deutsch**

| | | | ABSTRACT # |
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| 10.45 | Geoffray Labar, Franck Borel, Cédric Bauvois, Jean-Luc Ferrer, Johan Wouters and Didier M. Lambert | MOLECULAR INSIGHT INTO MONOGLYCERIDE LIPASE, THE ENZYME DEGRADATING 2-ARACHIDONOYLGLYCEROL | 44 |
| 11.00 | Jonathan Z. Long, Daniel K. Nomura and Benjamin F. Cravatt | MECHANISTIC CHARACTERIZATION OF SELECTIVE MONOACYLGLYCEROL LIPASE INHIBITION REVEALS DIFFERENCES IN CENTRAL AND PERIPHERAL ENDOCANNABINOID METABOLISM | 45 |
| 11.15 | Martin Kaczocha, Sherrye T. Glaser, Janiper Chae and Dale G. Deutsch | FAAH-2 IS A LIPID DROPLET-LOCALIZED ENZYME THAT MEDIATES N-ACYLETHANOLAMINE INACTIVATION | 46 |
| 11.30 | Patricia H. Reggio, Dow P. Hurst and Diane L. Lynch | THE ROLE OF FATTY ACID BINDING PROTEINS IN THE LIFE CYCLE OF ANANDAMIDE | 47 |
| 11.45 | Lina Thors, Staffan Tavelin and Christopher J. Fowler | TEMPERATURE-DEPENDENT AND SATURABLE UPTAKE OF ANANDAMIDE BY SYNTHETIC LIPOSOMES | 48 |
| 12.00 | Alessia Ligresti, Dolores Hernan Perez de la Ossa, Jesús Molpeceres, Rosario Aberturas, Mª Esther Gil, Aniello Schiano Moriello, Ana-Isabel Torres, Luciano De Petrocellis and Vincenzo Di Marzo | NANOPARTICLE-MEDIATED CELLULAR UPTAKE-CONTROLLED RELEASE AS A TOOL TO STUDY ANANDAMIDE MEMBRANE TRANSPORT | 49 |
| 12.15 | Leonie Wyffels, Giulio G Muccioli, Coco N Kapanda, Sylvie De Bruyne, Didier M Lambert and Filip De Vosa | RADIOSYNTHESIS AND IN VIVO EVALUATION OF [11C]- BIPHENYL-3-YL 4-METHOXYPHENYL-CARBAMATE AS PET TRACER FOR IN VIVO EVALUATION OF FAAH IN THE BRAIN | 50 |

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| 12.30 - 14.00 | LUNCH [ATRIUM] | |
| 13.00 – 14.00 | **NIDA CAREER DEVELOPMENT INFOLUNCHEON**<br>[MARSALIS]<br>"SPECIALTY RESEARCH SUPPLIES: HOW COMPANIES MANAGE PRODUCT SELECTION AND CUSTOMER SERVICE"<br>**Kirk Maxey and Jeff Johnson,** Cayman Chemicals<br>**Robert Zipkin,** Enzo Life Sciences | |
| 14.00 – 15.00 | **PLENARY LECTURE**<br>AN EVALUATION OF BRAIN ENDOCANNABINOID SIGNALING AND ADDICTION-RELATED BEHAVIORS<br>**LARRY PARSONS, PH.D.**<br>Scripps Institute | PS3 |
| 15.00 – 17.00 | **POSTER SESSION 3**<br>SNACKS | |
| 17.00 – 19.00 | **Rao Rapaka, Ph.D. and Vishnudutt Purohit, D.V.M., Ph.D.**<br>**NIDA presents:**<br>CANNABINOIDS AND LIVER DISEASES: MOLECULAR MECHANISMS AND DRUG DEVELOPMENT<br>**ENDOCANNABINOIDS, THE LIVER, AND THE METABOLIC SYNDROME**<br>George Kunos<br>**ENDOCANNABINOIDS AND HEMODYNAMIC CONSEQUENCES OF LIVER CIRRHOSIS**<br>Sándor Bátkai<br>**CANNABINOIDS, LIVER INFLAMMATION AND CANCER**<br>Prakash Nagarkatti<br>**THE ENDOCANNABINOID SYSTEM AS A REGULATOR OF THE HEPATIC WOUND HEALING PROCESS**<br>Sophie Lotersztajn | H1 – H4 |
| 19.00 | DINNER – ON YOUR OWN | |

# Day 4
# Saturday, July 11th

| | | | |
|---|---|---|---|
| 7.00 | Breakfast [Atrium] | | |
| **Oral Session 9.** Non-CNS Pharmacology / Physiology<br>**Moderators: Linda Parker and Vanessa Ho** | | | ABSTRACT # |
| 9.00 | Cheryl L. Limebeer, Kiran Vimuri, Holly Bedard, Klaus-Peter Ossenkopp, Alexandros Makriyannis and Linda A. Parker | NAUSEATING EFFECTS OF AM-251 MAY BE PRODUCED BY THE PERIPHERAL INVERSE-AGONIST PROPERTIES: EVIDENCE FROM THE CONDITIONED GAPING MODEL IN RATS | 51 |
| 9.15 | Vincenzo Di Marzo, An Verrijken, Antti Hakkarainen, Stefania Petrosino, Ilse Mertens, Nina Lundbom, Fabiana Piscitelli, Jukka Westerbacka, Aino Soro-Paavonen, Isabel Matias, Luc Van Gaal and Marja-Riitta Taskinen | INSULIN AS A NEGATIVE REGULATOR OF PLASMA ENDOCANNABINOID LEVELS IN OBESE AND NONOBESE SUBJECTS | 52 |
| 9.30 | Stefan Engeli, Anne-Christin Lehmann, Jana Böhnke, Verena Haas, Jürgen Janke, Anke Strauß, Alexander Zörner, Dimitrios Tsikas and Jens Jordan | INFLUENCES OF DIETARY FAT ON THE PERIPHERAL ECS IN LEAN AND OBESE HUMAN SUBJECTS | 53 |
| 9.45 | Yossef Tam, Jie Liu, Sándor Bátkai, Douglas Osei-Hyiaman, Alexandros Makriannis, V. Kiran Vemuri, Keith Sharkey and George Kunos | ANTI-OBESITY EFFECTS OF A NOVEL PERIPHERALLY ACTING NEUTRAL CANNABINOID 1 RECEPTOR ANTAGONIST IN MICE | 54 |

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|---|---|---|---|
| 10.00 | Nina L. Cluny, Catherine M. Keenan, Marnie Duncan, Alyson Fox, Beat Lutz and Keith A. Sharkey | THE ACTIONS OF A PERIPHERALLY RESTRICTED CANNABINOID CB1/CB2 RECEPTOR AGONIST ON EXPERIMENTAL COLITIS IN MICE | 55 |
| 10.15 | W.-S. Vanessa Ho and Neelam Parmer | N-ARACHIDONOYL GLYCINE ACTS AS A VASORELAXANT VIA NITRIC OXIDE AND LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS | 56 |
| 10.30 – 11.00 | COFFEE BREAK [POSTER AREA] | | |
| **ORAL SESSION 10.** ANALGESIA<br>**MODERATORS: STEPHEN ALEXANDER AND ANDREA HOHMANN** | | | ABSTRACT # |
| 11.00 | K. Starowicz, W. Makuch, M. Osikowicz, S. Petrosino, F. Guadagno, V. Di Marzo and B. Przewlocka | ANANDAMIDE INJECTED INTRATHECALLY INDUCES ANALGESIA MEDIATED BY A TRPV1-DEPENDENT MECHANISM IN NEUROPATHIC RATS | 57 |
| 11.15 | Mauro Dionisi, Leonie Norris, David Barrett, Stephen Alexander and Andrew Bennett | SUSTAINED INHIBITION OF FAAH ACTIVITY ELEVATES ENDOCANNABINOID TONE AND ACTIVATES PPAR NUCLEAR RECEPTORS IN HUMAN NEUROBLASTOMA CELLS | 58 |
| 11.30 | Steven G. Kinsey, Jonathan Z. Long, Scott T. O'Neal, Rehab A. Abdulla, Justin L. Poklis, Dale L. Boger, Benjamin F. Cravatt and Aron H. Lichtman | FAAH AND MAGL INHIBITION REDUCE NEUROPATHIC PAIN VIA DISCRETE RECEPTOR-SPECIFIC MECHANISMS OF ACTION | 59 |
| 11.45 | Kay Ahn, Douglas S. Johnson, Mauro Mileni, David Beidler, Jonathan Z. Long, Michele K. McKinney, Eranthie Weerapana, Nalini Sadagopan, Marya Liimatta, Sarah E. Smith, Scott Lazerwith, Cory Stiff, Satwik Kamtekar, Keshab Bhattacharya, Yanhua Zhang, Stephen Swaney, Keri Van Becelaere, Raymond C. Stevens and Benjamin F. Cravatt | DISCOVERY AND CHARACTERIZATION OF A HIGHLY SELECTIVE FAAH INHIBITOR THAT REDUCES INFLAMMATORY PAIN | 60 |

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| 12.00 | Ethan Russo, Stephen Wright, Philip Robson and Colin Stott | DOUBLE-BLIND, RANDOMIZED WITHDRAWAL CLINICAL TRIAL OF SATIVEX IN CENTRAL NEUROPATHIC PAIN IN MULTIPLE SCLEROSIS | 61 |
| 12.15 | Sabatino Maione, Enza Palazzo, Alessia Ligresti and Vincenzo Di Marzo | EFFECT OF PHYTOCANNABINOIDS ON SUPRA-SPINAL ANTINOCICEPTIVE DESCENDING PATHWAYS IN RATS: INVOLVEMENT OF CANNABINOID, TRP AND ADENOSINE RECEPTORS | 62 |
| 12.30 – 14.00 | LUNCH [ATRIUM] | | |
| 14.00 – 16.00 | **POSTER SESSION 4** | | |
| 16.00 – 17.00 | **YOUNG INVESTIGATOR AWARD**<br>"CANNABINOID RECEPTORS AND HUNTINGTON'S DISEASE"<br>**MICHELLE GLASS, PH.D.**<br>The University of Auckland | | PS4 |
| 17.00 | **ICRS BUSINESS MEETING** | | |
| 19.00 | **ICRS BANQUET** | | |

*Notes:*

# Poster Sessions 1 & 2 - Topics A - E

## July 8th (17.00 - 19.00) & July 9th (17.00 – 1900)

### *Topic A. Ligand Development / Receptor Structure*

| | | |
|---|---|---|
| Marilena Pira, Paolo Lazzari, Stefania Ruiu, Simone Tambaro, Christian Dessì and Luca Pani | SYNTHESIS AND BIOLOGICAL EVALUATION OF ANALOGUES OF THE CB1 ANTAGONIST N-PIPERIDINYL-5-(5-CHLORO-THIOPHEN-2-YL)-1-(2′,4′-DICHLOROPHENYL)-4-METHYL-1H-PYRAZOL-3-CARBOXAMIDE | P1 |
| Jianhong Chen, John W. Huffman, Jenny L. Wiley and Billy R. Martin | SYNTHESIS OF GEMINALLY DIMETHYL SUBSTITUTED ANALOGUES OF $\Delta^8$-THC | P2 |
| William Courtney | LIPINS, CANNABINS AND ENCANNINS: TOWARDS A RATIONAL NOMENCLATURE | P3 |
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| Toru Uyama, Jun Morishita, Xing-Hua Jin, Yasuo Okamoto, Kazuhito Tsuboi and Natsuo Ueda | THE TUMOR SUPPRESSOR H-REV107 FUNCTIONS AS PHOSPHOLIPASE A1/2 WITH A LOW N-ACYLTRANSFERASE ACTIVITY | P66 |
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| Stephen PH Alexander and Annie Patel | 4-NITROPHENYLACETATE AS A POTENTIAL SUBSTRATE FOR MAG LIPASE: INVESTIGATION OF AN ASSAY SUITABLE FOR HIGH-THROUGHPUT SCREENING USING RAT TISSUES | P68 |
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| Emmelie Björklund, Erika Norén, Johanna Nilsson, Yanlin Hu, and Christopher J. Fowler | THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ LIGAND TROGLITAZONE IS A POTENT INHIBITOR OF MONOACYLGLYCEROL LIPASE | P71 |
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| Hodaya Dahan, Aron Weller and Ester Fride | CANNABINOID CB1 RECEPTOR BLOCKADE AT BIRTH CAUSES GROWTH FAILURE DUE TO ORAL MOTOR WEAKNESS AND IS MEDIATED BY PERIPHERAL MECHANISMS | P86 |
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# KANG TSOU MEMORIAL LECTURE

# WEDNESDAY, JULY 8TH, 2009

## 16.00 - 17.00

## THE GLUTAMATE HOMEOSTASIS HYPOTHESIS OF ADDICTION


### PETER KALIVAS, PH.D.

Department of Neurosciences, Medical University of South Carolina, Charleston



The vulnerability to relapse is a defining characteristic of addiction to drugs of abuse, and constitutes a primary target for pharmacotherapeutic intervention. The neurocircuitry of relapse for many addictive drugs has been examined in animal models of drug seeking and using neuroimaging in humans. One common feature between drug-seeking in animals and the desire for drugs in humans is the activation of the projection from the prefrontal cortex to the nucleus accumbens. Considering this information in concert with the observation that the vulnerability to relapse arises in part from an inability of negative environmental contingencies to inhibit relapse, it has been proposed that communication between prefrontal cortex and striatal habit circuitry is impaired; thereby preventing the proper processing of environmental contigencies that would normally inhibit drug-seeking habits. In this presentation, it is hypothesized that this deficit results from a pathology in the prefrontal to accumbens projection, and we will explore the dysfunctional neuroplasticity that is produced in this projection using the reinstatement animal model of cocaine- and heroin-seeking. Thus, self-administration of these drugs changes the regulation of synaptic glutamate release in this projection by nonsynaptic glutamate, which impairs synaptic plasticity. The regulation of synaptic release and plasticity by nonsynaptic, extracellular glutamate is via extrasynaptic metabotropic glutamate receptors, and this process is termed glutamate homeostasis. The impairment in synaptic plasticity associated with dysregulation of glutamate homeostasis by drugs of abuse can be demonstrated in postsynaptic proteins, dendritic spines and electrophysiological responses of accumbens neurons to prefrontal input. These pathological effects of addictive drugs arise from down-regulated glial release and uptake of glutamate, which reduces glutamatergic modulation of pre- and postsynaptic extrasynaptic metabotropic glutamate receptors. Thus, glutamate release by the cystine-glutamate exchanger and glutamate uptake by the glial transporter (GLT-1) is down-regulated after drug self-administration. Importantly, the imbalance in glutamate homeostasis can be normalized by drugs that activate glutamate release through the cystine-glutamate exchanger (i.e. N-acetylcysteine) or stimulate glutamate uptake (i.e. ceftriaxone). The restoration of glutamate homeostasis by these drugs ameliorates the changes in synaptic plasticity induced by the self-administration of cocaine or heroin, including changes in dendritic spine morphology and the potentiation or depotentiation of synaptic strength (i.e. LTP and LTD, respectively). Also, restoring glutamate homeostasis inhibits the reinstatement of drug-seeking in animal models of relapse, and in double-blind pilot clinical trials N-acetylcysteine has been shown to inhibit cocaine cue reactivity, the number of cigarettes smoked and the desire to gamble.

PLENARY LECTURE

# THURSDAY, JULY 9TH, 2009

14.00 - 15.00

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# INDIVIDUAL DIFFERENCES IN RESPONSES TO DRUGS IN HUMANS

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HARRIET DE WIT, PH.D.

University of Chicago

Individuals vary widely in their behavioral and physiological responses to psychoactive drugs, in ways that may be related to the drugs' potential to be abused. We and others have studied the sources of these individual differences in controlled studies, measuring subjective, behavioral and neurophysiological responses to single doses of drugs in healthy volunteers. Many factors contribute to inter-individual variation in drug effects, including environmental factors such as expectancies and prior experience, as well as biological factors such as genetics and hormonal status. We will review recent findings describing individual variations in responses to a prototypic stimulant, d-amphetamine, and their associations with polymorphisms in genes involved in the drug's action. We will also describe recent findings relating to cannabinoids, including the involvement of endocannabinoid mechanisms in acute responses to amphetamine, and individual differences in the acute effects of $\Delta^9$-tetrahydrocannabinol.

PLENARY LECTURE

FRIDAY, JULY 10TH, 2009

14.00 - 15.00

# AN EVALUATION OF BRAIN ENDOCANNABINOID SIGNALING AND ADDICTION-RELATED BEHAVIORS


LARRY PARSONS, PH.D.

Associate Professor, Committee on the Neurobiology of Addictive Disorders,
The Scripps Research Institute, La Jolla, California, USA



Addictive drugs exert their behavioral and reinforcing effects through actions on multiple neurotransmitter systems including monoamines, endogenous opioids, and both excitatory and inhibitory amino acids. A growing body of literature also points to an involvement of the endogenous cannabinoid system in the etiology of drug addiction. For example there is evidence of a significant correlation between allelic variances in the cannabinoid-1 ($CB_1$) receptor gene and the incidence of drug dependence in humans. Similarly, a naturally occurring single nucleotide polymorphism in the gene encoding the endocannabinoid inactivating enzyme fatty acid amide hydrolase (FAAH) is associated with problem drug and alcohol use. A substantial amount of preclinical data also points to a $CB_1$ receptor influence in regulating the self-administration of alcohol, opiates and nicotine. However, little is known of the mechanisms through which endocannabinoids modulate drug-induced behaviors and there is even less understanding of the effects of prolonged drug exposure on endocannabinoid signaling. This lecture will provide *in vivo* evidence that voluntary drug intake alters extracellular endocannabinoid levels in the rodent brain in a manner that modulates the motivation for continued drug intake. Data demonstrating altered endocannabinoid function following chronic drug exposure will also be presented along with evidence that this dysfunction contributes to excessive drug intake through negative reinforcement mechanisms.

PLENARY LECTURE

# SATURDAY, JULY 11TH, 2009

16.00 - 17.00

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## CANNABINOID RECEPTORS AND HUNTINGTON'S DISEASE

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MICHELLE GLASS, PH.D.

Department of Pharmacology, School of Medical Sciences, University of Auckland.

Huntington's disease (HD) is a dominant inherited neurodegenerative disorder caused by a CAG repeat expansion resulting in a mutated polyglutamine tract in the expressed *huntingtin* (Htt) protein. Individuals with >39 CAG repeats develop HD; symptoms include a movement disorder and chorea, deficits in cognitive function and psychiatric changes. CB1 cannabinoid receptors are found at high densities in areas of basal ganglia circuitry that are specifically affected by cellular dysfunction and death in HD. These receptors are expressed pre-synaptically on GABAergic striatal terminals in the globus pallidus and substantia nigra and modulate inhibitory synaptic neurotransmission. When evaluated across a range of neuropathological grades in human post-mortem tissue, the loss of CB1 receptors from the basal ganglia output nuclei is one of the earliest neurological changes observed in HD, and unlike changes in co-localised dopamine receptors and neuropeptides, appears to precede cell death.

We have utilized two model systems, R6/1 mice and a Htt exon one PC12 cell model in order to better understand the role of the cannabinoid receptors in HD disease pathology. When exposed to a stimulating enriched environment from an early age, R6/1 mice showed improved performance in behavioural tests, that correlated with a delay in the neurodegenerative loss in cerebral volume (van Dellen et al., 2000). CB1 receptor ligand binding sites were examined in R6/1 mice and the benefits of environmental enrichment were found to correlate with an up-regulation of CB1 receptors in striatal output nuclei, when assessed at 20 weeks of age. R6/1 mice treated for 8 weeks from 12 weeks of age with daily injections of HU210 (0.01 mg/kg), URB596 (0.30 mg/kg) or THC (10.00 mg/kg) do not show any significant improvement in disease progression, furthermore, increased seizures and aggregates are observed in HU210-treated animals suggesting this drug treatment may actually be detrimental. In contrast with these findings, a small but significant improvement in cell viability in response to HU210 treatment is observed in PC12 cells expressing mutant exon one Htt protein. Inhibitor studies have shown that this effect is mediated via Gi activation of pERK1/2. Consistent with studies in R6/1 mice however, increased aggregates are observed in these cells, and this process appears to be mediated by activation of Gs by CB1. Thus activation of Gαi/ pERK by CB1R may be protective in HD, but promiscuous coupling of CB1R to Gαs may limit the therapeutic potential. Intriguingly, CB1 receptors appear to be selectively vulnerable in Htt cells compared to D1 dopamine receptors expressed under the same promoter, implying that loss of CB1 may be due to high intrinsic levels of constitutive activity and receptor turnover, or dysfunction of CB1-specific intracellular trafficking pathways.

# ENDOCANNABINOIDS, THE LIVER, AND THE METABOLIC SYNDROME

George Kunos[1], Jie Liu[1], Douglas Osei-Hyiaman[1], James Pickel[2]

[1]Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, and [2]Transgenic Core, National Institute on Mental Health, NIH, Bethesda, MD 20892

Diet-induced obesity is associated with the accumulation of ectopic fat in the liver, as well as insulin and leptin resistance and dyslipidemias. Endocannabinoids (ECs) and CB1 receptors (CB1R) have been implicated in these associated phenotypes because a) CB1R knockout (CB1-/-) mice are resistant to these diet-induced changes despite unchanged caloric intake, and b) the hormonal/metabolic profile of obese people is improved upon treatment with a CB1R antagonist. Functional CB1R are present in the liver where their activation leads to increased lipogenic gene expression and hepatic lipogenesis. To test whether hepatic CB1R may also be involved in diet-induced glucose intolerance and insulin resistance, we generated mice with hepatocyte-specific deletion of CB1R (LCB1-/- mice), as well as transgenic mice that express CB1R only in liver (CB1tg mice) on a C57Bl6 background. In vivo treatment with the CB1R agonist HU-210 or anandamide causes acute glucose intolerance and insulin resistance in wild-type (wt) and CB1tg mice, but not in CB1-/- or LCB1-/- mice. Similarly, wt or CB1tg mice on a high fat diet develop glucose intolerance and insulin resistance, whereas CB1-/- and LCB1-/- mice on the same diet remain glucose tolerant and insulin sensitive. In vitro exposure of mouse or human isolated hepatocytes to insulin causes increased akt/PKB phosphorylation. The insulin-induced increase in akt/PKB phosphorylation is attenuated by CB1 agonists in human hepatocytes and in hepatocytes from wt and CB1tg mice, but not in cells from CB1-/- or LCB1-/- mice. These findings indicate that both human and mouse hepatocytes have functional CB1R , activation of which inhibits insulin signaling via akt/PKB. Furthermore, hepatic CB1R activation is both necessary and sufficient to account for the glucose intolerance and insulin resistance of mice fed a high fat diet.

## ENDOCANNABINOIDS AND HEMODYNAMIC CONSEQUENCES OF LIVER CIRRHOSIS

Sándor Bátkai, Pál Pacher, George Kunos

Laboratory of Physiological Studies, NIAAA, National Institutes of Health, Bethesda, MD 20892-9413, USA

Hepatic cirrhosis, the end stage of chronic liver disease is associated with impaired hepatic function, portal hypertension and resultant cardiovascular complications including hemodynamic changes, altered systemic and splanchnic circulation, and cardiac dysfunction. Recent studies have demonstrated the involvement of the endocannabinoid (EC) system in the pathogenesis of cirrhosis and the associated cardiovascular abnormalities. Increased tissue levels of ECs have been detected not only in the liver, but also in cardiac tissue and circulating monocytes from cirrhotic rats. Moreover, in advanced cirrhosis, selective CB1 receptor antagonists reversed the hemodynamic alterations (systemic hypotension, decreased portal venous pressure, increased mesenteric flow and cardiac output; and increased total peripheral resistance). An advanced-stage complication of cirrhosis, the cirrhotic cardiomyopathy, has been documented in detail through altered parameters of contractile function using real-time intraventricular pressure and volume monitoring. The impaired cardiac contractility in cirrhosis could be attributed to increased activity of the EC system, as CB1 blockade was found to correct the contractile dysfunction in animal models of cirrhosis. By reversing the cardiovascular abnormalities, CB1 blockade also delays ascites formation and may reduce the likelihood of variceal rupture, a potentially fatal complication of advanced cirrhosis. CB1 receptor antagonism may therefore be therapeutically exploited in the medical management of advanced liver cirrhosis, helping patients to survive until a transplant becomes available.

# Cannabinoids and Liver Diseases:

## Molecular Mechanisms and Drug Development

### CANNABINOIDS, LIVER INFLAMMATION AND CANCER

Prakash Nagarkatti, Venkatesh L. Hegde, and Mitzi Nagarkatti

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University of South Carolina School of Medicine, Columbia, SC 29208, USA

The induction of CB1 and CB2 cannabinoid receptors during liver injury and the potential involvement of endocannabinoids in the regulation of this process have sparked significant interest in further evaluating the role of cannabinoid system during hepatic disease. Cannabinoid abuse has been shown to exacerbate liver fibrogenesis in patients with chronic hepatitis C infection involving CB1 receptor. Nonetheless, CB2 receptor activation may play a protective role during chronic liver diseases.

Our laboratory has focused on an acute model of murine hepatitis that mimics autoimmune hepatitis and investigated the effect of endogenous and exogenous cannabinoids in the treatment and pathogenesis of hepatitis. We investigated the effects of THC in a murine model of Concanavalin A(ConA)-induced hepatitis. Intraperitoneal administration of delta-9-tetrahydrocannabinol (THC) after ConA challenge, inhibited hepatitis as indicated by significant decrease in liver enzymes and less severe liver tissue injury. THC effect was mediated though CB1 and CB2 receptors and was found to suppress inflammation in the liver through multiple pathways that included induction of apoptosis in activated T cells, inhibition of inflammatory Th1 cytokines, induction of intrahepatic $CD4^{+}CD25^{+}$ Foxp3+ regulatory T cells and myeloid suppressor cells. Together, such immunosuppressive mechanisms were responsible for ameliorating hepatic injury. Interestingly, select CB1 or CB2 agonists were not able to block hepatitis either independently or in combination. However, CB1/CB2 mixed agonists were able to efficiently attenuate hepatitis similar to THC. We also noted that administration of CB1 or CB2 antagonists alone in mice undergoing hepatitis failed to inhibit liver injury.

In addition to exogenous cannabinoids, we found that anandamide was also able to reduce hepatitis and furthermore, deficiency or inhibition of endocannabinoid hydrolyzing enzyme fatty acid amide hydrolase (FAAH), resulted in decreased liver injury upon ConA challenge. Interestingly, cannabidiol, a major non-psychoactive constituent of Cannabis was also able to effectively inhibit liver injury.

While our studies demonstrate that cannabinoids can be an effective modality to treat hepatic inflammation and consequent injury, we have also shown that THC treatment can promote cancers that do not express cannabinoid receptors. In contrast, if the tumor cells express CB receptors, cannabinoids are highly effective in inducing apoptosis in such cancer cells. Thus, the use of cannabinoids against hepatocellular carcinoma may depend on the levels of expression of CB receptors. Together, our studies demonstrate that targeting cannabinoid receptors may constitute a novel treatment modality against inflammatory injury to the liver, particularly acute forms of T cell-mediated hepatitis.

Acknowledgements: Funded by NIH Grants P01AT003961, R01DA016545, R01AI053703, R01ES09098, R01AI058300, R01HL058641.

# CANNABINOIDS AND LIVER DISEASES:

## MOLECULAR MECHANISMS AND DRUG DEVELOPMENT

### THE ENDOCANNABINOID SYSTEM AS A REGULATOR OF THE HEPATIC WOUND HEALING PROCESS

Sophie Lotersztajn

Inserm U955, Hopital Henri Mondor, 94010, Creteil, FRANCE

Chronic liver disease is responsible for about 800,000 death/year due to cirrhosis and its complications. The most common causes of liver disease worldwide are viral hepatitis, chronic alcohol consumption and non alcoholic fatty liver disease associated with the metabolic syndrome. Following acute liver injury, restoration of normal architecture results from a wound healing response that associates an inflammatory reaction, hepatocyte proliferation and a matrix remodeling process driven by hepaticmyofibroblasts. In contrast, chronic liver injury is associated with prolonged and dysregulated wound healing response that leads to fibrogenesis. The cirrhotic endstage is is a major public health problem worldwide owing to life-threatening complications of portal hypertension and liver failure and to the risk of incident hepatocellular carcinoma. The frequent inability to eradicate the cause of chronic liver disease warrants the development of liver-specific antifibrotic strategies targeting fibrogenic cells, in order to inhibit their accumulation and/or reducing extracellular matrix accumulation. In addition, inhibition of parenchymal injury, or reduction of liver inflammation has also shown some beneficial antifibrogenic effects. However, despite encouraging experimental results, proof of efficacy of potential antifibrogenic molecules in a clinical setting is currently lacking.

Our recent studies have shown that the endocannabinoid system is a crucial regulator of the wound healing response. Thus, CB1 and CB2 receptors are up-regulated in the cirrhotic human liver, predominantly in liver fibrogenic cells. Moreover, endogenous activation of CB2 receptors limits progression of experimental liver fibrosis by reducing accumulation of liver fibrogenic cells. Finally, our recent data demonstrate that CB2 receptors reduce liver injury and promote liver regeneration, demonstrating that CB2 agonists elicit useful hepatoprotective properties, in addition to their antifibrogenic effects.

In contrast to CB2 receptors, CB1 is profibrogenic in the liver. Indeed, administration of rimonabant or genetic inactivation of CB1 receptors inhibits fibrosis progression in three models of chronic liver injury, by a mechanism involving reduced proliferation and increased apoptosis of liver fibrogenic cells. Moreover, daily cannabis use is an independent predictor of fibrosis severity in patients with chronic hepatitis C, suggesting that CB1 signaling predominates over CB2 in these patients.

These data demonstrate that the endocannabinoid system plays a dual opposite role during chronic liver injury, that associates antifibrogenic and hepatoprotective properties of CB2 receptors, and profibrogenic effects of CB1 receptors. These findings suggest that combined therapy with peripherally-restricted CB1 antagonists and/or CB2 agonists might open novel perspectives for the treatment of chronic liver disease.

# BIS[(DIALKYLTHIOCARBAMOYL)]DISULFIDE DERIVATIVES AS POTENT AND SELECTIVE MONOGLYCERIDE LIPASE INHIBITORS

Didier M. Lambert, Coco N. Kapanda,
Giulio G. Muccioli, Geoffray Labar, Jacques H. Poupaert

Louvain Drug Research Institute, Département de Chimie pharmaceutique (CMFA-cannabinoid and endocannabinoid research group), Université catholique de Louvain, 1200 Brussels, Belgium.

**Introduction**: Monoglyceride lipase received a great deal of attention over the last few years as it participates in regulating 2-AG levels. Along this line, inhibition of human monoglyceride lipase (hMGL) may offer a rational therapeutic approach in treating disease states in which a higher 2-AG tone would be beneficial. As our group recently discovered the MGL inhibitory properties of the marketed drug disulfiram (**1**), we present here the synthesis, pharmacological evaluation and SAR within a series of bis[dialkyl(thiocarbamoyl)]disulfide derivatives.

**Methods**: Target compounds were synthesized through an oxidative coupling of dithiocarbamic acid salts. Inhibition of MGL and FAAH was measured using pure human recombinant MGL and human recombinant FAAH.

**Results**: Bis(4-methyl-1-piperazinylthiocarbonyl)disulfide (**2**) was found to inhibit hMGL with a $pI_{50}$ of 6.97±0.05, along with a selectivity ratio of 1825 compared with FAAH inhibition. hMGL inhibition within this series was highly dependent on the presence of the disulfide group, as similar compounds lacking this functionality were less potent or unable to inhibit hMGL. The absence of substrate hydrolysis in diluted inhibitor-preincubated hMGL confirmed the irreversible nature of the inhibition (fig 1). Beside this, after the inactivation of the enzyme by compound **2,** DTT was able to restore enzyme activity (fig. 2). This suggests that these compounds inhibit MGL by reacting with the sulfhydryl moiety of Cys208 and/or Cys242, located in the vicinity of MGL active site, to form either a mixed adduct or an intramolecular disulfide bond.

**Conclusion**: We have demonstrated that bis[(dialkylthiocarbamoyl)]disulfide derivatives are hMGL inhibitors and that their ability to inhibit enzyme activity is closely related to the presence of thiocarbonyl and disulfide moieties. These compounds constitute therefore useful pharmacological tools to explore the endocannabinoid system.

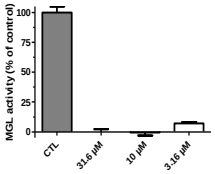
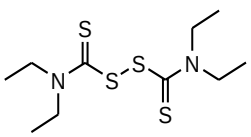



Fig.1



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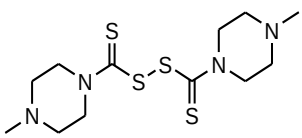

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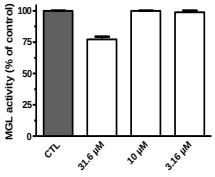


Fig.2

# STRUCTURE-ACTIVITY RELATIONSHIPS AT THE $CB_1$ AND $CB_2$ RECEPTORS FOR 1-ALKYL-3-(1-NAPHTHOYL-4 AND 8-HALOGEN SUBSTITUTED) INDOLES

John W. Huffman[1], Valerie J. Smith[1], Jianhong Chen[1], Jenny L. Wiley[2], and Billy R. Martin[2]

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We recently (Huffman *et al. Bioorg. Med. Chem.* **2005**, *13*, 89) reported structure-activity relationships (SAR) for a number of 1-alkyl-3-(1-naphthoyl)indoles with various substituents on the naphthalene portion of the molecule. These compounds contained either an unsubstituted naphthalene system or electron releasing substituents. To clarify the SAR for this class of cannabinoids we now describe the effect upon $CB_1$ and $CB_2$ receptor affinities of moderately electron withdrawing halogen substituents at C-4 and C-8 of the naphthalene group.

The syntheses of both groups of cannabimimetic indoles proceeded via acylation of the appropriate 1-alkylindole. The 4-halo-1-naphthoic acids, precursors for the corresponding aroyl chloride, were either known or commercially available compounds. The 8-halo-1-naphthoic acids were prepared from 1,8-naphthalic anhydride via several step syntheses.

The 1-pentyl-3-(1-naphthoyl-4-halo)indoles have uniformly high affinity for both receptors ($K_i$ = 1.2-14 nM) with no selectivity for either receptor. The 1-propyl analogs have modest affinities for the $CB_1$ receptor ($K_i$ = 93–530 nM), but high affinity for the $CB_2$ receptor ($K_i$ = 7-44 nM). 1-Propyl-2-methyl-3-(1-naphthoyl-4-iodo)indole (JWH-422) is 20-fold selective for the $CB_2$ receptor with $K_i$ = 20 nM at the $CB_2$ receptor and 501 nM at $CB_1$. The 4-fluoro (JWH-415) and 4-bromo (JWH-395) analogs are also selective for the $CB_2$ receptor (14 and 12-fold, respectively).

In the 8-halo series bromo, chloro and iodo analogs were prepared. $CB_1$ and $CB_2$ receptor affinities are in general weaker than for the 4-halo series. Two compounds, 1-pentyl-3-(1-naphthoyl-4-chloro)indole (JWH-457) and the corresponding bromo analog (JWH-424) have high affinity for the $CB_1$ receptor($K_i$ = 23 and 21 nM, respectively). 1-Pentyl-2-methyl-3-(1-naphthoyl-8-iodo)indole (JWH-417) is 40-fold selective for the $CB_2$ receptor with $K_i$ = 13 nM at the $CB_2$ receptor and 522 nM at $CB_1$. None of the 1-propyl compounds in this series have significant $CB_2$ receptor affinity.

In conclusion, a 4-halo-1-naphthoyl substituent has little effect upon $CB_1$ and $CB_2$ receptor affinity, however an 8-halogen attenuates affinity, probably due to steric effects, similar to those in the 2-methoxynaphthoyl series.

**Acknowledgements:** Supported by grants DA03590 and DA03672 from NIDA.

# DISCOVERY OF NEW CB2 LIGANDS WITH NOVEL CHEMICAL SCAFFOLD

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[1]Dept of Pharmaceutical Sciences, School of Pharmacy; Drug Discovery Institute; Departments of Computational & Structural Biology, University of Pittsburgh, USA ; [2]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zürich, Switzerland; [3]Dept of Pharmacology & Toxicology, Virginia Commonwealth University, USA

The identification of cannabinoid receptor subtype 2 (CB2) has led to renewed interest in the medicinal chemistry and pharmacology of cannabinoids. Unlike subtype CB1, found primarily in the brain and involved in CNS and psychotropic side effects, CB2 is predominantly found in the immune system. The fast emerging research suggests that CB2 ligands may have potential for treatment of inflammatory pain, neuropathic pain and autoimmune diseases as well as osteoporosis and other bone diseases. Recent studies reveal that CB2 receptors also occur in the central nervous system (CNS), thus the CB2 receptor may be an important drug target that has therapeutic promise for treating CNS disorders without psychotropic side effects. We have established a comprehensively combined approach of pharmacophore query generation, cheminformatics/structurally-diverse chemical library construction, 3D database in silico screening, and in vitro bioassay validation techniques for novel CB2 ligand design and discovery. The integrated in silico and in vitro GPCR chemical genomics approaches have allowed us successfully to identify several nanomolar-to-micromolar binding affinity CB2 ligands with new chemical scaffolds and high CB2 receptor selectivity. Our new CB2 ligands, in particular the CB2 antagonist XIE35 and its later generations, exhibit both chemical and pharmacological novelties in terms of new chemical scaffolds and high CB2 specificity (mouse CB2 HEK cell membrane Ki=32 nM and rat brain CB1 Ki=4185 nM). Structurally, XIE35 possesses a non-ring-type centroid moiety that is different from the structure of other reported CB2 antagonists. Pharmacologically, XIE35 appears to be a neutral antagonist with the ability to inhibit activity of CB2 agonists (e.g., WIN55212-2), but not an inverse agonist – a typical property of reported CB2 antagonists (e.g., SR144528). The new CB2 ligands provide us with chemical probes to explore CB2-mediated biological mechanisms and biological activity. The compounds are currently subject to chemical modification and SAR studies, as well as further biological studies. The developed in silico and in vitro chemical genomics approaches generate valuable outcomes in terms of 3D pharmacophore database virtual screening algorithm development and new CB2 chemical probes discovery, which will provide insight into better understanding of CB2 receptor biological mechanisms (Supported by NIH NIDA/NLM R01 015147.

*Sean Xie, email: xix15@pitt.edu

# A-1036654, A NOVEL ORALLY BIOAVAILABLE $CB_2$-SELECTIVE AGONIST EXHIBITING ANALGESIC ACTIVITY IN RODENT MODELS OF OSTEOARTHRITIC AND NEUROPATHIC PAIN

Michael Meyer, Betty Yao, Anthony Daza, Yihong Fan, Lanlan Li, Loan Miller, Odile El Kouhen, Gin Hsieh, Erica Wensink, Anita Salyers, Prasant Chandran, Michael Dart, Tongmei Li, and William Carroll

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**Introduction:** There is growing pre-clinical evidence that $CB_2$-selective agonists exhibit analgesic activity across a range of rodent models of nociceptive and neuropathic pain without eliciting the $CB_1$-mediated effects such as hypolocomotion, hypothermia, and catalepsy. However, the tool compounds available to the research community, such as AM1241, JWH-015 and JWH-133 are less than ideal *in vivo* probes for understanding the therapeutic potential of $CB_2$-selective agonists due to limitations with regards to subtype selectivity and poor pharmacokinetic profiles. Here we report the identification of A-1036654, a highly selective $CB_2$ agonist exhibiting excellent oral bioavailability and long half-life in rodents.

**Methods**: A-1036654 was evaluated for affinity, agonist efficacy and selectivity for the rat cannabinoid receptors, and in vivo efficacy was assessed in rodent models of osteoarthritic and neuropathic pain after single dose and sub-chronic administration.

**Results**: In radioligand binding assays, A-1036654 exhibits high affinity for the rat $CB_2$ receptor (Ki = 0.43 nM) and 9000-fold selectivity relative to the rat $CB_1$ binding site. Consistent with the radioligand binding profile, A-1036654 possesses high agonist potency (0.16 nM) and 27,000-fold selectivity for the rat $CB_2$ vs. $CB_1$ receptors in inhibition of adenylyl cyclase activities. *In vivo*, A-1036654 is fully effective in a monoiodoacetate-induced osteoarthritic pain (MIA-OA) model after oral administration, and exhibits a 10-fold leftward shift in the dose-response after sub-chronic administration (acute $ED_{50}$ = 11 mg/kg, p.o.; sub-chronic $ED_{50}$ = 1.0 mg/kg, p.o.). Additionally, A-1036654 displays efficacy comparable to gabapentin in the chronic constriction injury (CCI) model of neuropathic pain, and similarly to that seen in the MIA-OA pain model, a leftward shift in the dose-response is observed with sub-chronic administration. Importantly, full efficacy was achieved in this model without affecting spontaneous locomotor activity. Pharmacokinetic studies in the rat revealed high oral bioavailability (60%), moderate half-life (~4 hr), and excellent penetration into the CNS.

**Conclusion**: A-1036654 is a highly selective $CB_2$ ligand with agonist efficacy in *in vitro* functional assays. It exhibits analgesic efficacy across models of osteoarthritic and neuropathic pain, with enhanced potency after sub-chronic administration. A-1036654 shows excellent oral bioavailability and good CNS penetration, making it a highly useful tool for further exploration of the role of $CB_2$ receptor activation in the treatment of acute and chronic nociceptive and neuropathic pain conditions.

# COMPARISON BETWEEN THE $CB_2$ RECEPTOR AGONIST O-3223 AND THE POTENT, MIXED $CB_1/CB_2$ RECEPTOR AGONIST CP55,940 IN A BATTERY OF NOCICEPTIVE ASSAYS

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Although it is well established that $\Delta^9$-tetrahydrocannabinol (THC) and other mixed $CB_1/CB_2$ receptor agonists reliably elicit antinociceptive effects, their psychomimetic actions as well as their potential for abuse and dependence have dampened enthusiasm for their development as therapeutic agents. Conversely, $CB_2$ receptor selective agonists have been shown to reduce pain and inflammation, without eliciting any apparent cannabinoid behavioral effects. In the present study, we compared the pharmacological effects of the ethyl sulfonamide THC analog, O-3223, to the potent, mixed $CB_1/CB_2$ receptor agonist, CP55,940, in battery of preclinical models of pain, including the tail-flick test, hot plate test, formalin test, LPS model of inflammatory pain, and chronic constrictive injury of the sciatic nerve (CCI) model of neuropathic pain. Agonist-stimulated [$^{35}$S]GTPγS binding in $CB_1$ or $CB_2$ receptor transfected CHO cells revealed that O-3223 was approximately 50-fold more selective for $CB_2$ receptors than for $CB_1$ receptors. The $EC_{50}$ $\pm$ SEM values of O-3223 at $CB_1$ and $CB_2$ receptors were 431 $\pm$ 161nM and 8.6 $\pm$ 2.6 nM, respectively. O-3223 effectively reduced nociceptive behavior in both the early and late phases of the formalin test, reduced thermal hyperalgesia in CCI model, and reduced edema as well as thermal hyperalgesia elicited by an intraplantar injection of LPS into the paw. Each of these effects was completely blocked by pretreatment with the $CB_2$ receptor selective antagonist SR144528 (3 mg/kg), but not by pretreatment with the $CB_1$ receptor antagonist rimonabant (3 mg/kg). However, O-3223 did not elicit antinociceptive effects in either the tail-flick or hot-plate tests, acute models of thermal analgesia. Additionally, O-3223 did not elicit either hypothermia or motor disturbances, as assessed in the rotarod test. In contrast, CP55,940 elicited a $CB_1$ receptor mediated analgesic responses in each pain assays, as well as $CB_1$ receptor mediated hypothermia and motor disruption. Interestingly, SR144528 did not block any of the pharmacological actions of CP55,940. These data indicate that the $CB_2$ selective receptor agonist, O-3223, reduces inflammatory and neuropathic pain, without affecting normal pain perception or eliciting any apparent behavioral effects. Moreover, this compound can serve as a template to develop new $CB_2$ receptor agonists with increased receptor selectivity and increased potency in treating inflammatory and neuropathic pain.

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# PYRAZOLE CANNABINOIDS WITH NON-$CB_1$, NON-$CB_2$ ACTIVITY

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The prototypic $CB_1$ cannabinoid receptor antagonist/inverse agonist, rimonabant, is comprised of a pyrazole core surrounded by a carboxyamide with terminal piperidine group (3-substituent), a 2,4-dichlorophenyl group (1-substituent), a 4-chlorophenyl group (5-substituent) and a methyl group (4-substituent) (Fig 1). Results of previous research have suggested that the 3-position appears to be involved in receptor recognition and agonist activity [Wiley et al., 2001, J Pharmacol Exp Ther, 296:1013-1022]. Several of the 3-substituted analogs possess cannabinoid agonist properties *in vivo* [Martin tetrad effects: suppression of activity, antinociception, hypothermia, catalepsy; Martin et al., 1991, Pharmacol Biochem Behav, 40:471-478].

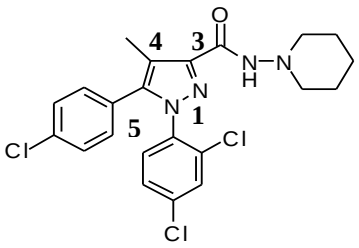


**Figure 1: Rimonabant substituents**

Recently, we characterized a series of rimonabant analogs with 3-substituent substitutions, in which the 3- carboxamide was retained and bromo, cyano and morpholine substitutions for the terminal piperidine were made. Several notable patterns emerged. First, similar to rimonabant, these pyrazole agonist analogs exhibited better affinity for the $CB_1$ receptor when rimonabant (vs. CP55,940) was used as the radioligand whereas other cannabinoid agonists showed better affinity when [$^3$H]CP55,940 was displaced. These results suggest structure, rather than function, is more influential in determining displacement of each radioligand. Second, all compounds had substantially better affinity for $CB_1$ receptors than for $CB_2$ receptors. Third, none of the compounds (at doses up to 30 mg/kg) were effective antagonists of the *in vivo* effects of $\Delta^9$-THC in the Martin tetrad. In fact, many of the compounds produced a profile of effects that were similar to those of $\Delta^9$-THC, although their potencies for doing so did not correlate well with their $CB_1$ receptor affinities. Unlike with $\Delta^9$-THC, however, rimonabant failed to reverse the tetrad effects of these compounds. Further, these compounds did not activate $CB_1$ receptors in a [$^{35}$S]GTPγS binding assay. Finally, evaluation of a selected compound (O-4332) in male $CB_1$ knockout and wildtype mice in three of the tetrad tests (antinociception was not measured) showed that a probe dose (30 mg/kg) of O-4332 (Fig 2) decreased activity and produced hypothermia and catalepsy in both $CB_1^{(-/-)}$ and $CB_1^{(+/+)}$ mice to a similar extent.

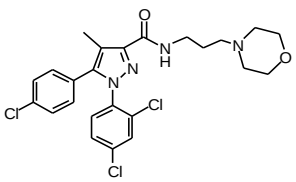


**Figure 2: O-4332**

Together, these results suggest that this series of pyrazole cannabinoids produce tetrad effects similar to other cannabinoid agonists; however, they do so by a different mechanism. Current research is focused on investigation of other possible non-$CB_1$, non-$CB_2$ receptor mechanisms.

This work was supported by NIH grants DA-03672 and DA-05488.

# DETERMINATION OF AN INTERNAL DISULFIDE BOND IN THE SECOND EXTRACELLULAR LOOP OF CANNABINOID CB2 RECEPTOR BY MASS SPECTROMETRY

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Most Class A G protein-coupled receptors (GPCRs) have a disulfide bond between the top of transmembrane helix 3 (TMH3) and second extracellular loop (ECL-2). In contrast to other Class A GPCRs, the cannabinoid CB2 receptor lacks the cysteine residue at the top of TMH3. Previous mutagenesis studies have suggested in-directly that there is a disulfide bond in the ECL-2 of the CB2 receptor. In this study, we showed that the specific binding of [$^{3}$H]CP55940 to the CB2 receptor was inhibited by dithiothreitol, suggesting that the integrity of a disulfide bridge is important for maintaining the active conformation of cannabinoid CB2 receptor. In this study, the disulfide bond linkage of cannabinoid CB2 receptor was determined directly through a combination of pepsin digestion, stable isotope alkylation of cysteine residues, matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and electrospray ionization tandem mass spectrometry (ESI-MS/MS). Our data demonstrated that the disulfide bond of cannabinoid CB2 receptor is formed by the linkage between Cys$^{174}$ and Cys$^{179}$. This work confirmed the proposed disulfide formation from mutagenesis studies. Because of this unique disulfide bond in the ECL-2, the structure of the CB2 receptor might be different from those of other Class A GPCRs.

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# TARGETING CANNABINOID RECEPTORS AS A NOVEL APPROACH TO PREVENT GRAFT-VERSUS-HOST DISEASE

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Inflammation plays a critical role in the pathogenesis of a wide range of diseases. Cannabinoids such as delta-9-tetrahydrocannabinol (THC) activate cannabinoid receptors on immune cells and mediate immunomodulation resulting in anti-inflammatory activity. Graft-*versus*-host disease (GVHD) is a major challenge to transplantation owing to associated morbidity and mortality. It is caused by activation of the donor-derived mature T cells that recognize the recipient's alloantigens and mount a strong inflammatory reaction. In the current study, we tested the hypothesis that activation of cannabinoid receptors on donor-derived T cells may help prevent GVHD. To this end, we tested an acute model of GVHD by transferring parental C57Bl/6 (B6) spleen cells into (C57B1/6 *X* DBA/2) F1 (BDF1) mice. Transfer of B6 cells into BDF1 mice produces lymphoid hyperplasia followed by immunosuppression, weight loss, a Thl pattern of cytokines and mortality. In this model, we tested the efficacy of natural cannabinoids, THC and cannabidiol (CBD) on the development of acute GVHD. THC administration led to early recovery from body weight loss, reduced tissue injury in the liver and increased survival. Impaired haematopoiesis seen during GVHD was rescued by treatment with THC. THC treatment suppressed the expansion of donor-derived T cells and blocked the decrease in the number of host cells. Moreover, the donor derived T cells from THC-treated GVHD mice failed to proliferate and mediate cytotoxicity against the recipient's cells. The ability of THC to reduce the clinical GVHD was reversed by administration of CB1 and CB2 antagonists thereby demonstrating that THC-mediated suppression of GVHD was cannabinoid receptor-dependent. Similarly, treatment with CBD also led to decreased GVHD. Our results demonstrate for the first time that use of cannabinoids may constitute a novel treatment modality against acute GVHD.

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# OSTEOBLASTIC CB2 CANNABINOID RECEPTOR ACTIVATES GI PROTEIN-ERK1/2-MAPKAPK2-CREB SIGNALING PATHWAY

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An increasing number of studies demonstrate the occurrence of a skeletal cannabinoid system. This system consists of CB1 cannabinoid receptors expressed in skeletal sympathetic nerve terminals and CB2 receptors in osteoblasts and osteoclasts. In addition, bone contains relatively high levels of anandamide and 2-arachidonoylglycerol (2-AG) and the endocannabinoid metabolic enzymes diacylglycerol lipases (DAGLs) and fatty acid amide hydrolase (FAAH). We have previously shown that activation of CB2 stimulates osteoblast proliferation. In the present study we characterized the pathway mediating the CB2 mitogenic signaling in the MC3T3 E1 osteoblastic cell line and in primary culture of newborn mouse calvarial osteoblasts (Nemco). Downstream of Gi-protein, specific and non-specific CB2 agonists (HU-308, AM1241, THC, anandamide) stimulated Erk1/2 phosphorylation. Interestingly, 2-AG failed to neither stimulate cell proliferation nor activate Erk1/2. The Erk1/2 stimulation persisted for 2 hours suggesting that tolerance does not develop at least during this time period. The activation of Erk1/2 was inhibitable by the MEK/Erk1/2 inhibitor PD98059. Activation of CB2 did not increase p38 phosphorylation. Downstream of Erk1/2, CB2 activation by $10^{-13}$-$10^{-7}$ M HU-308 lead to a dose-dependent increase of mRNA for the transcription factor mitogen activated protein kinase activated protein kinase 2 (Mapkapk2). The increase in Mapkapk2 mRNA could be blocked by PD98059. The increases in both Erk1/2 phosphorylation and Mapkapk2 mRNA were absent in CB2-deficient Nemco challenged with CB2 agonists. To assess signaling effects further downstream of Mapkapk2 we used MC3T3 E1 cells stably transfected with a $CRE_3$-luciferase chimera, a functional assay for the stimulation of CREB. Challenging these cells with HU-308 stimulated luciferase expression dose-dependently peaking at $10^{-8}$ M agonist dose. Taken together these results suggest that that the CB2 mitogenic signaling in osteoblasts involves a Gi-protein – Erk1/2 – Mapkapk2 – CREB signaling pathway.

# DELTA-9-TETRAHYDROCANNABINOL, $CB_2R$ AND IMMUNITY TO YEAST INFECTION

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It is known that delta-9-tetrahydrocannabinol (THC) suppresses immunity against bacterial, viral and protozoan infection. However, we do not know about THC's effects on the immune response to yeast infections, nor of the role, if any, $CB_2R$ plays during yeast infections. Thus, we set out to investigate the role of $CB_2R$ and the effects of THC using a *Candida albicans* (*C. albicans*) infection mouse model. We first investigated the role of $CB_2R$ on the early immune response to a systemic *C. albicans* infection. *C. albicans* ($1x10^7$ yeast cells/mouse) were injected i.v. into c57BL/6 (wild type) or $CB_2R$ knockout ($CB_2R^{-/-}$) mice 8 weeks of age. Three days after the infection, the mice were sacrificed and their kidneys, liver, spleen and serum were collected. The *C. albicans* fungal load was analyzed as colony forming units per gram of tissue (CFU/g) in the kidneys and liver. Using enzyme linked immune absorbent assay, the serum was analyzed for interleukin-6 (IL-6), a cytokine produced readily upon *C. albicans* infection. The spleen was used to obtain splenic macrophages (CD11b $Mac^+$ cells) that were to be used to assess transcript cytokine levels. Thus far, based on the parameters studied, our results show no significant difference in the early immune response to the yeast infection between wild type and $CB_2R^{-/-}$ mice. In addition to investigating the role of $CB_2R$ and the effect of THC on the early immune response to a systemic *C. albicans* infection, we studied the effect of THC on the memory response to the *C. albicans* infection. Wild type mice, 8 weeks of age were given THC (0, 4, 8, 16, 32 or 64 mg/kg, i.p.) on days 1 through 4, 8 through 11 and 15 through 19. *C. albicans* ($0.75 \times 10^6$ yeast cells/mouse, i.v.) was given on day 2. On day 19, the mice were challenged with a higher dose of the yeast ($5x10^6$ yeast cells/mouse). The mice were then monitored for an additional 15 days. Our preliminary findings have been surprising in that THC (16 mg/kg) increased the survival of mice in this paradigm. Thus, we investigated the antifungal property of THC via a minimum inhibitory concentration assay and found that, at all concentration tested, THC was not cytotoxic to *C. albicans*. In summary, thus far, we have found that, in the early immune response paradigm, mice deficient in the $CB_2R$ respond similarly to the *C. albicans* infection as wild type mice, suggesting the $CB_2R^{-/-}$ mice are immune competent with regards to this type of infection. Second, we have found that THC increases the survival rate of mice in the memory response paradigm.

# $CB_2$ RECEPTOR-MEDIATED ANTI-ALLODYNIC EFFECTS IN NEUROPATHIC PAIN MODELS IS LIKELY MEDIATED THROUGH NON-NEURONAL CELLS

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**Introduction**: Recent findings have demonstrated that selective activation of the $CB_2$ receptor produces anti-hyperalgesic and anti-allodynic effects in preclinical animal models of nociceptive and neuropathic pain, lacking the psychotropic side effects associated with $CB_1$ receptor activation. However, to date, the $CB_2$ mechanism in pain modulation has not been clearly defined. Here we report findings supporting a non-neuronal mechanism for $CB_2$-mediated anti-allodynic effects in neuropathic pain models.

**Methods**: $CB_2$ receptor-selective agonists were used to demonstrate anti-allodynic efficacy in the rat spinal nerve ligation (SNL) and chronic constriction injury (CCI) models of neuropathic pain. Microglia activation in the spinal cord were assessed using the immunohistochemistry method, and $CB_2$ gene expression levels in spinal cord and dorsal root ganglia (DRG) were quantified using the qPCR technique.

**Results**: Although the $CB_2$ receptor is predominantly expressed in the peripheral immune system under normal physiological conditions, we have demonstrated in this study that $CB_2$ gene expression is significantly increased in spinal cord and DRG tissues of rat neuropathic pain models. In the SNL model, direct injection of compounds into the spinal cord and DRG revealed that these are important sites for $CB_2$-mediated effects as assessed by both behavioral measures of allodynia and *in vivo* electrophysiological recordings of spinal wide dynamic range neuronal activity. A battery of studies designed to investigate if $CB_2$ receptor activation directly modulates neuronal activity failed to yield positive results, which led us to the hypothesis that the $CB_2$ receptor modulates neuronal activity indirectly through modulating the function of non-neuronal cells. We have demonstrated that the $CB_2$ receptor is expressed in spinal microglia, however, the normalized $CB_2$ expression level in these cells was not changed in animals of neuropathic pain models compared with naïve animals. The increase in $CB_2$ gene expression in the spinal cord is actually due to the increase of microglia cell number in the tissue induced by pain. In addition, sub-chronic treatments of animals with $CB_2$ receptor-selective agonists normalized the microglia cell number, as well as the $CB_2$ gene expression in the spinal cord that was increased in neuropathic pain conditions. Further, sub-chronic administration of $CB_2$ agonists significantly enhanced the anti-allodynic potencies and efficacies of these compounds.

**Conclusion**: The spinal cord and DRG are important sites for $CB_2$-mediated anti-allodynic efficacy. The mode of action for $CB_2$ agonists in modulation of neuropathic pain involves regulating the function and activation levels of glia cells in the spinal cord and DRG.

# CHRONIC RIMONABANT ALTERS BEHAVIOR AND GENE EXPRESSION IN HIPPOCAMPUS DURING LEARNING

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The cannabinoid receptor (CB1) antagonist, Rimonabant (Rmbt), is quite effective at countering neural and behavioral effects of exogenously applied cannabinoids (Hampson & Deadwyler, J. Neurosci 20:8932-8942, 2000). However, when Rmbt is applied alone, Rmbt appears to modulate only alterations in well learned behavior (Niyuhire et al., Psychopharmacology 191: 223-231, 2007; Deadwyler et al., Psychopharmacology 198:577-586, 2008). Since chronic CB1 agonists impair learning of a delayed-nonmatch-to-sample (DNMS) task and suppress the firing of hippocampal neurons correlated with DNMS task events (Goonawardena et al. Soc. Neurosci Abstr Prog # 485.11, 2008), we examined the effects of Rmbt exposure during DNMS learning to determine if the CB1 antagonist would facilitate acquisition of the operant behavior.

Long-Evans rats were partially trained in the delayed-nonmatch-to-sample (DNMS) behavioral task, surgically implanted with 16-channel hippocampal array recording electrodes, then DNMS training was resumed with receiving daily injections of Rimonabant (2.0 mg/kg, i.p.) or Pluronic F68 vehicle. Each animal was trained until performance exceeded 80% at 1-10 s delays on two successive days, then delays increased in increments of 10 s. Final criterion behavior was met when performance on sessions comprised of 1-30 s trials exceeded 80% for the subset of trials with delays of 1-10s. The number of days to acquire criterion performance at each delay increment was recorded for both vehicle and Rmbt-treated rats. Hippocampal neural activity was recorded every day and examined for the presence of neural firing correlated to DNMS task events (FCTs, Hampson et al., Nature 402;610-614, 1999). At the end of training, animals were sacrificed and hippocampi harvested for gene expression assays.

Surprisingly, Rmbt increased the number of days to criterion in the DNMS task, and reduced overall performance compared to controls. The number of hippocampal neurons exhibiting neural firing correlated to DNMS task events was likewise reduced. Gene expression assays showed no significant differences in expression for CB1 receptor. However, expression of AMPA receptor subunit, BDNF and Synapsin 1 were significantly increased, along with a slight increase in CREM, Synapsin 2 and GAP43. Other glutamate receptor subunits, GAD and GABA receptor subunits, BDNF receptor and CREB were not altered in Rmbt-animals. These results suggest that Rimonabant altered hippocampal synapses during the learning phase of the DNMS task, but that those alterations did not necessarily convey any improvement in performance of the task.

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# BETA-ARRESTIN 2 REGULATES CB1 RECEPTOR DESENSITIZATION AND THC-MEDIATED ANTINOCICEPTIVE TOLERANCE

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Chronic treatment with $\Delta^9$-tetrahydrocannabinol (THC) produces region-specific $CB_1$ receptor (CB1R) adaptations in the CNS, which occur in conjunction with tolerance to cannabinoid-mediated in vivo effects. These adaptations include uncoupling of CB1Rs from G-proteins (desensitization) and/or reduction of CB1R density (downregulation), which have been shown to involve phosphorylation of the receptor and recruitment of beta-arrestin. Our laboratory and others have found regional differences in CB1R adaptations, likely resulting from differential co-localization of CB1R with regulatory proteins, such as β-arrestin2. Previous studies using heterologous expression systems have shown that β-arrestin2 mediates agonist-dependent desensitization and internalization of CB1Rs. In addition, CNS distribution of β-arrestin2 partially overlaps with CB1Rs; however, its role in CB1R regulation in the CNS has not been elucidated. We therefore tested the hypothesis that mice lacking β-arrestin2 would exhibit reduced magnitude and/or regional distribution of CB1R desensitization and downregulation, which would be associated with attenuated tolerance to THC-mediated effects. Wild-type (WT) and β-arrestin2 knockout (KO) mice were treated for 6.5 days with 10 mg/kg THC twice daily and tested for THC-mediated effects, including thermal antinociception (tail-flick), catalepsy and hypothermia. Brain and spinal cord was then collected and membranes prepared for examination. To assess CB1R-mediated G-protein activation and levels, agonist-stimulated [$^{35}$S]GTPγS and [$^3$H]SR141716A receptor binding were performed. While both WT and β-arrestin2 KO mice developed tolerance to THC-mediated antinociception, hypothermia, and catalepsy, the β-arrestin2-KO mice developed a significantly lesser degree of tolerance to THC-mediated thermal antinociception. Data from membrane homogenate assays of spinal cord revealed that desensitization of CB1R-mediated G-protein activity was also attenuated in β-arrestin2-KO mice compared to WT controls. In contrast, no significant differences in CB1R levels were observed between the genotypes following treatment with vehicle or THC. These studies indicate that β-arrestin2 plays a role in chronic THC-induced desensitization of CB1Rs in spinal cord and spinally-mediated antinociceptive tolerance following chronic THC administration. Ongoing studies will investigate, at the whole brain level, the role of β-arrestin2 in regulating CB1R desensitization and downregulation using agonist-stimulated [$^{35}$S]GTPγS and [$^3$H]ligand autoradiography, respectively, in 3D reconstructed brain images. These results will provide new information on the regional regulation of CB1R signaling, which will extend our knowledge of the consequences of chronic treatment with cannabinoids.

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# DIFFERENTIAL REGULATION OF DSI TIME COURSE IN IDENTIFIED POPULATIONS OF AUTAPTIC HIPPOCAMPAL NEURONS

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Depolarization-induced suppression of excitation and inhibition (DSE/DSI) appear to be important forms of short-term retrograde neuronal plasticity involving endocannabinoids, the activation of presynaptic cannabinoid CB1 receptors, and the suppression of neurotransmitter release. In the course of whole cell patch recording from ~2000 autaptic hippocampal neurons we opportunistically examined cannabinoid signalling in inhibitory neurons (~10% of cultured neurons). We have distinguished five populations of autaptic inhibitory neurons that exhibit differential cannabinoid responses, including two temporally distinct forms of DSI. Two remaining populations responded to cannabinoids but did not have DSI while a fifth had neither DSI nor cannabinoid responses.

Of the two chief candidate endocannabinoids (eCBs), 2-AG inhibition reversed fully while anandamide irreversibly inhibited IPSCs, the latter's action inconsistent with a role as a *bona fide* eCB mediator of DSI. The duration of depolarization necessary to elicit DSI (Effective dose (ED-50) ~130ms; ~280ms) was far less than for DSE. However the nearly identical concentration response for 2-AG to inhibit EPSCs and IPSCs indicates that this difference is not due to differential cannabinoid receptor sensitivity.

Interestingly, of the two populations exhibiting DSI, one had a substantially faster recovery time course both after DSI and 2-AG, this despite being cultured under identical conditions. Several enzymes have been proposed to play a role in 2-AG breakdown, presumably determining the time course of DSI: fatty acid amide hydrolase (FAAH), cyclooxygenase-2 (COX-2), monoacyl glycerol lipase (MGL), α/β-hydrolase domain 6 and 12 (ABHD6 and ABHD12). We tested the impact on DSI time courses by blockers for FAAH, COX-2, MGL and ABHD6. Interestingly, the population with slow DSI was regulated only by MGL, whereas the fast DSI population was regulated by both MGL and COX-2. This suggests that the faster DSI time course may occur as a result of the concerted action of multiple enzymes. This may represent a more general mechanism for regulation of time courses of different forms of DSI and DSE.

# EXPRESSION, LOCALIZATION, AND DRUG MODULATION OF CANNABINOID CB1 AND DOPAMINE D2 RECEPTOR HETERODIMERS IN CATH.A DIFFERENTIATED NEURONAL CELLS

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Increasing evidence suggests that G protein-coupled receptors (GPCRs) may function as receptor dimers or higher order oligomer complexes. One set of receptors that has received significant attention relevant to oligomerization is the $CB_1$ cannabinoid ($CB_1$) receptor and $D_2$ dopamine ($D_2$) receptor pair. $CB_1$ and $D_2$ receptors are co-expressed in the basal ganglia; an area of the brain involved in such processes as cognition, motor function, and emotional control. Several lines of suggest that $CB_1$ and $D_2$ receptors may oligomerize providing a unique pharmacology *in vitro* and *in vivo*. However, limited information exists on the regulation of $CB_1$ and $D_2$ receptor dimers. We employed a novel technique, multicolor bimolecular fluorescence complementation (BiFC) to examine initially the subcellular localization of $D_2$-$D_2$ homomers and $CB_1$-$D_2$ heteromers in a neuronal cell model (CAD cells). Multicolor BiFC allows for the detection of two separate protein-protein complexes in living cells by visualizing the fluorescence complementation of two distinct spectral variants of GFP. Multicolor BiFC was also used to explore the effects of chronic ligand treatment on receptor dimerization at the plasma membrane and intracellularly. Our results revealed that the BiFC-tagged receptors retained appropriate function and provided additional evidence that $CB_1$ and $D_2$ receptors oligomers were appropriately localized and trafficked. We also revealed that chronic $CB_1$ or $D_2$ receptor agonist treatment favors the formation of the $CB_1$-$D_2$ heterodimer relative to the formation of a $D_2$-$D_2$ homodimer. However, the mechanisms and patterns of these agonist-induced changes appeared to differ. The $D_2$ ligand-mediated effects may involve a receptor-expression-dependent component, whereas, the $CB_1$ agonist-mediated changes in heterodimer formation appeared to involve primarily $CB_1$ receptor activation. These results provide further insight into the dynamic nature of GPCR oligomerization and suggest that their may be physiological implications for agonist modulation of receptor dimerization in disorders such as Parkinson's disease and drug abuse.

# BEHAVIORAL AND FUNCTIONAL ADAPTATION OF THE ENDOCANNABINOID SYSTEM FOLLOWING REPEATED MONOACYLGLYCEROL LIPASE (MAGL) INHIBITION

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The recent development of JZL184, a highly potent and selective inhibitor of monoacylglycerol lipase (MAGL), the primary enzyme responsible for 2-arachidonoylglycerol (2-AG) hydrolysis, has provided a powerful tool to elucidate the role of 2-AG in the endogenous cannabinoid system. With the ability to elevate 2-AG levels over 8-fold in whole brain, the goal of our studies was to examine the behavioral responses and molecular adaptations that occur as a result of continual elevations in 2-AG signaling. Because 2-AG content is orders of magnitude greater than that of anandamide (AEA), it was hypothesized that prolonged treatment with JZL184 would elicit down-regulation and/or desensitization of the $CB_1$ receptor and result in a decreased response to exogenous cannabinoids.

We examined both behavioral and biochemical alterations that occur to the endocannabinoid system following once-daily dosing of JZL184 for six days. First, C57BL/6 mice treated with repeated vehicle, acute JZL184, or repeated JZL184 were examined for cross-tolerance to THC on day 7 in a subset of the cannabinoid tetrad assay. Mice treated with repeated JZL184 did not exhibit THC-induced antinociceptive or hypothermic effects (3-100 mg/kg), suggesting substantial behavioral cross-tolerance. Second, we investigated the impact of subchronic JZL184 on whole brain $CB_1$ receptor desensitization using CP55,940 stimulated [$^{35}$S]GTPγS activation assays, as well as downregulation of $CB_1$ receptor density measured via [$^{3}$H]SR141716 binding. Following repeated JZL184 administration, both receptor desensitization and downregulation were comparable (~25-30%) to repeated dosing of low-dose THC. Finally, whole brain AEA and 2-AG levels were quantified 24 h following the final injection. While there were no AEA level perturbations, 2-AG content of mice treated with JZL184 were significantly elevated above vehicle treatment, with repeated treatment showing significant elevation above that of acute administration [Veh: 8.2±0.4 nm/g, Acute JZL184: 79.7±6.7 nm/g, Repeated JZL184: 111.1±5.3 nm/g]. These data suggest repeated MAGL inhibition led to an accumulation of almost 40% in 2-AG content compared to acute MAGL inhibition. These results, taken together, suggest that repeated blockade of MAGL results in cumulative enhancement of 2-AG concentrations in the brain, tolerance to exogenous cannabinoids, and biochemical alterations that closely mimic those of global cannabinoid agonists. The observation that prolonged blockade of MAGL has profound consequences on $CB_1$ receptor function is in marked contrast to previous studies examining FAAH (-/-) mice, which demonstrated normal $CB_1$ receptor number and functional responses to exogenous cannabinoids compared to wild-type animals.

# LONG-TERM DEPRESSION IN VENTRAL TEGMENTAL DOPAMINE NEURONS MEDIATES LEARNING TO ASSOCIATE CANNABINOID EXPOSURE WITH ENVIRONMENTAL CUES

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**Introduction:** Cannabis (i.e. marijuana or cannabinoids) has been the most commonly used illicit drug worldwide and the lifetime prevalence of cannabis addiction is the highest of all illicit drugs in the USA. However, there is no effective treatment for cannabis addiction in humans, largely because its underlying mechanism remains not clear. Since virtually all abused drugs, including cannabis, activate dopamine neurons in the ventral tegmental area (VTA) and thereby lead to drug rewarding response, long-term enhancement of synaptic strength, i.e. long-term potentiation (LTP), in these neurons is thought to be essential in drug addiction. It is entirely unknown, however, whether cannabis is able to induce LTP or long-term depression (LTD) in these neurons. More importantly, whether such alterations in synaptic plasticity in VTA dopamine circuitry causatively contribute to drug addictive behavior has not previously been addressed. We explored these essential questions with combination of *in vitro* electrophysiological, molecular biological, morphological and behavioral strategies.

**Results:** We report that chronic cannabinoid exposure *in vivo* (i.e. once daily i.p. injections for 5 days of HU210 at 100 μg/kg) induces long-term weakening of synaptic strength (i.e. LTD) in VTA dopamine neurons through activation of astrocytic CB1 receptors, increased release of astrocytic glutamate, activation of extra-synaptic NR2B-containing NMDA receptors and facilitated endocytosis of AMPA receptor GluR2 subunits in VTA dopamine neurons. Blockade of LTD induction with the GluR2-derived peptide TAT-GluR2 prevents HU210-induced conditioned place preference (CPP), i.e. learning to associate cannabinoid exposure with a specific environment. While nicotine exposure facilitates LTP induction in the VTA and induces CPP, co-administration of cannabinoid and nicotine neutralizes not only their distinct effects on synaptic plasticity but also their common effects on CPP.

**Conclusions:** Our results, together with previous findings, suggest a signaling pathway in the midbrain VTA that mediates LTD induction to produce cannabis addiction: while activating CB1 receptor on VTA afferent axonal terminals to decrease glutamate release into synaptic cleft, cannabiniod exposure *in vivo* activates VTA astrocytic CB1 receptor to increase astrocytic glutamate release, leading sequentially to NMDA receptor activation, GluR2 endocytosis and LTD induction in VTA dopamine neurons. Our findings also indicate a novel therapeutic strategy for treating cannabis addiction with the TAT-GluR2 peptide, and the alleviating effects of cannabis on addiction to nicotine, cocaine, amphetamine, morphine and ethanol.

# REPEATED HOMOTYPIC STRESS ELEVATES 2-AG AND ENHANCES DEPOLARIZATION-INDUCED SUPRESSION OF INHIBITION IN BASOLATERAL AMYGDALA

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Psychosocial stress is a major risk factor for development and exacerbation of neuropsychiatric illness. We have previously shown that repeated stress causes biochemical adaptations in endocannabinoid signaling that contribute to stress-response habituation, however, the synaptic correlates of these adaptations have not been examined.

ICR male mice were exposed to 0 or 10 days of restraint stress for 1 hour per day. We used isotope-dilution, electrospray-ionization, LC/MS/MS in the selective reaction monitoring mode to analyze 2-AG, diacylglycerol, and arachidonic acid from basolateral amygdala (BLA) micropunches. Whole-cell electrophysiology was used to study DSI in control and stressed mice.

Here we show that the synthetic enzyme for 2-AG, diacylglycerol lipase-alpha, is heterogeneously expressed in the amygdala and that levels of 2-AG are transiently increased in the BLA after 10 days of restraint stress. We also observed a decrease in the 2-AG degradation product arachidonic acid, while 2-AG precursors were significantly elevated after 10 days of restraint stress. We show that DSI is mediated by 2-AG in the BLA, and its duration limited by endocannabinoid degradation. After 10 days of restraint stress, DSI duration, but not magnitude, was significantly enhanced in BLA pyramidal neurons.

These data indicate that exposure to repeated homotypic stress produces neuroadaptations that confer BLA neurons with an enhanced capacity to elevate 2-AG content and engage in 2-AG-mediated short-term retrograde synaptic signaling. We suggest stress-induced enhancement of endocannabinoid-mediated suppression of inhibitory transmission in the BLA could contribute to affective dysregulation associated with chronic stress.

# URB597 RECOVERS SOME OF THE SIGNS PRESENT IN THE DEPRESSIVE PHENOTYPE INDUCED BY ADOLESCENT EXPOSURE TO THC

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Our studies have recently shown that chronic administration of THC to adolescent rats induces subtle but long-lasting alterations in the emotional circuit, which result in depressive-like behavior in adult female rats only. This was paralleled by alterations in expression and activity of CREB and BDNF, two key proteins in neuronal plasticity that are considered prototypical molecular markers for depression (Rubino et al., 2008). Since it is increasingly recognized that cognitive dysfunction accompanies mood symptoms in depression, we then checked different aspects of memory in adult female rats exposed to THC during adolescence (Rubino et al., 2009). No alteration was found in aversive memory, conversely, in the radial maze THC pretreated animals exhibited a deficit in spatial working memory that was paralleled by a significant decrease in synaptophysin and PSD95 proteins in the prefrontal cortex. A deeper examination of prefrontal cortex synaptosomes through proteomic analysis revealed a reduction in mitochondrial proteins, energetic metabolism enzymes and proteins belonging to the proteasome machinery, pointing to the presence of less active synapses characterized by reduced ability in maintaining normal synaptic efficiency (Rubino et al., 2009).

Since it has been reported that deficits in adult hippocampal neurogenesis may underlie the cognitive dysfunction seen in depression, as well as that impairments in this event could lead to the development of the depressive state, while the antidepressants could prevent it by improving neurogenesis, to further characterize our model of depression we studied neurogenesis in the dentate gyrus of adult female rats pre-exposed to THC during adolescence. Adolescent THC exposure significantly reduced the number of BrdU-positive cells in THC treated rats as well as hippocampal volume. This result joins the complex picture we have already depicted regarding the depressive phenotype induced by adolescent THC exposure and further supports the validity of this model in representing depression.

Finally, to test whether the increase in the endocannabinoid system could ameliorate the depressive phenotype, adult female rats pre-exposed to THC were injected with URB597 (0.3 mg/kg ip) and then tested in behavioural and biochemical assays. URB597 reduced behavioural despair in the forced swim test as well as anhedonia measured in the sucrose preference test. URB597 was also able to reduce the alteration seen in CREB activation after adolescent exposure to THC.

These results confirm the importance of the endocannabinoid system in the regulation of emotionality and support the use of URB597 as a new therapeutic approach with antidepressant properties.

# LACK OF SPECIFICITY FOR CANNABINOID CB1 RECEPTOR ANTAGONISTS: INTERACTIONS WITH GPR55

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Recently it has been suggested that the orphan G protein-coupled receptor, GPR55, is a novel endocannabinoid receptor that may also be activated by the lysophospholipid, L-α-lysophosphatidylinositol (LPI). GPR55 mRNA is expressed widely throughout the body with high levels found in the adrenal glands, spleen, CNS and the gut, suggesting that, like the $CB_1$ receptor, it may play a role in regulating a wide range of physiological processes.

GPR55 signaling in HEK293 cells is associated with $Ca^{2+}$ release from internal stores via the induction of $G_{\alpha 13}$, RhoA and PLC but so far a number of inconsistencies have been documented with respect to GPR55 pharmacology. Interestingly, the $CB_1$ inverse agonist AM251 has been shown to activate GPR55, but its close structural analogue SR141716A is though to be either an agonist or an antagonist.

We set out to investigate whether a series of cannabinoid antagonists act at GPR55 in a HEK293 cell line stably over-expressing human GPR55. In these cells we find that the diarylpyrazole ligands AM251 and SR141716A stimulate $Ca^{2+}$ release at relatively low concentrations (100nM-3μM) and induce ligand-dependent receptor trafficking. The related ligand AM281 generated a similar response, but at higher concentrations (3μM-30μM). In contrast, the structurally-distinct $CB_2$ antagonist AM630 produced no $Ca^{2+}$ response or receptor trafficking at concentrations up to 30μM. Next we evaluated ERK MAP-kinase activation in HEK293 cells expressing GPR55. Although LPI was highly effective in stimulating phospho-ERK, the diarylpyrazole ligands were much less efficacious, possibly indicating agonist bias in GPR55 signaling.

Thus prototypic $CB_1$ receptor antagonists may not be as specific as first thought and it will be interesting to establish whether any of the physiological effects of these compounds that have been previously ascribed to $CB_1$ inhibition involve GPR55 activation.

## LPI-EVOKED INCREASES IN INTRACELLULAR CALCIUM INCREASES IN MICROGLIAL CELLS IN CULTURE

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Activation and migration of microglial cells are key elements in a variety of neuroinflammatory CNS disorders such as stroke and multiple sclerosis. Cannabinoid compounds have well known immunomodulatory properties, via their actions predominantly on $CB_2$ cannabinoid and, as yet unidentified atypical cannabinoid receptor, which are regarded as promising pharmaceutical targets. Recent reports suggest that the orphan G protein-coupled receptor 55 (GPR55) is an atypical cannabinoid receptor, that might account for some of the non-$CB_1$, non-$CB_2$ effects reported for certain cannabinoid ligands. The purpose of the present study was to test the effect of cannabinoid ligands, in comparison with lysophosphatidylinositol (LPI, the putative endogenous ligand for GPR55) on $Ca^{2+}$ levels in BV2 cells, a microglial cell line commonly used to study microglial function.BV2 cells were seeded in black-walled, clear-bottomed, 96-well microtitre plates to monitor $Ca^{2+}$ release using Fluo-4, measured using a FlexStation plate-reader. Addition of vehicle evoked a small, inconsistent change in $[Ca^{2+}]_i$ relative to the response evoked in the presence of 100 µM ATP (6 %). The endocannabinoids, anandamide and 2AG, as well as synthetic cannabinoid ligands, HU210, CP55940, WIN55212-2 and rimonabant (all at 10 µM) were unable to induce a significant change in $[Ca^{2+}]_i$ ($\leq$10 % ATP response). The phytocannabinoids, cannabidiol and THC produced modest increases in $[Ca^{2+}]_i$ (15 and 16 %, respectively).. In comparison, LPI induced a much larger elevation in $[Ca^{2+}]_i$ to 51-57 % ATP response, with calculated $pEC_{50}$ values of 4.8-4.9. Quantitative RT-PCR showed a moderate level of expression of GPR55 mRNA in BV2 cells in comparison to mouse brain tissue. Whilst these results are consistent with LPI-induced $Ca^{2+}$ mobilisation via GPR55 in these cells, further pharmacological and biochemical investigation is required.

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## GPR55: *TO BE OR NOT TO BE* A CANNABINOID RECEPTOR

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GPR55 was first identified a decade ago as a novel human G protein coupled receptor. GPR55 has since been reported to be activated by exogenous and endogenous cannabinoid compounds and been presented as one of the missing candidate cannabinoid receptor subtypes. GPR55 has also been demonstrated to be activated by the endogenous non cannabinoid mediator lyso-phosphatidylinositol (LPI). Whether the orphan G-protein coupled receptor GPR55 is also a cannabinoid receptor family member remains unclear as a result of the conflicting chemical space of agonists that recognize GPR55. To resolve these inconsistencies in classification, an alternative approach for identifying GPR55 ligands that is insensitive to the endogenous complement of cellular receptors could circumvent many of the challenges that have arisen in the measurements of G protein signaling.

We examined the effects of numerous exogenous and endogenous cannabinoid ligands on GPR55 using a beta-arrestin-green fluorescent protein biosensor as a direct readout of receptor activation. β-arrestins are intracellular proteins that bind and desensitize activated GPCRs and in the process form stable receptor/arrestin signaling complexes. β-arrestin redistribution to the activated membrane bound receptor represents one of the early intracellular events provoked by agonist binding, making it attractive to monitor. In this study, we used stably expressed GPR55 in U2OS cells to determine GPR55 responsiveness to a representative panel of cannabinoid ligands and LPI in the presence of a β-arrestin2-green fluorescent protein (βarr2-GFP) biosensor.

Our β-arrestin trafficking and receptor internalization data demonstrate that of the compounds examined only LPI, the $CB_1$ inverse agonist/antagonists SR141716A, and AM251 are equi-efficacious GPR55 agonists; and the $CB_1$ agonist CP55940 is a potent GPR55 antagonist at nanomolar concentrations.

These data together with our inability to observe activation of GPR55 by $\Delta^9$-THC and endocannabinoids indicate that GPR55 should be classified as an atypical cannabinoid receptor at best.

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# FUNCTIONAL SELECTIVITY OF CANNABINOIDS ON THE NOVEL CANNABINOID RECEPTOR GPR55

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**Introduction:**
We have recently shown that the human G protein-coupled Receptor 55 (GPR55) mediates intracellular effects of lysophosphatidylinositol (LPI). Here we show that some of the $CB_1R$ antagonists activate GPR55 and the signaling pathway depends on the agonist.

**Results:**
HEK-293 cells stably expressing the GPR55 receptor were characterized in terms of signaling and receptor trafficking properties. To this end, NFAT, NF-kB and CREB reporter gene assays, transcription factors translocation, antibody feeding and dynamic mass redistribution (DMR) assay in the Corning® Epic® System have been performed. We tested a panel of known cannabinoid ligands and discovered that the CB1R antagonists, SR141716A (Rimonabant), AM251 and AM281 were able to induce GPR55-mediated signaling. While LPI and AM251 are efficacious inducers of NFAT and NF-kB, AM281 is the most efficacious activator of CREB. Moreover, the suitability of Corning® Epic® System was shown for GPR55 because DMR data corroborated transcription factor data for this receptor.

**Conclusion:**
In summary, we have screened a panel of cannabinoids and non-cannabinoids compounds for GPR55 activation in different assays. We found that some of the $CB_1R$ antagonists are agonists for GPR55 and they show functional selectivity in terms of signaling pathway they can trigger.

# LYSOPHOSPHATIDYLINOSITOL INDUCES RAPID CYTOSKELETAL REARRANGEMENTS AND MORPHOLOGICAL CHANGES IN HEK293 CELLS EXPRESSING GPR55: FURTHER EVIDENCE THAT LYSOPHOSPHATIDYLINOSITOL IS THE NATURAL LIGAND FOR GPR55

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GPR55 is a putative novel cannabinoid receptor. Recently, we started the search for the endogenous ligand for GPR55 using HEK293 cells which express GPR55. Finally, we found that lysophosphatidylinositol (LPI) acted as an agonist toward GPR55. LPI induced rapid phosphorylation of ERK and a $Ca^{2+}$ transient in GPR55-expressing cells. These results strongly suggest that GPR55 is a specific and functional receptor for LPI (*Biochem. Biophys. Res. Commun*., 362, 928-934, 2007). We also obtained evidence that the biological activity of 2-arachidonoyl LPI is the highest among those of other molecular species (*J. Biochem.*, 145, 13-20, 2009). In the present study, we explored possible biological activities of 2-arachidonoyl LPI using GPR55-expressing cells. We found that 2-arachidonoyl LPI induces rapid morphological changes (rounding) in HEK293 cells expressing GPR55. The effect of 2-arachidonoyl LPI was detectable at 1 min and reached a peak at 5 min following the addition of 2-arachidonoyl LPI. No apparent effect was observed with vector-transfected cells. The activity of 2-arachidonoyl LPI was markedly higher than that of 1-stearoyl LPI. On the other hand, 2-arachidonoylglycerol, free arachidonic acid and lysophosphatidylcholine did not exhibit any activity. These results provide further evidence that LPI, especially 2-arachidonoyl LPI, is the natural ligand for GPR55. The morphological changes induced by 2-arachidonoyl LPI was abrogated by Y-27632 or C3 toxin, suggesting that the Rho-ROCK pathway is closely involved in LPI-induced morphological changes. We then examined the effects of LPI on the intracellular signaling molecules in GPR55-expressing cells. We found that the addition of 2-arachidonoyl LPI caused the activation of Rho in GPR55-expressing cells. Such an effect was not observed in vector-transfected cells. We also found that 2-arachidonoyl LPI provoked actin stress fiber formation in GPR55-expressing cells. These results suggest that 2-arachidonoyl LPI induces rapid actin filament rearrangement via the GPR55- and Rho-ROCK-dependent pathway thereby eliciting various biological responses in GPR55-expressing cells.

# CANNABIGEROL DISPLAYS SIGNIFICANT POTENCY AS A 5-HT$_{1A}$ RECEPTOR ANTAGONIST

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We have recently discovered that the phytocannabinoid cannabigerol (CBG) behaves as a potent $\alpha_2$-adrenoceptor agonist in vitro in both the mouse isolated vas deferens and in the [$^{35}$S]GTPγS assay using mouse brain membranes (Cascio et al., 2009). There is also published evidence that there is overlap in selectivity of ligands for $\alpha_2$ and 5HT$_{1A}$ receptors (Newman-Tancredi et al., 1998). Both the adrenergic and serotonergic systems have been implicated in disorders such as depression, interestingly it has also been shown that CBG has antidepressant properties in vivo (Musty et al, 2006). The initial aim of this investigation was to determine if CBG would behave as an agonist or antagonist of the 5HT$_{1A}$ receptor.

Experiments were performed using the [$^{35}$S]GTPγS assay, [$^{3}$H]CP55940 displacement binding and dissociation kinetics assays in mouse whole brain membranes. Protocols for these assays have been published previously (Thomas et al., 2007; Price et al., 2005). CBG was obtained from GW pharmaceuticals and all other compounds are commercially available. All compounds were dissolved in DMSO.

Our results show that CBG (1 μM) shares the ability of the established 5HT$_{1A}$-selective antagonists WAY100135 (100 nM) and WAY100635 (100 nM), to oppose stimulation of [$^{35}$S]GTPγS binding to brain membranes induced by the 5HT$_{1A}$-selective agonist, 8-hydroxy-2-dipropylaminotetralin hydrobromide (DPAT). All three compounds appeared to produce antagonism that was competitive in nature with apparent $K_B$ values of 31.2 nM, 10.3 nM and 0.7 nM respectively.

CBG also behaves as a CB$_1$ receptor antagonist/inverse agonist although with less potency than observed for 5HT$_{1A}$ (Gauson et al., 2008). There is also a growing body of evidence that suggest interactions between 5HT$_{1A}$ and CB$_1$ exist (Egashira et al., date., 2008 ) It was of interest to investigate whether DPAT targets the CB$_1$ receptor. In mouse brain membranes, DPAT partially displaced 80.1% of [$^{3}$H]CP55940 from specific binding sites. A displacement which is significantly less than 100% can be indicative of allosteric modulation. To test this hypothesis a dissociation kinetics assay was performed. At concentrations of 10μM and 1μM, DPAT significantly decreased the half life of [$^{3}$H]CP55940 on the orthosteric binding site in brain membranes, thus acting as an allosteric antagonist at CB$_1$.

Further experiments are required to explore the therapeutic potential of CBG-induced 5HT$_{1A}$ antagonism, and to seek other unknown pharmacological actions of CBG. The manner in which DPAT and other 5HT$_{1A}$ ligands interact with the CB$_1$ receptor remains to be elucidated.

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# CANNABIDIOL AS A NOVEL INHIBITOR OF ID-1 GENE EXPRESSION IN AGGRESSIVE BREAST CANCER CELLS

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Invasion and metastasis of aggressive breast cancer cells is the final and fatal step during cancer progression. Clinically, there are still limited therapeutic interventions for aggressive and metastatic breast cancers available. Clearly, effective, targeted and non-toxic therapies are urgently required. Id-1, an inhibitor basic helix-loop-helix transcription factors, has recently been shown to be a key regulator of the metastatic potential of breast and additional cancers. We previously reported that cannabidiol (CBD), a cannabinoid with a low toxicity profile, could down-regulate Id-1 gene expression in aggressive human breast cancer cells. The CBD concentrations effective at inhibiting Id-1 expression correlated with those used to inhibit the proliferative and invasive phenotype of breast cancer cells. We now show that CBD inhibits human cancer cell proliferation and invasion through differential modulation of the ERK and ROS pathways, and that sustained activation of the ERK pathway leads to down-regulation of Id-1 expression. Moreover, we demonstrate that CBD up-regulates the pro-differentiation agent, Id-2. Using the mouse 4T1 cell line and a syngeneic model of metastatic breast cancer, we also show that CBD significantly reduced metastatic spread, therefore, CBD may represent a promising non-toxic agent for the treatment of breast cancer patients with secondary tumors.

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# ANTICONVULSANT EFFECTS OF CANNABIDIOL UPON SPONTANEOUS EPILEPTIFORM ACTIVITY IN ACUTE HIPPOCAMPAL BRAIN SLICES

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**Introduction**: Cannabis has been associated with contradictory effects upon seizure states despite numerous epilepsy sufferers using cannabis medicinally for seizure control. However, it remains to be determined whether cannabis represents a yet to be unmasked therapeutic anti-convulsant or, conversely, a potential risk factor to recreational and medicinal users of cannabis. Preliminary clinical trials have suggested the potential use of the phytocannabinoid cannabidiol (CBD) as an anti-epileptic drug, either by itself, or in combination with existing anti-epileptic medication (Cunha *et al*., 1980).

**Methods***:* We investigated the effects of CBD (10 nM - 100 μM) in two adult rat hippocampal acute brain slice models of epileptiform activity ($Mg^{2+}$-free and 100 μM 4-AP-induced). Activity in specific CA1, CA3 and dentate gyrus (DG) regions was recorded using 60 channel extracellular multi-electrode array (MEA) electrophysiology (Egert *et al*., 2002) to assess the effect of CBD on local field potential (LFP) burst amplitude and duration. All presented data were obtained from $n$=6 slices per model and statistical testing was performed using a two-tailed Mann-Whitney U test. In all cases, $P \leq 0.05$ was considered significant.

**Results***:* In the $Mg^{2+}$-free model, CBD at >1 μM and >10 μM significantly attenuated LFP burst amplitudes in CA1 and DG respectively, whereas CBD at >10 nM significantly shortened LFP burst duration in all hippocampal regions. In the 4-AP model, CBD at 100 μM significantly attenuated LFP burst amplitudes in CA1, whereas CBD at >10 nM and >100 nM significantly shortened burst duration in DG and CA3 respectively.

**Conclusion***:* CBD exerts significant anti-epileptiform effects in two chemically-induced rat hippocampal brain slice models, *in vitro*. These data, combined with a reported absence of associated psychoactive effects, suggest that CBD exhibits promise as a legitimate therapeutic treatment for epilepsy. Future work will ascertain the anti-convulsant potential of CBD in standardised *in vivo* seizure models to further examine the clinical potential of CBD in epilepsy.

# CANNABIDIOL AS A NOVEL ANTI-ACNE AGENT? CANNABIDIOL INHIBITS LIPID SYNTHESIS AND INDUCES CELL DEATH IN HUMAN SEBACEOUS GLAND-DERIVED SEBOCYTES

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We have previously shown that prototypic endocannabinoids, i.e. anandamide (AEA) and 2-arachidonoylglycerol, are produced in the human sebaceous gland. Using human immortalized SZ95 sebocytes, we have furthermore shown that the locally produced endocannabinoids constitutively stimulated lipid production and induced cell death, hallmarks of sebocyte differentiation and hence a model of holocrine sebum production. These effects were mediated by $CB_2$-coupled signaling mechanisms involving the MAPK pathway and key genes regulating lipid synthesis (e.g. PPAR transcription factors) (Dobrosi et al, *FASEB J*, 22:3685, 2008).

In this study, we investigated the effects of Cannabidiol (CBD), a non-psychoactive component of *Cannabis sativa*, on biological functions of SZ95 sebocytes. In contrast to the above effects of the endocannabinoids, CBD (up to 10-20 μM) did not affect basal lipid synthesis of the sebocytes. However, of great importance, CBD markedly abrogated the effects of AEA or arachidonic acid (AA, another know lipogenetic factor in sebocytes) to stimulate lipid synthesis. Intriguingly, higher concentrations (30-50 μM) of CBD induced apoptosis in SZ95 sebocytes, similar to the action of AEA (actually, AEA augmented the effect of CBD).

We then investigated the involvement of various signaling mechanisms in the action of CBD. Inhibition of $CB_1$ (AM251) or $CB_2$ (AM630) did not prevent the effect of CBD. However, the decrease of extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_e$) markedly suppressed the action of CBD to inhibit lipid synthesis. CBD was previously shown to modulate $Ca^{2+}$-permeable transient receptor potential (TRP) channels, namely TRPV1, V2, M8, and A1. Among these, we have identified the expression of TRPV1 and V2 on SZ95 sebocytes and have shown that the activation of TRPV1 inhibits lipid synthesis of the cells (Tóth el al, *J Invest Dermatol*, 129:329, 2009). Indeed, CDB increased $[Ca^{2+}]_i$ which was prevented by ruthenium red, a non-specific inhibitor of TRP channels. Intriguingly, capsazepine, a blocker of TRPV1, was unable to inhibit the action of CBD to suppress lipid synthesis. These findings (at least indirectly) suggest that the cellular effects of CBD on SZ95 sebocytes are mediated by $Ca^{2+}$-permeable channels, most probably by TRPV2 (our currently running siRNA experiments intend to unambiguously identify the CBD-target molecule).

Collective, our data introduce CBD as a novel molecule with a significant sebostatic function. Since CBD was shown to penetrate the skin, our findings may encourage one to systematically explore whether CBD can be exploited in the management of such a common skin disorder as acne, which is characterized by pathologically elevated lipid (sebum) production of the sebaceous glands.

# CANNABIDIOL (THE NON-PSYCHOACTIVE COMPONENT OF CANNABIS) MAY ACT AS A 5-$HT_{1A}$ AUTO-RECEPTOR AGONIST TO REDUCE TOXIN-INDUCED NAUSEA AND VOMITING

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Cannabidiol (CBD), the non-psychoactive component of cannabis has been shown to suppress acute cisplatin-induced vomiting in shrews (Kwiatkowska et al., 2004) and reduce lithium-induced conditioned gaping to a flavour (Parker & Mechoulam, 2003), but little is known about its mechanism of action, as CBD has very low affinity for the $CB_1$ and $CB_2$ receptors. Evidence suggests the 5-$HT_{1A}$ auto-receptor in the neuroprotectant and anxiolytic properties of CBD, but no such study has investigated the mediation of CBD's anti-emetic properties.

Experiments 1-3 investigated the effect of WAY100135 (a 5-$HT_{1A}$ antagonist) and CBD on nicotine- (Experiment1), lithium- (Experiment 2), and cisplatin- (Experiment 3) induced emesis in shrews. Experiment 4 explored the ability of WAY100135 and CBD to interfere with the establishment of lithium-induced conditioned gaping in rats.

At 5 mg/kg, CBD reduced nicotine- and lithium-induced vomiting in shrews and interfered with the establishment of conditioned gaping in rats. WAY100135 prevented these suppressant effects. At 10 mg/kg, CBD did reduce low dose (20 mg/kg) cisplatin-induced vomiting in the shrews, but not at a higher dose (40 mg/kg), nor did it interfere with lithium-induced conditioned gaping in rats. WAY100135 also prevented this low dose suppressant effect on vomiting.

The anti-emetic and anti-nausea effects of CBD appear to be mediated by agonism of 5-$HT_{1A}$ receptors. Most likely, CBD acts as an agonist on somatodendritic 5-$HT_{1A}$ receptors on the raphe nuclei, serving to reduce the firing rate of 5-$HT_{1A}$ afferents.

# CANNABIDIOL CONTROLS INTESTINAL INFLAMMATION THROUGH THE MODULATION OF ENTERIC GLIAL CELLS

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**Introduction**. Abnormalities of the enteric nervous system (ENS), i.e. the degeneration and neuronal number reduction, are essential elements in the pathogenesis of gastrointestinal disorders. Recent findings indicate that cells of the ENS, such as enteric glial cells (EGCs), actively contribute to develop an inflammatory reaction through antigen presentation and cytokine synthesis. During inflammation of the gut, EGCs proliferate and release neurotrophines, growth factors, and pro-inflammatory cytokines which, in turn, can amplify the immune response. EGC thus represent an important link between the nervous and the immune systems of the gut. Therefore, the discovery of molecules that are able to control ECG-activation and in turn modulate the inflammatory response is of high therapeutic interest. Cannabinoids represent such molecules because several evidences have shown that cannabinoids are able to control neurogenic inflammation, both in CNS and ENS. Especially cannabidiol (CBD), which is a Cannabis derivative that is devoid of psychotropic effects, represents a promising agent with high therapeutic prospective.

Therefore, the aim of our study was to investigate the effect of cannabidiol on the development of intestinal inflammation in an experimental model of acute and chronic intestinal inflammation and on human biopsies of patients with UC.

**Methods.** Acute intestinal inflammation was induced by intra-peritoneal administration of LPS for 18 h in mice. Chronic intestinal inflammation was studied in mice with DNBS induced colitis. In the acute model, CBD was administrated 15 minutes before and 2 h after administration of LPS. In the chronic model, CBD was administered daily for 96 h after DNBS. Intestinal tissues were processed for biochemical, histological and immunohistochemical analysis. Finally CBD was administrated to cultured biopsies of ulcerative colitis patients.

**Results.** Treatment of mice with LPS induced a significant increase of S100B protein in the small intestine. This increased expression of S100B was prevented when mice were pre-treated with CBD. CBD also prevented glial cell hyperactivation in LPS-mice. Parallel to the decline in S100B expression, CBD considerably decreased the number of mast cells and macrophages in the intestine of LPS-mice. This was confirmed by histological, biochemical and immunoistochemical analysis. Moreover CBD treatment of LPS-mice also reduced TNF-alfa expression and this was accompanied by the expression of cleaved caspase-3. This most likely accounted for the protection against intestinal damage in the intestine of CBD-treated LPS mice. DNBF colitis resulted in an enhanced expression of S100B and glial cell hyperactivation. Treatment of DNBF mice with CBD decreased glial hyperactivation and reduced the general inflammatory scenario and intestinal damage: CBD reduced the expression of S100B, iNOS protein and prevented the fragmentation of intestinal DNA.

Finally, in human colon biopsies of UC patients, we found that CBD treatment of cultured biopsies prevented the overproduction of nitrite.

**Conclusion.** Our study indicates that CBD modulates the neuro-immune axis in the gut during intestinal inflammation. By controlling glial cell hyperactivation, CBD regulates the inflammatory environment and protects against intestinal damage. Our results open the way to new therapeutic potential for cannabinoids, and especially CBD, to treat inflammatory bowel disorders.

## $GABA_A$ MODULATION OF THE DISCRIMINATIVE STIMULUS EFFECTS OF $\Delta^9$-THC IN HUMANS

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Controlled clinical trials of putative medications for cannabis-use disorders have met with limited success. Data from preclinical and clinical research suggest that agonist replacement treatment is an effective means to manage drug-use disorders, and that this strategy would be viable for cannabis-use disorders as well. Few cannabinoid compounds are currently available for clinical use, however, so non-cannabinoid drugs with an "agonist-like" profile could be valuable alternatives. There is overlap in the effects produced by cannabinoids and drugs that are positive $GABA_A$ modulators, and neuroanatomical, neurochemical and behavioral studies support a functional link between GABA and cannabinoid systems. However, there are limited data regarding the effects of $GABA_A$ positive modulators on cannabinoid-mediated effects in humans, and whether their effects overlap with those of cannabinoids in cannabis users. This ongoing experiment is testing the effects of $GABA_A$ modulators in cannabis users and determining their ability to alter the behavioral and physiological effects of $\Delta^9$-THC. The primary outcome for this experiment is the discriminative stimulus produced by $\Delta^9$-THC. Healthy subjects who report moderate cannabis use, but do not meet criteria for cannabis dependence, are enrolled as outpatients at the University of Kentucky Residential Research Facility and learn to discriminate oral $\Delta^9$-THC. During a sampling phase, subjects receive 30 mg $\Delta^9$-THC, which is identified as by a unique letter code (e.g., Drug X). During a control phase, subjects are required to correctly identify when they receive placebo (i.e., Not Drug X) or 30 mg $\Delta^9$-THC. Finally, a test phase is conducted in which $\Delta^9$-THC (0, 5, 15 and 30 mg) is administered alone and in combination with diazepam (0, 5 and 10 mg). In addition to the drug-discrimination task, several other measures are collected to determine a profile of $\Delta^9$-THC and diazepam effects, including physiological indices, self-report questionnaires, a time estimation procedure, as well as psychomotor performance tasks. To date, 4 subjects have completed the study and 3 are currently enrolled. Preliminary results indicate that the discriminative-stimulus effects of $\Delta^9$-THC are attenuated by diazepam. In contrast, the peak self-reported, performance and physiological effects of these drugs generally appear to be enhanced when given in combination. To the extent that the discriminative-stimulus effects of drugs set the occasion for drug use and therefore might be involved in relapse, the ability of a $GABA_A$ modulator to block this effect of $\Delta^9$-THC could be useful for treatment. Moreover, the enhancement of $\Delta^9$-THC's other effects by diazepam suggests that there are overlapping effects produced by cannabinoid and GABAergic drugs, consistent with an agonist-replacement strategy.

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# CANNABIS AS A SUBSTITUTE FOR ALCOHOL AND OTHER DRUGS

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**Introduction**: The personal health practice of substitution among medical cannabis patients can provide information concerning non-traditional and alternative means used by individuals to personally address their health issues, and practice harm reduction; potentially without official involvement in a formal health care system. Furthermore, examining the rate of substitution among this population might translate into addiction insight and improved treatment practices.

**Methods**: This study was conducted in 2008 at the Berkeley Patient's Group (BPG), a medical cannabis dispensary in Berkeley, California. Anonymous, self-report surveys (N=350) were collected between August and October 2008 via primary data collection. The survey consisted of questions addressing: demographic characteristics, medical status, cannabis use patterns, drug and alcohol use and/or treatment, and service utilization. The survey sample for the BPG study consisted of 350 medical cannabis patients between the ages of 18 and 81. The sample was 68.4% male, 66.2% White and 14.6% Multi-racial.

**Results**: Fifty three percent (N=180) of respondents reported that they currently drink alcohol. Among current drinkers, the average number of drinking days per week was 2.63, and the average number of drinks on drinking days was 2.88. Sixteen percent reported previous treatment for alcohol or substance use, and 2.4% are currently in a 12-step program. Eleven percent of the sample reported using a drug other than cannabis, a prescription or over the counter drug in the past 30 days. Of the 38 participants who listed the illicit drugs or drugs used without a prescription by participants in the past 30 days, cocaine, MDMA and Vicodin were reported most frequently (N=5), followed by LSD (N=4), mushrooms and Xanax (N=3). Forty percent of respondents reported using cannabis as a substitute for alcohol, 26% reported using it as a substitute for illicit drugs, and 65.8% use it as a substitute for prescription drugs. Sixty five percent of respondents reported using cannabis as a substitute because it has less adverse side effects than alcohol, illicit or prescription drugs, 34% use it as a substitute because it has less withdrawal potential, 17.8% use it as a substitute because its easier to obtain cannabis than alcohol, illicit or prescription drugs, 11.9% use it as a substitute because cannabis has greater social acceptance, 57.4% use it as a substitute because cannabis provides better symptom management, and 12.2% use it as a substitute for some other reason.

Conclusion: Research on medical cannabis patients has alluded to the use of cannabis as a substitute for alcohol, illicit or prescription drugs (Harris et al., 2000; Charlton, 2005; Mikuriya 1970, 2004; Mikuriya and Mandel, 2001; Reiman, 2007). Substitution for alcohol and illicit drugs has even been touted as a harm reduction treatment for addiction. Given that over 2% of the sample is currently in a 12-step program, this study also generates questions about the suitability of traditional 12-step programs for those who might be using cannabis as a substitute for alcohol or other drugs. The use of cannabis as a substitute for alcohol and other drugs has implications for both the fields of addiction, and the field of medicinal cannabis.

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# MORE AROUSED, LESS FATIGUED: FAAH GENE POLYMORPHISMS INFLUENCE ACUTE RESPONSE TO AMPHETAMINE

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**Introduction***:* Amphetamine is a stimulant drug that enhances attention and feelings of alertness. Amphetamine's effects are known to be modulated by endogenous cannabinoids, which are degraded by the enzyme fatty acid amide hydrolase (FAAH). In this study we investigated inter-individual differences in mood response to amphetamine in relation to four polymorphisms in the FAAH gene, including the FAAH missense variant Pro129Thr (rs324420C/A), which was previously found to be associated with street-drug use and addictive traits.

**Methods***:* 159 healthy Caucasian volunteers participated in a three session double-blind crossover design receiving either placebo or oral d-amphetamine (10 and 20 mg). Associations between individual genotypes and levels of self-reported Arousal (POMS) after d-amphetamine ingestion were investigated using two way ANOVAs/ANCOVAs. Association analyses for haplotypes were performed using the adaptive permutation approach implemented in PLINK.

**Results***:* Genotypes at rs3766246 and 2295633 were significantly associated with increased ratings of Arousal ($p< 0.05$) and Fatigue ($p<0.01$) after the 10 mg dose. Fatigue levels were also found to be associated with the haplotypes CCC and TAT formed from rs3766246, Pro129Thr and rs2295633 ($p<0.05$).

**Conclusion***:* These data suggest that the endocannabinoid system influences variation in the subjective response to amphetamine. This has important implications for understanding the role of endogenous cannabinoids in response to amphetamine, studies of poly-substance abuse and understanding the genetic determinants of inter-individual differences in stimulant effects and risk of abuse.

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# NEURAL BASIS OF ANXIOLYTIC EFFECTS OF CANNABIDIOL (CBD) IN GENERALIZED SOCIAL ANXIETY DISORDER

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**CONTEXT**: Animal and human studies indicate that cannabidiol (CBD), a major constituent of cannabis has anxiolytic properties.

**OBJECTIVES:** To investigate whether CBD has anxiolytic effects in patients with Generalised Social Anxiety Disorder (SAD), and to use functional neuroimaging to examine the neural basis of these effects.

**DESIGN:** Double-blind repeated measures administration of CBD (400mg) and placebo prior to measurement of resting regional cerebral blood flow.

**SETTING:** Clinical research centre.

**PARTICIPANTS:** Ten treatment naïve patients with SAD, randomly divided into two groups of 5 subjects. Subjects were recruited from an epidemiological survey of SAD from 2320 university students.

**MAIN OUTCOME MEASURES:** Resting regional cerebral blood flow (rCBF), measured using $^{99m}$Tc-ECD SPECT (rCBF tracer ethyl-cisteinate-dimer (ECD) labeled with technetium-99m (99mTc-ECD).. The Visual Analogue Mood Scale (VAMS) was used to assess subjective states.

**RESULTS:** Relative to placebo, CBD was associated with significantly decreased subjective anxiety ($p<0.001$), reduced ECD uptake in the left parahippocampal gyrus, hippocampus, and inferior temporal gyrus ($p<0.001$, uncorrected), and increased ECD uptake in the right posterior cingulate gyrus ($p<0.001$, uncorrected). Following administration of CBD condition, there was a negative correlation between subjective anxiety and EDC uptake in the amygdala bilaterally ($p<0.001$, uncorrected).

**CONCLUSIONS:** CBD reduces anxiety in SAD and this is related to its effects on activity in limbic and paralimbic brain areas.

# SEX AND CANNABINOID CB1 GENOTYPE DIFFERENTIATE PALATABLE FOOD AND COCAINE SELF-ADMINISTRATION IN MICE

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**Rationale:** Both cannabinoid CB1 receptor knockout and antagonism produce well-established attenuation of palatable food and drug self-administration behavior. Although CB drugs have received attention as pharmacotherapeutics for various disorders, including obesity and addiction, it is unclear whether these agents produce equivalent behavioral effects in females and males.

**Methods:** In the present study, acquisition of 10% Ensure or 32% corn oil self-administration and maintenance of Ensure, corn oil, or 0.56 mg/kg/inf cocaine self-administration under both an FR-1 and PR schedule of reinforcement was compared in male and female WT and CB1 KO mice. Also, the effect of pretreatment with the CB1 antagonist SR141716 (0.3 – 3.0) on Ensure self-administration in male and female WT and CB1 KO mice was assessed.

**Results:** CB1 genotype and sex significantly interacted to produce an attenuation of acquisition and maintenance of Ensure self-administration and motivation to self-administer both Ensure and cocaine in male CB1 KO mice. In contrast, male CB1 KO mice showed no deficit in acquisition and maintenance of FR-1 responding or in PR responding maintained by corn oil. Sex differences also arose within genotypes for responding maintained under all three reinforcers. Lastly, pretreatment with SR141716 attenuated Ensure self-administration in WT and CB1 KO mice but was approximately five-fold more potent in WT mice than in CB1 KOs.

**Conclusion:** The present data add to a small but growing literature suggesting that the cannabinoid system may be a more sensitive modulator of appetitive behavior in males versus females.

# THE EFFECT OF CANNABIDIOL AND $\Delta^9$-TETRAHYDROCANNABINOL ON SOCIAL INTERACTION OF RATS

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While $\Delta^9$-tetrahydrocannabinol (THC) is the main psychoactive constituent of the cannabis plant, a non-psychoactive constituent is cannabidiol (CBD). CBD has been implicated as a potential treatment of a number of disorders including schizophrenia and epilepsy and has been incorporated with THC in a 1:1 combination for the treatment of conditions such as neuropathic pain. This study investigated the effect of THC and CBD, alone or in combination, on some objective behaviours of rats in the open field.

Pairs of rats were injected i.p. with CBD or vehicle 20 min prior to THC or vehicle and placed in an open field for 10 min 20 min later. The rats' behaviour was recorded and then analysed by SMART® video tracking software to assess several aspects of social interaction. In vehicle pretreated rats THC (1 mg/kg) significantly reduced social interaction between rat pairs. Treatment with CBD had no significant effect alone, but pretreatment with CBD (20 mg/kg) reversed the THC-induced decrease in social interaction. A higher dose of THC (10 mg/kg) produced no significant effect on social interaction. However, the combination of high dose CBD and high dose THC significantly reduced social interaction between rat pairs, as well as producing a significant decrease in locomotor activity.

This data suggests that CBD can reverse social withdrawal induced by low dose THC, but the combination of high dose THC and CBD impairs social interaction, possibly by decreasing locomotor activity.

## GENETIC BASIS OF MARIJUANA USE

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A major breakthrough in marijuana-cannabinoid research has been the discovery of a previously unknown but elaborate endogenous endocannabinoid system (ECS) composed of endocannabinoids and the enzymes for their biosynthesis and degradation with genes encoding two distinct cannabinoid (CB1 and CB2) receptors (CBRs) that are activated by endocannabinoids, cannabinoids and marijuana use. Physical and genetic localization of the cannabinoid receptor *CNR1* and *CNR2* genes have been mapped to human chromosome 6 and 1 respectively. A number of variations in CBR genes have been associated with human disorders including drug dependency, obesity and depression. The ubiquitous abundance and differential distribution of the ECS in the human body and brain along with the coupling to many signal transduction pathways may explain the effects in most biological system and the myriad behavioral effects associated with smoking marijuana. This remarkable progress in understanding the biological actions of marijuana and cannabinoids have provided a much richer than previously appreciated cannabinoid genomics and raised a number of critical issues on the molecular mechanisms of cannabinoid induced behavioral and biochemical alterations. The objective of the study is to determine the genetic basis of marijuana use. Therefore, multidisciplinary approaches involving RT-PCR, immoblotting, genotyping, immunoelectron microscopy and analysis of human cannabinoid CBR gene structures and isoform specific expression patterns, behavioral modification in mouse models of emotionality and motor function tests were utilized to study the genetic basis of marijuana use. The data indicate that the mouse cannabinoid Cnr1 and Cnr2 genes are syntenic with the human *CNR1* and *CNR2* genes on chromosome 1 and 6 respectively. Additional evidence is provided for the complex *CNR1* and *CNR2* gene structures and their associated regulatory elements and variants. The post-synaptic localization of neuronal CB2-Rs warrants a re-evaluation of the role of CB2-Rs and their interaction with CB1-Rs in the mammalian brain. Our data also indicate a number of polymorphisms and sub-type cannabinoid receptor specificity indicating that marijuana use may not only be coded in our genes but may be exploited in disorders associated with disturbances of the endocannabinoid system. It is concluded that genetic variants and haplotypes in *CNR1* and *CNR2* genes associated with obesity or addiction phenotypes may help identify specific targets in conditions of endocannabinoid dysfunction. Thus, understanding the ECS in the human body and brain will contribute to elucidating this natural regulatory mechanism in health and disease.

## CANNABIS FOR THE MANAGEMENT OF PAIN: ASSESSMENT OF SAFETY STUDY (COMPASS)

Ware MA, Wang T, Shapiro S, Collet JP for the COMPASS study team

**Introduction**
An estimated 10-15% of patients with chronic noncancer pain self-administer cannabis to manage symptoms. Little is known of the long-term safety of such use, including adverse events (AEs), endocrine, pulmonary and cognitive function.

**Methods**
We conducted a cohort study to compare patients with chronic pain using cannabis (cases) with those who were not cannabis users (controls). Standardized herbal cannabis was dispensed for one year. Adverse events were reported monthly. A subset of patients underwent cognitive, pulmonary, hematological, biochemical, liver, renal and endocrine testing.

**Results**
A total of 215 cases and 216 controls were recruited from 7 clinics across Canada; 67 cases and 35 controls dropped out. Cases had higher pain, were younger, more likely to be male, disabled and to have used tobacco compare to controls. The average cannabis dose was 1.89g/day. No difference in serious AEs was noted [Crude IRR: 0.78 (0.44, 1.39)], though there were more non-serious AEs among cases than controls [Crude IRR: 1.64 (1.36, 1.98)]. Neurocognitive testing improved for both groups over one year with no differences between groups. A median of 30-mL decrease in $FEV_1$ (p=0.02) with a median 1% decrease in $FEV_1$/FVC ratio was observed (p=0.01) among cases. No differences were noted for other parameters.

**Discussion**
Cannabis use for chronic pain over one year is not associated with major changes in lung, endocrine, cognitive function or serious adverse events. The increase in non-serious adverse events is consistent with those for pharmaceutical cannabinoids. Longer-term studies are required to determine effects over more than one year.

# CANNABIDIOL REDUCES LIPOPOLYSACCHARIDE-INDUCED VASCULAR DYSFUNCTION IN THE MOUSE BRAIN: AN INTRAVITAL MICROSCOPY STUDY

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**Introduction:** Endotoxic shock (ES) is a severe complication of infectious diseases associated with high mortality; in this condition, lipopolysaccharide (LPS) released from gram negative bacteria induces an inflammation-mediated widespread vascular dysfunction leading to massive edema, hypotension and death. There is no current available therapy for this condition. In this regard, cannabinoids with vascular and anti-inflammatory effects are of great interest. In particular, investigation on the role of cannabidiol (CBD) has grown lately, because CBD adds to its neuroprotective and anti-inflammatory properties the lack of psychoactive effects. In the present work we tested the brain vascular effects of CBD in a model of ES, by using intravital microscopy that allows the real-time observation of pial vessel events.

**Methods:** A craniotomy was performed in anaesthetised C57BL6 adult mice to directly observe surface brain venules and arterioles with a Nikon 90i upright microscope coupled to a Nikon C1 confocal system; to improve vessel observation, 70000MW dextrane conjugated with fluorophores was administered iv. Then, vehicle (Tween-saline) (VEH, n=7), LPS (2 mg/kg) (LPS, n=8) or LPS plus CBD (3 mg/kg) (LPS+CBD, n=7) were administered i.v. (t0). Changes in diameter of 3 selected arterioles and 3 venules in each animal were studied every 15 min up to 180 min (t180). Leukocyte margination (appearing as dark spots on the vessel wall) was similarly observed in 3 segments 200 μm long of the widest venule present in the field. Arteriolar and venular diameter remained stable throughout the experiment in VEH.

**Results:** LPS induced a sustained arteriolar vasodilation of up to 30±3%, starting at t15 and peaking at t75; in agreement, LPS induced a venular vasodilation of up to 15±2% starting at t75. CBD blunted the vasodilator effect of LPS, so that in LPS+CBD arteriolar and venular dilation accounted only for 10±2% and 5±2%, respectively (ANOVA $p<0.05$ vs. LPS). Leukocyte margination was not observed in VEH, whereas in LPS a density of marginated leukocytes of 19.1±0.5 cells/200 μm was observed at t180; in LPS+CBD leukocyte margination was reduced to 1.8±1.8 cells/200 μm (ANOVA $p<0.05$).

**Conclusion:** We concluded that CBD shows strong anti-inflammatory and vessel stabilizing properties in brain after LPS administration, warranting further investigation to consider its future therapeutic use in ES.

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## BLOOD ENDOCANNABINOID AND PALMITOYLETHANOLAMIDE LEVELS IN HUMAN ISCHEMIC STROKE

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**Background and Purpose:** Endocannabinoids have shown neuroprotective properties in murine and cell culture models of stroke, acting on NMDA-mediated excitotoxicity and modulating the neuroinflammatory response. Our aim was to determine endocannabinoid and palmitoylethanolamide peripheral levels in acute stroke patients and normal controls, considering possible correlations with clinical disability or stroke volume.

Ten patients with a first ischemic stroke in Middle Cerebral Artery (MCA) territory and eight age- and metabolic function-matched control subjects were included. All control subjects underwent a blood sample collection for anandamide (AEA), 2-arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) measurement; patients admitted within 6 hours since stroke onset underwent the same procedure on admittance (T0), after 12 (T1) and after 24 (T2) hours since symptom onset. Patients' neurological impairment was assessed using the NIH Stroke Scale (NIHSS) and Fugl-Meyer Scale arm subitem (FMS) scores; stroke volume was determined on 48-hours follow-up brain CT scans. Blood samples were analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry.

Blood T0 AEA levels were significantly higher in stroke patients compared to control subjects. Stroke patients showed also a significant inverse correlation between their FMS score and T0 AEA levels. Blood T0 PEA levels showed a significant direct correlation with the NIHSS score. A positive correlation trend was found between T0 AEA levels and stroke volume at 48-hours follow-up brain CT scans. Finally, a correlation between BMI, cholesterol, glucose and triacylglycerol plasmatic levels and 2-AG levels was found.

This is the first demonstration of elevated peripheral AEA levels in stroke patients; moreover, in line with previous murine studies, there is a significant relationship between AEA and PEA levels and neurological impairment at 6 hours since stroke onset. The findings corroborate the hypothesis that AEA and PEA might play distinct roles during stroke, whereas 2-AG might be instead related to the metabolic risk factors associated with, and potentially leading to, this pathological condition.

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# THE COMBINATION TREATMENT WITH A $CB_2$ AGONIST AND $CB_1$ ANTAGONIST ATTENUATED CEREBRAL ISCHEMIC INJURY THROUGH DIFFERENT MECHANISMS

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Cannabinoids have been found to influence cerebral ischemic injury but the mechanisms responsible are still not clear. We have previously reported that a selective $CB_2$ agonist (O-1966), and a $CB_1$ antagonist (SR141716) both provide protection for the brain in a mouse model of stroke. The most striking protection was achieved by combined treatment with these two compounds which was found to increase cerebral blood flow during ischemia. The goal of the current study was to investigate the mechanisms through which this combination treatment worked. Transient cerebral ischemia was induced in the wide type (wt) C57BL/6 mice, $CB_2$ -/- mice and $CB_1$-/- mice by intravascular occlusion of the middle cerebral artery for 60 minutes. The cerebral blood flow was monitored by Laser Doppler Flowmetry. Cranial windows were implanted to evaluate the pial microvascular diameter change during ischemia. The results indicated that the combination treatment of a $CB_2$ agonist and a $CB_1$ antagonist did not change cerebral blood flow in wt mice without ischemia but the this combination treatment increased blood flow in wt mice during cerebral ischemia compared to vehicle treated mice. The increase of cerebral blood flow was not diminished in $CB_1$-/- mice or in wt mice pretreated with Capsazepine (a TRPV1 antagonist) but was attenuated in either $CB_2$-/- mice or in wt mice pretreated with WAY100135 (a 5HT1A antagonist). In vivo observation of the cerebral pial microcirculation indicated that the combination treatment induced arteriolar dilation during ischemia and this vasodilation could be diminished by pretreatment with WAY100135. The histological evaluation showed that the combination treatment decreased cerebral infarction by 75%, and this protective effect was attenuated in either $CB_2$-/- mice or pretreatment of wt mice with WAY100135 but not in $CB_1$-/- mice or Capsazepine treatment. In conclusion, our study indicated that a combined treatment with a $CB_2$ agonist and $CB_1$ antagonist attenuated cerebral ischemic injury partially through the elevation of cerebral perfusion and vasodilation during ischemia, which could be mediated by both the $CB_2$ receptors and 5HT1A receptors.

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# CAPSAICIN AFFECTS BRAIN FUNCTION IN A MODEL OF HEPATIC ENCEPHALOPATHY ASSOCIATED WITH FULMINANT HEPATIC FAILURE IN MICE

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Hepatic encephalopathy is a neuropsychiatric syndrome caused by liver failure. In view of the effects of cannabinoids in a thioacetamide-induced model of hepatic encephalopathy and liver disease and the beneficial effect of capsaicin (a TRPV1 agonist) in liver disease, we assumed that capsaicin may also affect hepatic encephalopathy.

Fulminant hepatic failure was induced in mice by thioacetamide administration and 24 hours later, the animals were injected with one of the following compound(s): 2-arachidonoylglycerol ($CB_1$, $CB_2$ and TRPV1 receptors agonist), HU308 ($CB_2$ receptor agonist), SR141716A ($CB_1$ receptor antagonist), SR141716A+2-arachidonoylglycerol, SR144528 ($CB_2$ receptor antagonist), capsaicin and capsazepine (TRPV1 receptor agonist and antagonist respectively). Their neurological effects were evaluated on the basis of activity in the open field, cognitive function in an eight arm maze and a neurological severity score. The mice were killed 3 or 14 days after the thioacetamide administration. 2-Arachidonoylglycerol, catecholamines and serotonin levels were determined by either GC-MS or HPLC-ECD.

Capsaicin had a neuroprotective effect in the above animal model as shown by the neurological score, activity and cognitive function. The effect of capsaicin was blocked by capsazepine. Thioacetamide induced astrogliosis in the hippocampus and the cerebellum and enhanced brain serotonin levels, which were decreased by capsaicin, SR141716A and HU-308. Thioacetamide lowered brain 2-arachidonoylglycerol levels, which were reversed by capsaicin.

Capsaicin improves both liver and brain dysfunction caused by thioacetamide. These results suggest that both the endocannabinoid and the vanilloid systems play important roles in hepatic encephalopathy. Their modulation may have therapeutic value.

# ENDOCANNABINOID AND CB1 RECEPTOR-MEDIATED REGULATION OF NEUROGENESIS IN ZEBRAFISH

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CB1 cannabinoid receptor (CB1R) has been showed to stimulate adult neurogenesis. However, the role of the CB1R in the developmental neurogenesis is poorly characterized. The objective of this study is to determine the role of endocannabinoid anandamide and its G-protein coupled receptor (GPCR)-mediated molecular signaling in the regulation developmental neurogenesis under physiological and pathophysiological conditions. We used green fluorescence protein (GFP)-expressing transgenic zebrafish ISLET-1 embryos to determine the role of zebrafish CB1 cannabinoid receptor (zCB1R) in developmental neurogenesis. We found that zCB1R mRNA transcript was expressed in the different regions of zebrafish central nervous system (CNS) including diencephalon (D), telencephalon (T), hypothalamus (H), and trigeminal ganglion at 48 and 72 hpf (hour post-fertilization). We showed that CB1R agonist CP55940 (1-10 μM) stimulates the neurogenesis in diencephalon, telencephalon, hypothalamus, and trigeminal ganglion and this response was attenuated when embryos were co-treated with CB1R antagonist SR141716 (1-10 μM). We also found that Kainic acid treatment of zebrafish embryos (at 6 hpf-48 hpf) increased zCB1R expression and stimulate neurogenesis. Further, we showed that knocking down CB1R using morpholino or by blocking CB1R by CB1R antagonist SR141716 attenuated kainic acid-mediated increase in neurogenesis. Together, results from this study showed for the first time that CB1R plays a vital role in the regulation of neurogenesis in developing zebrafish under physiological and excitotoxicity-mediated pathophysiological conditions.

# MOLECULAR INSIGHT INTO MONOGLYCERIDE LIPASE, THE ENZYME DEGRADATING 2-ARACHIDONOYLGLYCEROL

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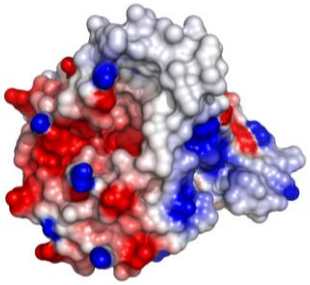
**Introduction.** Among the endocannabinoid system, monoglyceride lipase (MGL) constitutes a promising target for the development of drugs able to increase 2-AG. Such a modulation could be therapeutically relevant in the treatment of pain, inflammation disorders and in order to regulate tumoral proliferation. Our laboratory, as well as other groups in the field of cannabinoid research, is involved in the identification and the development of novel MGL inhibitors. In this context, the knowledge of the tridimensional structure of MGL would constitute a powerful tool for the onset of a rational drug design process.

**Results**. Here we present the X ray structure of human MGL. Beside the well known gastric lipase, hormone sensitive lipase and pancreatic lipase structures, enzymes mostly acting on di- and triglycerides, this work constitutes the first insight into the structure of a lipase aimed at the catabolism of a monoglyceride (i.e. 2-AG). As expected, MGL fold closely matches that of the wide $\alpha/\beta$ hydrolase superfamily. However, despite a strong homology with several esterases and haloperoxidases, MGL distinguishes itself by different properties and structural organization of the cap domain. These constitute key features allowing MGL to access and bind its lipid substrate.



**Conclusion**. There is an urgent need for the design of valuable inhibitors of 2-AG degradation for both pharmacological and therapeutic purposes, with a special emphasis on improving potency and selectivity versus other serine hydrolases (FAAH and other carboxylesterases). In this context, and beside the gain for the fundamental knowledge of the catabolic pathway that regulates 2-AG signalling, it constitutes a promising tool for future medicinal chemistry endeavours.

# MECHANISTIC CHARACTERIZATION OF SELECTIVE MONOACYLGLYCEROL LIPASE INHIBITION REVEALS DIFFERENCES IN CENTRAL AND PERIPHERAL ENDOCANNABINOID METABOLISM

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Monoacylglycerol lipase (MAGL) is a principal degradative enzyme for the endocannabinoid 2-arachidonoylglycerol (2-AG). We recently reported a piperidine carbamate, JZL184, that inhibits MAGL with high potency and selectivity. Here, we describe a comprehensive mechanistic characterization of JZL184. We provide evidence that JZL184 irreversibly inhibits MAGL via carbamoylation the enzyme's serine nucleophile. Functional proteomic analysis of mice treated with JZL184 revealed that this inhibitor maintains good selectivity for MAGL across a wide range of central and peripheral tissues. Interestingly, MAGL blockade produced marked, tissue-specific differences in monoglyceride metabolism, with brain showing the most dramatic elevations in 2-AG and peripheral tissues often showing greater changes in other monoglycerides. Collectively, these studies indicate that MAGL exerts tissue-dependent control over endocannabinoid and monoglyceride metabolism and designate JZL184 as a selective tool to characterize the functions of MAGL in vivo.

## FAAH-2 IS A LIPID DROPLET-LOCALIZED ENZYME THAT MEDIATES N-ACYLETHANOLAMINE INACTIVATION

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Fatty acid amide hydrolase (FAAH) is the principal enzyme in mice that degrades the endocannabinoid anandamide (AEA) and the anti-inflammatory lipid palmitoylethanolamide (PEA), among other N-acylethanolamines (Deutsch and Chin, Biochem Pharmacol (1993) 46; 791-6; Cravatt et al., Proc Natl Acad Sci U S A (2004) 101; 10821-6). Recently, a novel FAAH enzyme was identified (termed FAAH-2) that is expressed in higher mammals but not in mice and rats (Wei et al., J Biol Chem (2006) 281; 36569-78). FAAH-2 hydrolyzes the N-acylethanolamines, AEA and PEA, with lower rates than FAAH *in vitro*. Apart from this report on FAAH-2, little is known about its subcellular localization or its capacity to catabolize AEA and PEA in intact cells.

The capacity of FAAH-2 to hydrolyze internalized AEA and PEA was examined in transfected HeLa cells. Despite its lower expression level, FAAH-2 hydrolyzed AEA and PEA with rates approximately one third that of FAAH. This suggests that FAAH-2 is an efficient AEA and PEA inactivating enzyme in intact cells. Immunofluorescence studies indicated that in contrast to FAAH, FAAH-2 did not localize to the endoplasmic reticulum (ER). Instead, FAAH-2 was found primarily on cytoplasmic lipid droplets/adiposomes, which serve as sites of triglyceride and cholesteryl ester storage and liberation. The distribution of FAAH-2 to lipid droplets was confirmed by subcellular fractionation. The putative N-terminal transmembrane helix of FAAH-2 was identified as a lipid droplet targeting sequence and was both necessary and sufficient for mediating adiposome localization. Re-localization of FAAH from the ER to lipid droplets did not affect the rate of anandamide inactivation, confirming that AEA is efficiently delivered to lipid droplet membranes for hydrolysis. These findings are in agreement with a recent report demonstrating that AEA partitions to lipid droplets in cells (Oddi et al., Cell Mol Life Sci. (2008) 65(5); 840-50).

A minor pool of FAAH-2 proteins bearing luminally-facing membrane orientations were observed in cells. However, FAAH-2 chimeras localizing to the cytoplasmic or luminal faces of ER membranes were either inactive or not well expressed. These data confirm that FAAH-2 is not a resident ER protein, and instead suggest that FAAH-2 may traverse the ER lumen prior to reaching the lipid droplet surface. Collectively, these data demonstrate that FAAH-2 is an efficient AEA and PEA inactivating enzyme with a unique localization pattern among N-acylethanolamine degrading enzymes. This study also establishes lipid droplets as functional sites of endocannabinoid and N-acylethanolamine inactivation. **Acknowledgements**: NIH grants DA09374 and DA16419.

# THE ROLE OF FATTY ACID BINDING PROTEINS IN THE LIFE CYCLE OF ANANDAMIDE

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Anandamide (AEA), an endogenous ligand for the Cannabinoid Type 1 (CB1) receptor, is an uncharged lipid-like molecule. After AEA binding at the CB1 receptor, a transmembrane protein located on the plasma membrane, AEA is inactivated via cellular uptake and then catabolized by fatty acid amide hydrolase (FAAH), a membrane associated protein in the endoplasmic reticulum. Given the hydrophobic nature of AEA, free diffusion from the plasma membrane to the ER is unlikely. In fact, recent experiments [1] point to select members of the fatty acid binding protein (FABP) family as carriers for AEA across the cytosol to the ER, in an assisted transport mechanism.

We have begun to study in atomic detail, the binding mechanism of AEA to FABP. The crystal structures of human brain-FABP (FABP7) in complex with oleic or docosahexanoic acid (DHA) have been reported.[2] The general structure of FABP7 is very similar to that of the other fatty acid-binding proteins. The overall structural motif of the family is represented by a 10-strand β barrel. The first and second β strands are connected by a helix-turn-helix motif. Fatty acids are believed to enter the protein through a "dynamic portal" formed by the helix-turn-helix motif. The helices at the portal are designated αI and αII. Backbone hydrogen bonds typical of antiparalleled beta-pleated sheets are found between all strands, with the exception of beta-strands 4 and 5. These strands are separated, and the space between these two strands is filled with protein side chain atoms.

A multinanosecond NAMD2 [3] molecular dynamics simulation of apo-FABP7 was first conducted in water. These studies revealed that water can enter and leave FABP7 either through the portal region or between beta-strands 4 and 5. After equilibration of the apo-FAPB7 in water, the Glide docking program (Schrodinger, San Diego, CA) was used to conduct a series of docking experiments of AEA in apo-FAPB7. Parallel studies were performed with DHA and compared to the crystal structure.[2] Docking experiments were guided by the location of key amino acids (Y128, R106 and R126) in FABP7 as revealed by the oleic- and DHA- FABP7 crystal structures.[2] Resultant docks were used for multinanosecond NAMD2 molecular dynamics simulations in water in order to probe the structure/dynamics for AEA uptake. [Support: DA003934 and DA021358 to PHR].

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# TEMPERATURE-DEPENDENT AND SATURABLE UPTAKE OF ANANDAMIDE BY SYNTHETIC LIPOSOMES

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There is considerable controversy concerning the mechanism(s) governing the cellular uptake of anandamide (AEA). Three separate observations that have been interpreted to indicate a facilitated diffusion mechanism are that the uptake shows temperature-dependency, saturability, and can be inhibited pharmacologically. However, it has been suggested that the temperature-dependency and saturability can be explained without the need to invoke a transporter (Fowler & Thors, *Br J Pharmacol* 149 [2006] 73-81; Bojesen *et al.*, *J Lipid Res* 47 [2006] 561-70). In the present study, we have investigated whether the properties of AEA retention by cultured cells can be mimicked in liposomes made from synthetic lipids.

Unilamellar liposomes were made from a combination (9:1 w/w) of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (phosphatidylcholine) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (phosphatidylglycerol) using a LiposoFast-Basic extruder (http://www.avestin.com/lf.html#lfbasic). The liposomes were incubated with [$^3$H]AEA and the tritium retained was determined by rapid filtration using a cell harvester following washing of the liposomes.

Initial experiments indicated that the retention of tritium label by the liposomes was dependent on ***incubation time*** (***up*** to 60 ***min***) and an incubation time of 30 min was used for further experimentation. Under these conditions, the retention of label showed a marked temperature-dependency. Thus, for example, at both 100 nM and 3 µM added AEA concentrations, the observed retention of tritium at an incubation temperature of 4 °C was only 6% of that seen at 20 °C. At 20 °C, the retention of tritium showed both linear (at added AEA concentrations of >100 nM) and saturable (≤100 nM) components. At an added AEA concentration of 100 nM, the retention of label was not inhibited by either AM404 (0.1-30 µM), VDM11 (0.1-30 µM) or URB597 (0.1-30 µM).

These data mimic rather well the observed retention of AEA by RBL2H3 cells seen at short incubation times, where both saturable and non-saturable components have been reported (Ligresti *et al.*, *Biochem J* 380 [2004] 265-72) and where VDM11 does not inhibit the uptake (Kaczocha *et al.*, *J Biol Chem* 281 [2006] 9066-75). This would be consistent with the suggestion that that initial rapid cellular retention of AEA is independent upon the presence of designated proteins in the plasma membrane.

# NANOPARTICLE-MEDIATED CELLULAR UPTAKE-CONTROLLED RELEASE AS A TOOL TO STUDY ANANDAMIDE MEMBRANE TRANSPORT

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Nanoparticles are ultra-finely dispersed, solid particles, nm in size and possessing a high specific surface to volume ratio, that are currently used for imaging, biosensing and gene/drug delivery. To put it in perspective, a DNA molecule is 2 nm wide and red blood cells are thousands of nm in diameter. Because of their ultra-small size, nanoparticles can penetrate cell membranes and integrate into larger intracellular molecular structures. This system is used to protect drugs from chemical and enzymatic degradation and to allow them to enter the cell and rapidly reach the cytoplasm, thus by-passing any possible drug-specific membrane transporter system. In particular, poly-ε-caprolactone nanoparticles (PLC-NP's) are used because of their biocompatibility, lipophilicity, cost-effectiveness and capability to support passive uptake processes, compared with other polyesters. Given their physicochemical nature, the hypothesized mechanism for intracellular penetration of PLC-NP's is via endosomal entrapment followed by cytoplasmatic release. Although the mechanism by which this process occurs is not fully understood, one possible explanation is that detergents used in colloid formulations as particle stabilizers induce endosomal membrane disruption once inside the cell. We used a PLC-NP-mediated cellular uptake-controlled release system as a tool to test the hypothesis of the existence of an anandamide (AEA) membrane transporter. The activation of TRPV1 receptors, which possess a cytosolic binding site for AEA, was selected as an indirect measure of the entry into cells of either free or PLC-NP-incorporated AEA (NP-AEA).

PLC-NP's were prepared by the nanoprecipitation technique and used for incorporating AEA. Nanoparticles with a mean particle size of 80 nm were obtained. Drug loading was confirmed by routine particle size, zeta potential, microscopic imaging, loading and release assays. AEA loading into the nanoparticles was 466 µg/mL, with a >97 % entrapment efficiency. A fluorometric test was used to compare the effect of NP-AEA and free AEA on intracellular $Ca^{2+}$ in HEK-293 cells overexpressing the human recombinant TRPV1 receptor, in the presence or absence of BSA or the selective AEA cellular uptake inhibitor, OMDM-1. The capability of NP-AEA to rapidly enter HEK-293 cells was ascertained using nanoparticles loaded with rhodamine instead of AEA, followed by fluorescence microscopy.

Both NP-AEA and free AEA induced elevation of intracellular $Ca^{2+}$ in TRPV1-expressing HEK293 cells, with similar kinetics, potency and efficacy, although only the effect of the former was preserved after storage at room temperature for one week. Importantly, the effect of NP-AEA was significantly less sensible than that of AEA to inhibition by OMDM1 (and BSA), at all tested doses of OMDM-1 and AEA.

These findings suggest that incorporation into PLC-NP's: 1) protects AEA from chemical degradation; 2) allows AEA to enter directly and quickly into the cells to then be released into the cytosol and bind TRPV1 intracellular binding site; 3) prevents AEA from being recognized by the putative membrane transporter system or by other proteins facilitating AEA cellular uptake, and hence impairs BSA and OMDM-1 inhibition of TRPV1 activation by AEA. Therefore, these experiments provide further evidence for the existence of a specific process for AEA membrane transport and/or cellular uptake.

# RADIOSYNTHESIS AND IN VIVO EVALUATION OF [$^{11}$C]-BIPHENYL-3-YL 4-METHOXYPHENYLCARBAMATE AS PET TRACER FOR IN VIVO EVALUATION OF FAAH IN THE BRAIN

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**Introduction:** The existence of a link between the endocannabinoid system and more specifically FAAH, and several neurological and neuropsychiatric disorders has been the subject of increasing interest in recent years. A deeper insight in the relationship between the FAAH-endocannabinoid system and those disorders could be achieved by visualization of the enzyme in vivo using molecular imaging and positron emission tomography (PET). Non-invasive in vivo detection of enzymes can be obtained by using a radiolabeled enzyme inhibitor. Since no PET tracer for in vivo visualization of cerebral FAAH has been reported so far, the aim of this study was the synthesis of an URB-597 analogue, [$^{11}$C]-biphenyl-3-yl 4-methoxyphenylcarbamate ($^{11}$C-**1**), and the evaluation of its usefulness as tracer for mapping brain FAAH in vivo.
**Methods:** Inhibition of FAAH by **1** was evaluated using recombinant rat FAAH and [$^{3}$H]-AEA. Synthesis of $^{11}$C-**1** was done by methylation of desmethyl-**1** using $^{11}CH_3I$ followed by RP-HPLC purification. Biodistribution of $^{11}$C-**1** was studied in wild type (WT) and FAAH knock-out (KO) mice after i.v. injection of 3.7 MBq $^{11}$C-**1**. At 1, 10 and 30 min post injection (pi) (18 - 37 MBq of $^{11}$C-**1**), metabolite analysis in WT and KO mice was performed. Plasma and brain proteins were precipitated with $CH_3CN$ and supernatant was analyzed by RP-HPLC.
**Results: 1** inhibits hydrolysis of [$^{3}$H]-AEA by rFAAH with an $IC_{50}$ value of 0.44 µM. $^{11}$C-**1** was obtained in a radiochemical yield of 35% with a radiochemical purity of > 99%. $^{11}$C-**1** demonstrated high brain uptake in WT and KO mice with retention of radioactivity in brain (8.58% ± 2.32 ID/g at 1 min and 0.55% ± 0.20 ID/g at 1 h in WT and 8.34% ± 0.16 ID/g at 1 min and 0.42% ± 0.07 ID/g at 1 h in KO mice). The uptake was not statistically different in WT compared to KO mice. $^{11}$C-**1** demonstrates hepatobiliary and urinary clearance with a higher uptake in small intestines in WT compared to KO indicating different metabolic profiles. Metabolite analysis revealed complete metabolisation of $^{11}$C-**1** in plasma of WT and KO at 30 min pi, while slower metabolisation was detected in KO brain (57% intact tracer in KO and 26% in WT at 30 min) indicating possible FAAH mediated metabolisation of $^{11}$C-**1**.
**Conclusion: 1** effectively inhibits [$^{3}$H]-AEA hydrolysis in vitro. We have successfully labelled **1** with $^{11}$C and demonstrated its brain uptake. The metabolic profile should be further evaluated. Together these data suggest that **1** can serve as lead for the preparation of inhibitor based FAAH imaging agents.

# NAUSEATING EFFECTS OF AM-251 MAY BE PRODUCED BY THE PERIPHERAL INVERSE-AGONIST PROPERTIES: EVIDENCE FROM THE CONDITIONED GAPING MODEL IN RATS

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**Introduction**: Although rats are not capable of vomiting, they display characteristic conditioned gaping reactions when exposed to a flavor previously paired with a nausea-inducing treatment, such as Lithium Chloride (LiCl). Cannabinoid compounds modify conditioned gaping in rats in a manner that suggests they reduce nausea as well as vomiting. CB1 receptor agonists reduce LiCl-induced conditioned gaping reactions and the CB1 receptor antagonist/inverse agonist, SR141716, potentiates LiCl-induced conditioned gaping at a low dose that does not produce gaping on its own. Here we evaluated the potential of the CB1 antagonist/inverse agonist, AM251, and the silent CB1 antagonists, AM6527 (centrally and peripherally active) and AM6545 (peripherally active) to produce conditioned gaping and to potentiate LiCl-induced conditioned gaping.

**Methods**: All rats were surgically implanted with an intra-oral cannula to facilitate infusion of saccharin directly to the oral cavity. Rats in the centrally administered groups were implanted with a unilateral guide cannula directed at the lateral ventricle. All AM compounds were prepared in a vehicle of 45% 2-hydroxypropyl-β-cyclodextrin at a concentration of 1 mg/ml. With the exception of microinfusion to the lateral ventrical, all AM compounds were administered intraperitoneally (i.p). LiCl was prepared in a 0.15M solution with sterile water and administered i.p. at a volume of 20 ml/kg.

**Results**: At 8 mg/kg, only AM251 produced conditioned gaping reactions when paired with saccharin solution. Furthermore, at a dose that does not produce conditioned gaping on its own, systemically administered AM251 potentiated LiCl-induced conditioned gaping; however even doses as high as 8 mg/kg of AM6545 and AM6527 did not potentiate LiCl-induced conditioned gaping in rats. Intracerbroventricular (icv) infusions of AM251 did not potentiate LiCl-induced conditioned gaping reactions, but both doses attenuated saccharin palatability.

**Conclusions**: These results suggest that the nausea producing effects of AM251 appear to be mediated by its inverse agonist properties and may be peripherally mediated.

# INSULIN AS A NEGATIVE REGULATOR OF PLASMA ENDOCANNABINOID LEVELS IN OBESE AND NONOBESE SUBJECTS

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Endocannabinoids control energy intake and processing via cannabinoid $CB_1$ receptors. Their plasma levels are elevated in overweight type 2 diabetes (T2D) and in obese patients, and decrease post-prandially in normoweight individuals. Defective insulin signalling has been proposed as one of the possible causes of endocannabnoid overactivity in animal models of hyperglycemia and obesity. We investigated, in two different cohorts of nonobese or obese volunteers, if oral glucose in glucose tolerance tests (OGTT), or acute insulin infusion during euglycemic hyperinsulinemic clamps, affect plasma endocannabinoid levels.

OGTT was performed in ten obese hyperinsulinemic patients (BMI=35.8 kg/m$^2$, fasting insulin=14.83 mU/l), and ten normoweight normoinsulinemic volunteers (BMI=21.9 kg/m$^2$, fasting insulin=7.2 mU/l). Insulin clamp was performed in 19 mostly nonobese men (BMI=25.8 kg/m$^2$) with varying degree of liver fat and plasma triglycerides, with (N=7) or without (N=12) T2D. Plasma levels of endocannabinoids (anandamide and 2-arachidonoylglycerol) were measured by liquid chromatography-mass spectrometry, before and 60 and 180 min after OGTT, and before and 240 and 480 min after insulin or saline infusion.

Oral glucose load decreased plasma endocannabinoid levels to an extent inversely correlated with: 1) BMI, 2)waist circumference, 3)subcutaneous fat, 4) fasting insulin levels, and 5) glucose and insulin areas under the curve during the OGTT. Oral glucose load decreased 2-arachidonoylglycerol levels to a lower extent and in a way inversely correlated only with fasting insulin levels. Oral glucose load did not decrease plasma endocannabinoid levels in obese volunteers. Insulin infusion decreased anandamide, but not 2-arachidonoylglycerol, levels to an extent negatively correlated with triglycerides, liver fat and fasting insulin, and positively with HDL-cholesterol.

Our results represent the first clear evidence that insulin down-regulates the peripheral endocannabinoid tone in humans. We propose that insulin reduces endocannabinoid, and particularly anandamide, levels in a way inversely related to anthropometric and metabolic predictors of insulin resistance and dyslipidemia, and that, because of this, post-prandial endocannabinoid overactivity might occur also in nonobese dyslipidemic subjects.

## INFLUENCES OF DIETARY FAT ON THE PERIPHERAL ECS IN LEAN AND OBESE HUMAN SUBJECTS

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**Introduction:** Endocannabinoid concentrations in blood and adipose tissue are increased in obese subjects whereas FAAH is down regulated. Similar changes occur in the liver in mice on a high fat diet. We hypothesized that endocannabinoid dysregulation in human obesity might be secondary to increased fat ingestion.

**Methods:** 18 lean and 15 obese healthy women and men entered this randomized cross-over study. A stabilizing period (30% of caloric intake from fat) was followed by a period of either 15% or 45% fat intake. Afterwards, subjects entered a second stabilization period and then crossed over to a period with 45% or 15%. Each period lasted for 2 weeks. At the end of high and low fat periods, we measured anandamide in blood before and after a test meal by stable isotope dilution GC-MS/MS. We determined ECS gene expression in subcutaneous adipose tissue and skeletal muscle.

**Results:** Body weight and body composition did not change throughout the study. Compared with lean subjects, fasting anandamide was increased by 24% in the obese group ($p = 0.01$). Anandamide did not respond to high fat intake. With test meal ingestion, anandamide decreased similarly in both groups (-31% at 1h, -44% at 2h, $p < 0.001$). In obese subjects, adipose tissue DAGL$\alpha$ expression was increased and MGL and FAAH expression were decreased. ECS genes did not respond to changes in dietary fat. Skeletal muscle ECS gene expression was similar in lean and obese subjects. Yet, high fat intake reduced $CB_1$-R and MGL gene expression in skeletal muscle.

**Conclusion:** Differences in circulating anandamide concentrations and adipose tissue ECS expression between lean and obese subjects cannot be solely explained by excessive dietary fat intake. However, dietary fat intake changes ECS gene expression in skeletal muscle.

## ANTI-OBESITY EFFECTS OF A NOVEL PERIPHERALLY ACTING NEUTRAL CANNABINOID 1 RECEPTOR ANTAGONIST IN MICE

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Cannabinoid 1 receptor (CB1R) antagonists reduce body weight and improve the associated hormonal/metabolic abnormalities in obese rodents and humans. However, their value as anti-obesity agents is limited by side effects mediated at CB1R in the CNS, such as anxiety, depression and suicidality, as revealed in recent phase III clinical trials with the benchmark CB1R antagonist rimonabant. CB1R and endocannabinoids are also present in liver, adipose tissue and skeletal muscle, and several lines of evidence suggest that activation of peripheral CB1R contributes to diet-induced obesity and its hormonal/metabolic consequences.

Here we describe a novel CB1R neutral antagonist, AM6545, and its effects in mice with high fat diet-induced obesity (DIO). AM6545 and rimonabant have similar CB1R binding affinity, but AM6545 has markedly reduced brain penetrance and, unlike rimonabant, fails to inhibit the centrally mediated hypothermic response to the CB1R agonist HU-210 or to induce a CB1R-mediated increase in locomotor activity seen with rimonabant. Measured by indirect calorimetry, AM6545 (10 mg/Kg, ip) or rimonabant (10 mg/Kg, ip) caused a similar and immediate decrease in both respiratory quotient and carbohydrate oxidation, and a long lasting increase of fat oxidation. Chronic 28-day treatment of DIO mice with AM6545 (10 mg/Kg/day, ip) did not affect total caloric intake despite a transient reduction in food intake. Yet, AM6545 caused a lasting reduction in body weight, pointing to a metabolic effect independent of energy intake. Serum leptin, glucose and insulin levels were significantly reduced, while adiponectin levels were increased (-41%, -14%, -52% and + 95% respectively, $p<0.05$ vs. vehicle-treated group). AM6545 treatment ameliorated glucose tolerance and insulin sensitivity, and also reduced cholesterol levels. Finally, AM6545 prevented diet-induced hepatic steatosis and hepatocellular damage, as reflected by blunting the rise in hepatic triglycerides and serum alanine and aspartate aminotransferases. These chronic effects were very similar to the effects of chronic treatment of DIO mice with rimonabant, as documented in several published studies.

In conclusion, in a rodent model of obesity, chronic AM6545 treatment produces a marked and sustained decrease in body weight and hepatic steatosis associated with improved plasma lipid profile and glucose tolerance, without eliciting effects associated with blockade of CB1R in the CNS. Peripherally restricted CB1R neutral antagonists have therapeutic potential in the management of fatty liver, dyslipidemia, impaired glucose homeostasis and obesity.

# THE ACTIONS OF A PERIPHERALLY RESTRICTED CANNABINOID $CB_1/CB_2$ RECEPTOR AGONIST ON EXPERIMENTAL COLITIS IN MICE

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**Background**: The endocannabinoid system is involved in the regulation of inflammation in the gastrointestinal tract. Cannabinoid agonists reduce the degree of inflammation. Treatment of intestinal inflammation in patients is limited by the central side-effects of cannabinoids. Recently, naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (NVP) was shown to be a peripherally restricted, dual cannabinoid $CB_1/CB_2$ receptor agonist. We therefore investigated whether NVP would alter the development of colitis in two, well-characterized animal models of inflammatory bowel disease, dextran sulphate sodium (DSS)-induced colitis and trinitrobenzene sulfonic acid (TNBS)-induced colitis.

**Methods**: Male CD1 mice were administered DSS (5% DSS in drinking water, days 0-5) and body weight was recorded daily. On days 3 - 8 post-DSS initiation, NVP (0.1 and 1.0 mg/kg), WIN 55,212-2 (2 mg/kg) or vehicle (4% DMSO, 2% Tween 80 in 0.9% physiological saline) was administered intraperitoneally twice daily (9:00 and 17:00). On day 8 mice were killed for the macroscopic and biochemical assessment of colitis. In additional studies, colitis was induced in male CD1 mice by the intra-rectal administration of 100 µl 4% TNBS in 30% ethanol 1 hr after receiving NVP (0.1 and 1.0 mg/kg), WIN 55,212-2 (2 mg/kg) or vehicle. NVP, WIN 55,212-2 or vehicle was injected 8 and 24 hr post-TNBS administration. Body weight was recorded for 3 days. At day 3, the mice were killed for the macroscopic and biochemical assessment of colitis.

**Results**: DSS and TNBS induced colitis in vehicle treated mice. DSS caused a reduction in body weight in all treatment groups which was sustained for the duration of the study following administration of vehicle, 0.1 and 1.0 mg/kg NVP. WIN 55,212-2 treated animals did not lose further weight after day 6. WIN 55,212-2 significantly ($P < 0.05$) attenuated the total damage score in both DSS-treated and TNBS-treated CD1 mice. The administration of NVP at a dose of 0.1 mg/kg significantly potentiated DSS-induced colitis, with significant changes in body weight scores, colon length score and total damage scores compared to those in vehicle treated mice. This trend was also observed in TNBS-induced colitis. Biochemically WIN 55,212-2 tended to improve colitis whereas NVP was largely without effect. A dose of 1.0 mg/kg NVP did not modify the development of inflammation in either DSS or TNBS treated mice.

**Conclusions**: These data suggest that the peripherally restricted cannabinoid agonist NVP is not an effective therapeutic in animal models of intestinal inflammation. Our data suggests that central actions of cannabinoids might be important in the anti-inflammatory effects of exogenously administered cannabinoids.

# *N*-ARACHIDONOYL GLYCINE ACTS AS A VASORELAXANT VIA NITRIC OXIDE AND LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS

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A number of *N*-arachidonoyl amino acids including *N*-arachidonoyl glycine (NAGly) has been identified in mammalian tissues but their physiological functions remain poorly understood. NAGly is structurally similar to the endocannabinoid, *N*-arachidonoyl ethanolamide (anandamide) but, unlike anandamide, it does not activate cannabinoid $CB_1$ receptors. Given that anandamide is a well-known vasorelaxant, we aimed to examine the vascular effects of NAGly.

Male Wistar rats (200-350g) were killed by cervical dislocation. The third-order branches of the superior mesenteric artery (2mm long) were mounted in a wire myograph and maintained at 37$^{o}$C in gassed (95% $O_2$/5% $CO_2$) Krebs-Henseleit solution. Relaxations to cumulative addition of NAGly to vessels precontracted with methoxamine (3-10 µM) were expressed as mean±s.e.m ($n \geq 4$ rats) and analysed by two-way analysis of variance of the whole data set. Inhibitors were incubated with vessels for 30min (or 2h for pertussis toxin) before determination of a concentration-response curve. $P<0.05$ was considered statistically significant.

In endothelium-intact vessels, NAGly induced concentration-dependent relaxation, which was reduced by the nitric oxide synthase inhibitor, L-NAME (control: $pEC_{50}$=5.8±0.2, $E_{max}$=98±1%; + 300µM L-NAME: $pEC_{50}$=5.1±0.1, $E_{max}$=77±8%) or the selective inhibitor of large conductance calcium-activated potassium channels ($BK_{Ca}$), iberiotoxin (+ 50nM iberiotoxin: $pEC_{50}$=5.0±0.1, $E_{max}$=63±17%). The combination of L-NAME and iberiotoxin caused a larger inhibition than with L-NAME alone (+ L-NAME + iberiotoxin: $pEC_{50}$=4.8±0.1, $E_{max}$=62±19%). Endothelial removal reduced the potency of NAGly as a vasorelaxant (without endothelium, $pEC_{50}$=5.1±0.1; $E_{max}$=82±5%) and the resultant NAGly responses were inhibited by iberiotoxin but not L-NAME (data not shown). NAGly relaxations were also attenuated by O-1918, a novel endothelial cannabinoid receptor antagonist, and pertussis toxin, which uncouples interactions of $G_{i/o}$ with their receptors (with endothelium, control: $pEC_{50}$=5.5±0.2, $E_{max}$=91±3%; + 3µM O-1918: $pEC_{50}$=4.9±0.1, $E_{max}$=48±18%; + 400ng/ml pertussis toxin: $pEC_{50}$=5.3±0.2, $E_{max}$=87±4%). However, antagonists for $CB_1$ and $CB_2$ receptors or Transient Receptor Potential Vanilloid type 1 receptors had no significant effect (data not shown).

These data suggest that NAGly acts as a vasorelaxant predominantly via activation of $BK_{Ca}$ in rat small mesenteric arteries. It is hypothesised that NAGly activates an unknown $G_{i/o}$-coupled receptor, stimulating endothelial release of nitric oxide which in turn activates $BK_{Ca}$. In addition, NAGly might also directly activate $BK_{Ca}$, as has been suggested for *N*-arachidonoyl serine.

# ANANDAMIDE INJECTED INTRATHECALLY INDUCES ANALGESIA MEDIATED BY A TRPV1-DEPENDENT MECHANISM IN NEUROPATHIC RATS

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The recognition of the chemical similarity between anandamide (AEA) and capsaicin marked the beginning of studies correlating the endocannabinoid and vanilloid signaling systems. Lately, intrathecal (i.t.) administration of AEA was shown to reduce pain behaviour via TRPV1 receptors during carrageenan-induced thermal hyperalgesia. However, no data exist on the potential role of spinal TRPV1 activation by AEA in neuropathic pain models.

Rats chronically implanted with intrathecal (i.t.) catheters underwent sciatic nerve ligation (CCI model), and mechanical allodynia and thermal hyperalgesia were measured. Seven days after CCI, we tested the effect of AEA (5, 50, 100 µg; i.t.); or the inhibitor of AEA enzymatic hydrolysis, URB597; we evaluated the involvement of TRPV1 or cannabinoid $CB_1$ receptors by blocking these receptors with I-RTX or AM251, respectively; and we determined the levels of AEA in the spinal cord of CCI rats. All experiments were carried out according to IASP rules. AEA displayed a dose-depended antiallodynic (von Frey test) and antihyperalgesic (paw withdrawal test) action. The analgesic effect of AEA (50 µg) was abolished by pretreatment with I-RTX but not AM251, suggesting the involvement of spinal TRPV1 receptors. URB597 dose-dependently enhanced spinal AEA levels and, depending on the administered dose (100-200 µg i.t.), reduced thermal and tactile nociception via $CB_1$ or TRPV1 receptors as demonstrated by the attenuation of its effects by pretreatment with the respective antagonists, AM251 or I-RTX.

We suggest that i.t. AEA reduces neuropathic pain by acting as an endovanilloid, possibly by activating/desensitizing TRPV1 receptors co-localized with $CB_1$ receptors in the superficial lamina of the spinal cord. When endogenously up-regulated with URB597, AEA exerts analgesia via both receptors. AEA- and TRPV1-based therapies might be useful for the control of ascending pain pathways.

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# SUSTAINED INHIBITION OF FAAH ACTIVITY ELEVATES ENDOCANNABINOID TONE AND ACTIVATES PPAR NUCLEAR RECEPTORS IN HUMAN NEUROBLASTOMA CELLS

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Inhibition of FAAH activity and subsequent elevation of the endocannabinoid tone has been reported to elicit analgesia in a rat model of inflammatory pain through activation of the nuclear receptor PPARα (Jhaveri et al., 2008). However, the mechanism underlying this effect is still under scrutiny. In order to test this hypothesis and simplify the experimental setup, we have investigated the effects of sustained inhibition of FAAH activity in SH-SY5Y human neuroblastoma cells.

FAAH activity was assessed by monitoring liberation of [$^3$H]-ethanolamine from labelled anandamide (Boldrup et al., 2004). FAAH protein and RNA expression were measured by immunoblotting and qRT-PCR respectively. Endocannabinoid levels were assessed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In order to evaluate PPAR activation, a PPRE-linked luciferase construct (Peroxisome Proliferator Response Element) was transiently transfected into SH-SY5Y cells.

24 hours treatment with URB597 1μM resulted in significant FAAH activity inhibition sustained after 1, 12 and 24 hours of exposure ($P<0.001$). However, URB597 treatment had no effect on either FAAH protein or RNA levels. Monitoring of the endocannabinoid tone showed significant augmentation of anandamide ($P<0.01$), palmitoylethanolamine ($P<0.05$) and 2-arachidonoylglycerol ($P<0.01$) levels after URB597 10μM treatment, while oleoylethanolamine levels were unaffected. Both URB597 10μM and OL135 10μM, an FAAH inhibitor structurally and functionally different from URB597, elicited PPAR transactivation after 24 hours treatment ($P<0.01$ and $P<0.001$ respectively).

In conclusion, we were able to induce activation of PPAR nuclear receptors *in vitro* by inhibition of FAAH activity and subsequent augmentation of endocannabinoid tone. These data suggest that at least in a model setup, it is possible to modulate the endocannabinoid tone without any previous external stimulus of their synthesis and trigger a functional and potentially analgesic effect.

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**FAAH AND MAGL INHIBITION REDUCE NEUROPATHIC PAIN VIA DISCRETE RECEPTOR-SPECIFIC MECHANISMS OF ACTION**

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Phytocannabinoids, such as $\Delta^9$-THC, and synthetic cannabinoid receptor agonists have analgesic effects, but their psychoactive side effects have hindered their therapeutic development. Alternatively, inhibition of enzymes responsible for catabolism of the endogenous cannabinoids reduces nociception in a variety of preclinical assays, with minimal behavioral effects. Published reports indicated that inhibiting fatty acid amide hydrolase (FAAH), the principal enzyme responsible for the degradation of the anandamide (AEA), reduces mechanical allodynia and thermal hyperalgesia. The recent development of a highly selective inhibitor of monoacylglycerol lipase (MAGL), which degrades 2-arachydonoyl glycerol (2-AG), has made it possible to examine the role of 2-AG in pain modulation. This MAGL inhibitor, JZL184, causes an eight fold increase in brain 2-AG levels and increases withdrawal latencies in the tail immersion test[1]. In the present study, we tested whether MAGL inhibition also blocks neuropathic pain. We hypothesized that JZL184 would attenuate mechanical allodynia, as well as acetone-induced cold allodynia, in mice subjected to chronic constriction injury (CCI) of the sciatic nerve. Acute administration of JZL184 decreased allodynia in both tests. This attenuation was completely blocked by pretreatment with the $CB_1$ selective receptor antagonist SR141716, although the $CB_2$ receptor specific antagonist SR144528 had no effect on the anti-allodynic effects of MAGL inhibition. On the other hand, although the reversible FAAH inhibitor, OL-135, also reduced allodynia, this effect was prevented by pretreatment with either the $CB_1$ or $CB_2$ receptor antagonist. In FAAH (-/-) mice, which did not show any phenotypic allodynia vs. wildtype mice, OL-135 did not elicit anti-allodynic effects, whereas the effects of JZL184 were FAAH-independent. These data indicate that inhibition of FAAH or MAGL attenuate neuropathic pain via discrete cannabinoid receptor-mediated mechanisms of action.

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**DISCOVERY AND CHARACTERIZATION OF A HIGHLY SELECTIVE FAAH INHIBITOR THAT REDUCES INFLAMMATORY PAIN**

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Endocannabinoids are lipid signaling molecules that regulate a wide range of mammalian behaviors, including pain, inflammation, and cognitive/emotional state. The endocannabinoid anandamide is principally degraded by the integral membrane enzyme fatty acid amide hydrolase (FAAH), and there is currently much interest in developing FAAH inhibitors to augment endocannabinoid signaling *in vivo*. Despite considerable advances in the development of FAAH inhibitors, agents that combine high target selectivity with exceptional *in vivo* efficacy are still needed. Here, we report the discovery and detailed characterization of PF-3845, a piperidine urea FAAH inhibitor that satisfies these criteria. Mechanistic and structural studies confirm that PF-3845 is a covalent inhibitor that carbamylates FAAH's serine nucleophile. Kinetic studies demonstrate that PF-3845 inhibits FAAH with a 10- to 20-fold higher *in vitro* potency compared to previously described inhibitors (URB597, PF-750). PF-3845 displays remarkable *in vivo* selectivity for FAAH as determined by activity-based protein profiling and raises brain anandamide levels for up to 24 hrs, resulting in profound cannabinoid receptor-dependent anti-hyperalgesic effects in the complete Freund's adjuvant (CFA) model of inflammatory pain. PF-3845 has excellent pharmacokinetic properties including high oral bioavailability. These data thus designate PF-3845 as a valuable pharmacological tool for *in vivo* characterization of the FAAH-regulated endocannabinoid system.

# DOUBLE-BLIND, RANDOMIZED WITHDRAWAL CLINICAL TRIAL OF SATIVEX IN CENTRAL NEUROPATHIC PAIN IN MULTIPLE SCLEROSIS

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**Introduction:** Central neuropathic pain (CNP) in multiple sclerosis (MS) produces significant morbidity (prevalence 17-52%) and available medicines are only partially effective and often relatively toxic. Sativex® (Nabiximols) is an oromucosal cannabinoid spray that combines a $CB_1$ partial agonist (THC), alongside an endocannabinoid system modulator (CBD) with analgesic and anti-inflammatory properties. Sativex is indicated in Canada for symptomatic relief of neuropathic pain in MS, and for treatment of cancer pain unresponsive to optimized opioid therapy. Previous studies of Sativex in CNP have demonstrated efficacy in both a short-term 5-week study (Rog et al. *Neurology* 2005) and in an open-label Safety-Extension study of greater than 1 year (Wade et al. *Multiple Sclerosis* 2006).

**Methods:** A 12-week randomized double-blind placebo-controlled clinical trial of Sativex in 339 patients with MS demonstrated that over 50% of subjects achieved a greater than 30% decrement in CNP over baseline, but this effect was obscured by an unusually high placebo response. A subset of 53 subjects continued onto a subsequent 12-week open label study from which 42 were re-randomized to administer an identical number of daily sprays of either Sativex or placebo without further dose titration for an additional 4 weeks (i.e. a 4-week randomized withdrawal period). The rate of treatment failure (worsening of pain by 20% or requirement to increase other analgesic medication) was the primary outcome measure.

**Results:** The mean number of sprays per day was 7.4 in the Sativex® group (N=21), and 8 for placebo subjects (N=21). The treatment failure rate significantly favored Sativex (24%) over placebo (57%) (p=0.04). Numerical rating scales (NRS) of pain for Sativex® subjects improved slightly (3.83 to 3.72, -3.5%), while those on placebo worsened (3.75 to 4.51, +23.7%) (p=0.028). Similarly, Neuropathic Pain Scale composite, scores improved on Sativex® by 3.3% (34.24 to 33.1) while placebo scores deteriorated by 19.2% (31.71 to 36.7), although this was not statistically significantly (p=0.15). Sleep disturbance NRS scores were stable on Sativex® (2.12 to 2.05, or -3.3%) while those on placebo deteriorated (1.66 to 2.59, or +56%) (p=0.015). Other symptom scores all favored Sativex, over placebo. During randomized withdrawal, 2 Sativex subjects experienced adverse events (depression, mucosal erosion), while 5 did on placebo (severe injury, increased LFTs, extremity pain, MS relapse, tremor, dry skin). Only 1 subject withdrew early due to relapse (placebo). No subjects who abruptly ceased Sativex administration experienced consistent withdrawal-type symptoms.

**Conclusion: Sativex was effective** in long-term treatment for CNP in MS and abrupt discontinuation produced deterioration in MS symptoms without withdrawal sequelae.

# EFFECT OF PHYTOCANNABINOIDS ON SUPRA-SPINAL ANTINOCICEPTIVE DESCENDING PATHWAYS IN RATS: INVOLVEMENT OF CANNABINOID, TRP AND ADENOSINE RECEPTORS

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The study of the descending control of pain in the brainstem dates back to 1962, when the gate hypothesis of pain was postulated with the periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) being key players. It was demonstrated that electrical stimulation of the PAG produces antinociception: PAG-induced nociception involves modulation of the activity of two main populations of RVM neurons projecting to the spinal dorsal horn: ON and OFF cells. ON cells are characterized by a burst of activity associated with nocifensor withdrawal reflexes. Their sustained activation produces hyperalgesia, whereas reduction in their threshold of cell burst is associated with a decrease in reflex latency. OFF cells are defined by a pause in firing associated with withdrawal reflexes and are generally thought to exert antinociception. Several receptors, including ionotropic and metabotropic glutamate, μ-opiod, $CB_1$ cannabinoid, A1 adenosine and TRPV1 receptors, are expressed in the VL-PAG and their activation inhibits pain perception. Mechanisms of action include disinhibition or stimulation of PAG output neurons projecting to the RVM and the resulting inhibition and stimulation, respectively, of ON and OFF neurons, with subsequent inhibition of nociceptive transmission in the spinal cord. We have studied here the effect of intra-VL-PAG injection of two phytocannabinoids that can act as direct or "indirect" agonists of $CB_1$ cannabinoid, A1 adenosine and/or TRP receptors, i.e. cannabidiol (CBD) and cannabichromene (CBC).

Drugs were administered into the VL-PAG and ON and OFF cells were identified by the characteristic OFF cell pause and ON cell burst of activity just before tail flick responses (Fields et al. 1983; Xu et al., 2007) in the RVM. To monitor the antinociceptive effects, tail flicks were elicited every 4-5 min for at least 15-20 min prior to microinjecting drugs, the respective vehicle, or their combination.

CBD (1.5, 3 and 6 nmol) inhibited the tail flick response with a bell-shaped dose-response curve, the maximal effect being observed at 3 nmol)., and at the same time inhibited paradoxically both ON and OFF cell activity. The effects CBD (3 nmol) were antagonised partially by the TRPV1 antagonist, I-RTX (1 nmol) and fully by the TRPA1 antagonist, AP18 (6 nmol), the $CB_1$ antagonist, AM251 (0.5 nmol), and the A1 antagonist, DPCPX (0.05 nmol). CBC (6 nmol) also elicited similar effects in a way antagonized by AP18 (12 nmol) and AM251 (0.5 nmol).

These findings indicate that CBD and CBC stimulate descending antinociception by unusual multi-receptor-mediated mechanisms leading to inhibition of both ON and OFF cell activity. Therefore, phytocannabinoids can exert analgesic actions at both peripheral, spinal and supra-spinal levels.

This work was partly supported by GW Pharmaceuticals.

# SYNTHESIS AND BIOLOGICAL EVALUATION OF ANALOGUES OF THE $CB_1$ ANTAGONIST N-PIPERIDINYL-5-(5-CHLORO-THIOPHEN-2-YL)-1-(2',4'-DICHLOROPHENYL)-4-METHYL-1*H*-PYRAZOL-3-CARBOXAMIDE

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As shown in our Patent Application US2005/0261281, N-piperidinyl-5-(5-chloro-thiophen-2-yl)-1-(2',4'-dichlorophenyl)-4-methyl-1*H*-pyrazol-3-carboxamide and several analogues have proved to be new ligands for $CB_1$ receptors. Attempting to improve the $CB_1$ selectivity inside this class of derivatives, we have synthesized a series of analogue compounds changing the halogen atom on the thienyl or on the phenyl substituent in position 1 or 5 of the pyrazole ring, respectively. Moreover, the replacement effect of the 5-(5-halo-2-thienyl) with an appropriate 5-(5-aryl-2-thienyl) moiety has been assayed (Figure 1).

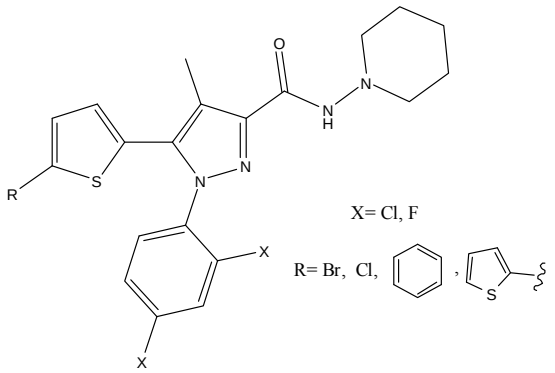


***Figure 1***

Suzuki reaction was adopted to convert the 5-(5-halo-2-thienyl)-substituted derivatives to the corresponding aryl-thienyl compounds.

The receptor profile towards both CB1 and $CB_2$ receptors was determined through radioreceptor binding assays according to the procedure reported by Ruiu et al. in *J.P.E.T.,* 2003, 306, 363−370.

Potency and activity of the synthesized compounds were evaluated by *ex vivo* assays (isolated organs) using the method described by Pertwee et al. (*Br. J. Pharmacol.*, 1993, 110, 1483–1490) on mice vas deferens. Organs were exposed to cumulative increasing concentrations of WIN 55,212-2 to obtain concentration-response curves either in the absence (control) or in the presence of the cannabinoid antagonit compounds to be assayed added at a fixed concentration 20 min before the first concentration of WIN 55,212-2.

Significant effects were highlighted on $CB_1$ receptor affinity due to the different adopted substitutions. In addiction, a marked $CB_1$ selectivity was evidenced through the replacement of the halogen atom with an aryl (phenyl or thienyl) substituent on the thienyl in 5 position of the pyrazole ring.

# SYNTHESIS OF GEMINALLY DIMETHYL SUBSTITUTED ANALOGUES OF $\Delta^8$-THC

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It is known that a *gem*-dimethyl group appended to C-1 of the alkyl side chain of a traditional cannabinoid, such as $\Delta^8$-THC, enhances $CB_1$ receptor affinity and *in vivo* potency. This effect is mirrored in the $CB_1$ and $CB_2$ receptor affinities of 1-methoxy and 1-deoxy-$\Delta^8$-THC analogs (Huffman *et al. Bioorg. Med. Chem.* **1999**, *7*, 2905; **2002**, *10*, 4119). Several years ago we reported the synthesis and pharmacology of the methylheptyl analogs of $\Delta^8$-THC and found that a methyl substituent at C-1' to C-3' enhanced $CB_1$ receptor affinity relative to 3-heptyl-$\Delta^8$-THC, while substitution at C-4' or C-6' had little effect, but a C-5'-methyl group attenuated $CB_1$ receptor affinity (Huffman *et al. Bioorg. Med. Chem.* **1998**, *6*, 2383). To continue the investigation of the effect of side chain substitution on $CB_1$ receptor affinity in the $\Delta^8$-THC series and the possible development of new $CB_2$ selective ligands we have instituted a program to prepare 2', 2'- to 6', 6'-dimethylheptyl-$\Delta^8$-THC and the corresponding 1-methoxy and 1-deoxy analogues.

Currently, the synthesis of 2', 2'-, 4', 4'- and 6', 6'-dimethylheptyl-$\Delta^8$-THC and their 1-methoxy and 1-deoxy analogues has been completed and the receptor affinities have been determined. 2', 2'-, 4', 4'- And 6', 6'- dimethylheptyl-$\Delta^8$-THC all have high affinity for the $CB_1$ receptor ($K_i$ = 1.5 ± 0.3, 10.3 ± 0.7 and 9.4 ± 1.2 nM, respectively). These compounds have comparable affinities for the $CB_2$ receptor. None of the corresponding 1-methoxy-dimethyl compounds have measurable affinity for the $CB_1$ receptor. The 1-methoxy-2', 2'-dimethyl analog has modest affinity for the $CB_2$ receptor ($K_i$ = 120 ± 12 nM), but the 4', 4'- and 6', 6'- isomers have little $CB_2$ receptor affinity ($K_i$ = 550 ± 46 and 1368 ± 53 nM, respectively). None of the 1-deoxy analogs have significant affinity for the $CB_1$ receptor, however 1-deoxy-2', 2'-dimethylheptyl-$\Delta^8$-THC has moderate affinity for the $CB_2$ receptor ($K_i$ = 76 ± 1 nM). The 4', 4'- and 6', 6'-dimethyl isomer have little affinity for the $CB_2$ receptor ($K_i$ = 887 ± 31 and 479 ± 42 nM, respectively).

The chemical synthesis and pharmacological evaluations of other compounds in this series is in progress. In conclusion it is apparent that a 3-(1', 1'-dimethylheptyl) substituent imparts enhanced affinity for the $CB_2$ receptor in the 1-methoxy and 1-deoxy $\Delta^8$-THC series, while a *gem* dimethyl substituent at the 2', 4' and 6'-position greatly attenuates affinity for this receptor.

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# LIPINS, CANNABINS AND ENCANNINS: TOWARDS A RATIONAL NOMENCLATURE

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As cannabinology evolves into a specialty, chronologically accurate nomenclature will facilitate education.

Labeled THC led to the discovery of the first cannabinoid receptor / CB1, which then led to the discovery of the ligand anandamide (AEA), thereby creating the core elements of the Endogenous Cannabinoid System (ECS).

The serpentine receptor, with its critical role in extra-cellular and then inter-cellular communication, originated in members of the Kingdom Archea, which appeared 4.2 billion years ago. The architecture of this receptor has been conserved over four billion years and now serves as the backbone of the protein receptors that signal, target, and modulate cell physiology from proliferation through apoptosis.

Depending on the evolutionary timeline of the hominids and their Sapiens sapiens branch, the Endogenous aspect of the ECS is one to two million years old. The Cannabinoid component of the ECS first appeared 34 million years ago, while the 'System' part of ECS first arose with the need for extra-cellular autocrine regulation billions of years ago.

The use of membrane lipids to construct 'on the fly' autocrine lipid messengers laid the groundwork for the advent of early paracrine-like exchanges, essential to the evolution of symbiotic associations. Subsequent incorporation of one symbiotic member by another, exemplified by Geosiphon, an Endo-symbiont, is a lipin-mediated process responsible for the structural complexity of today's eukaryotes. Lipins were essential in the evolution of multi-cellular life forms hundreds of millions, if not a billion, years before Cannabis, the vascular plant bearing exogenous cannabinoids, first appeared.

Endorphins, now a household term, is distinct for most laypersons from its progenitor Endogenous Morphine. Similarly, Encannins may someday stand alone from Cannabis. Encannins will give the medical student a contraction of Endogenous Cannabinoids, which will allow them to more easily converse with cannabiphobic patients. Cannabinology, the basis of a new medical specialty, will benefit from a language consistent across the 4 billion year old evolution of the membrane synthesized messengers, from Archea's autocrine to the eukaryotic paracrine modulators.

In summary, the proposed chronological nomenclature includes, **Lipins**, those membrane messenger molecules, which appeared 4 billion years ago. **Cannabins,** i.e. the Exogenous Cannabinoids, that arose 34 million years ago; and are the product of lateral evolution mediated by host-plasmid bearing broad host viruses between vascular plants and a variety of animals. **Encannins,** or Endogenous Cannabinoids, which were last on the scene, appearing only 1 to 2 million years ago.

# ISOTHIAZOLYLIDENE AMIDES: A NEW SERIES OF SELECTIVE $CB_2$ AGONISTS FOR PAIN MANAGEMENT

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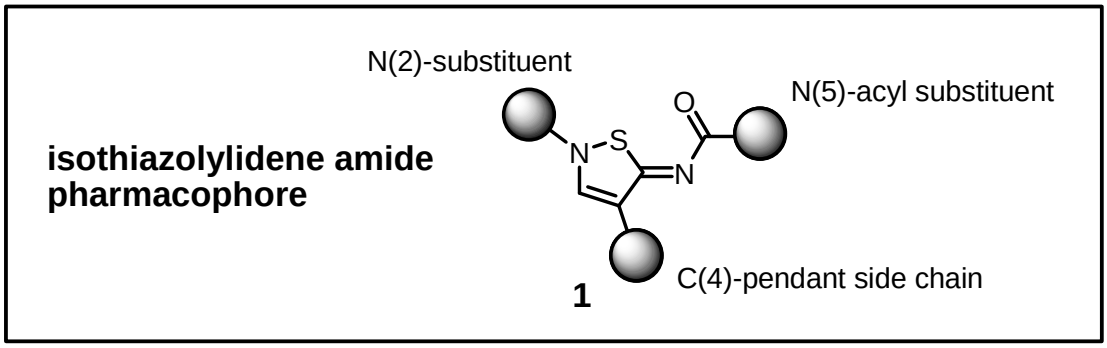
**Introduction:** Research targeting $CB_2$ receptor agonists for the treatment of pain continues to gain increased attention. Highly selective $CB_2$ agonists are anticipated to minimize $CB_1$-mediated CNS events such as sedation, euphoria, and appetite stimulation that have plagued the clinical utility and development of non-selective cannabinoid agonists for managing pain. Here we describe the design and initial SAR studies of a new series of selective $CB_2$ agonists based on a novel isothiazolylidene amide core (**1**).

**Methods**: A variety of isothiazolylidene amide derivatives were synthesized and characterized in assays to evaluate cannabinoid receptor binding affinity, selectivity, and agonist activity. Select compounds were assessed for in vivo efficacy in rodent pain models.

**Results:** SAR investigations on the isothiazolylidene pharmacophore, including modification of the acyl amide moiety and C(4)-pendant side chain, provided numerous ligands with high affinity for the $CB_2$ binding site. Several analogs possess high levels of selectivity (>1000-fold) for the $CB_2$ versus the $CB_1$ receptor as determined in adenylyl cyclase functional assays. Medicinal chemistry optimization led to the identification of the $CB_2$-selective agonist A-1036654, which exhibits in vivo efficacy in multiple rodent models of nociceptive and neuropathic pain.

**Conclusion:** We have designed, synthesized and characterized a series of selective $CB_2$ agonists based on a novel isothiazolylidene amide pharmacophore. Several highly selective isothiazolylidene $CB_2$ ligands have been identified, including the potent agonist A-1036654 that possesses robust efficacy in animal pain models.



# THIOPHENE BISAMIDE $CB_2$ AGONISTS

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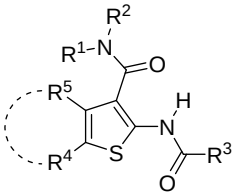
*Introduction*. The cannabinoid G-protein coupled receptor (GPCR) family consists of two well-characterized subtypes. Cannabinoid 1 ($CB_1$) receptors exist primarily in the central nervous system (CNS) and also in peripheral tissues. Activation of $CB_1$ receptors produces a variety of effects, including sedation, euphoria, and appetite stimulation. Cannabinoid 2 ($CB_2$) receptors are expressed peripherally in tissues associated with the immune system. Activation of $CB_1$ or $CB_2$ receptors can produce analgesia, but agonists that lack selectivity for $CB_2$ over $CB_1$ cause the undesirable effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) in marijuana. Abbott initiated a program to pursue $CB_2$-selective agonists for pain management, and here we report a new series of $CB_2$ agonists.

*Results*. A high-throughput screen (HTS) of the Abbott compound collection yielded several $CB_2$ agonists containing the thiophene bisamide pharamacophore. Medicinal




chemistry efforts were initiated to determine the structural features required for ligand affinity and cannabinoid receptor function. New compounds were evaluated using competitive radioligand binding methods as well as *in vitro* functional assays. The assays utilized recombinant cannabinoid receptors from human and rat expressed in cells devoid of endogenous cannabinoid function. Activity at $CB_2$ and $CB_1$ were determined to ascertain selectivity levels required for separation of analgesic effects from side effects. Selected compounds advanced to pharmacokinetic (PK) studies in rats and behavioral pain models. Compound A-889419 emerged as a lead and demonstrated the analgesic potential of the series. The selectivity profile and PK properties of A-889419 limited development, but structure-activity relationship (SAR) studies around the thiophene pharmacophore addressed some of these limitations. Examples of thiophene bisamides with enhanced metabolic stability and/or improved selectivity and pharmacokinetics include A-939766 and A-968880.

*Conclusion*. Thiophene bisamide derivatives can exhibit high levels of affinity for the $CB_2$ receptor and function as agonists. Selectivity for $CB_2$, as well as ADME and PK profiles, can be modified by minor structural variation of the pharmacophore. Several examples of this structural series demonstrate efficacy in behavioral pain models.

# SYNTHESIS AND PHARMACOLOGICAL CHARACTERIZATION OF QUATERNIZED SR141716 ANALOGS THAT ANTAGONIZE CANNABINOID RECEPTOR 1

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**Introduction:** Peripherally expressed endocannabinoid receptors, particularly the CB1 receptor has recently emerged as a promising candidate for targeted therapy in liver fibrosis and other disorders. The majority of CB1 receptor antagonists that are currently available are unsuitable for use in disorders with peripheral CB1 receptor involvement due to their deleterious effects on the central nervous system (CNS). The goal of these studies was to rationally design, synthesize and test CB1 receptor antagonists for peripheral selectivity, thereby eliminating problems associated with inhibition of CB1 receptors expressed in the CNS.

**Methods:** Peripheral selectivity of a compound is dictated by its ability to cross the blood-brain barrier (BBB). In general, charged compounds do not cross the BBB, unless transported by specific transporters, whereas neutral lipophilic molecules (e.g. SR141716) can often penetrate the BBB by simple diffusion. Size of the compound is also a determinant of permeability across the BBB. With these factors in mind, several analogs of SR141716 were synthesized. The types of compounds synthesized include quaternary salts of SR141716 at the hydrazide position and unquaternized SR141716 analogs bearing aminoalkyl chains. A Gα16-coupled intracellular calcium mobilization assay was used to functionally characterize these compounds. Finally, abbreviated pharmacokinetic (PK) evaluation of one of the compounds was performed in rats to determine its distribution in plasma and brain following intraperitoneal injection.

**Results:** Several of the synthesized analogs inhibited CB1 receptor function at pharmacologically relevant concentrations (Ke < 10 μM). Some of the unquaternized aminoalkyl-linked SR141716 analogs had Ke < 200 nM. In general, introduction of charge or an aminoalkyl chain at the hydrazide position of the parent compound decreased activity. Most interestingly, the quaternized SR141716 analog that was tested in PK studies was excluded from the brain and had a half-life of ~30 minutes.

**Conclusions:** Analogs of SR141716 bearing aminoalkyl linkers or quaternized at the hydrazide position were synthesized and tested. Many of these compounds were pharmacologically relevant. Further, PK studies were conducted with a quaternized analog that indicated exclusion of the test compound from the CNS. Studies will be conducted in the future to synthesize and test additional peripherally selective analogs of SR141716 for antagonism towards CB1 receptor and activity in appropriate disease models.

**DEVELOPMENT OF BIVALENT SR141716 LIGANDS FOR CB1 RECEPTOR DIMERS**

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The CB1 receptor belongs to the G protein-coupled receptors (GPCRs) super family, the largest class of cell surface receptors. While GPCRs were traditionally described as monomeric, it is generally accepted that many of these receptors including the CB1 receptor can form functional homodimers, heterodimers or even oligomers. More importantly, GPCR dimers display unique pharmacological properties and represent novel targets for medication development mediated by these receptors. The importance of GPCR dimerization and oligomerization *in vivo*, however, remains to be exploited and appreciated, largely due to a lack of selective pharmacological tools. Bivalent ligands, defined as two pharmacophores linked by spacers, represent a unique and promising approach to probe the function and mechanism of receptor dimers. This approach is based on the premise that bivalent ligands will selectively bind with greatly enhanced affinity to ligand recognition sites on heterodimers or oligomers due to the formation of thermodynamically stable complexes. A series of bivalent ligands, composed of two SR141716 units linked by alkylamine spacers of various lengths, were synthesized. For comparison, the corresponding monovalent controls were also prepared. The affinities of these bivalent ligands at CB1 and CB2 receptors were determined using competitive radioligand binding assays. Their functional activities were determined using GTPγS binding and intracellular calcium mobilization assays. In vivo activity of a select number of compounds was determined in a tail-flick assay. Most compounds demonstrated Ki or Ke values that were pharmacologically relevant in the nanomolar range. The data also suggested that both the nature of the linker and its length were crucial factors for optimum binding and activity. Finally, selected compounds could attenuate the antinociceptive response to CP55940 in mice following intraperitoneal injection as measured in the tail-flick assay. These novel compounds will serve as the basis to better understand the function and mechanism of receptor dimerization and oligomerization and further elucidate their physiological roles.

# LIPID MEMBRANE STRUCTURE OF THE CANNABINOID $CB_1$ RECEPTOR SECOND EXTRACELLULAR LOOP

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Structural determination of the 18-residue second extracellular loop (E2) of the brain cannabinoid ($CB_1$) receptor is quite challenging due to its conformational flexibility, which is beyond the limit that any theoretical approach can handle (de Bakker et al., *Proteins,* **2003**, 51:21–40). Taking advantage of the E2 residue boundary accurately defined by the recently determined helical bundle structure of the $CB_1$ receptor (Shim, *Biophys. J*. **2009**, 96:3251–3262) and an α-helical segment in the middle of E2 suggested by reliable secondary prediction programs, the simulated annealing (SA) simulations were performed for an extensive conformational sampling of E2 in an environment mimicking the physiological condition (i.e., lipid bi-layer). After clustering analysis and the molecular dynamics (MD) simulation refinement of 166 energy-minimized, diverse E2 conformations, the final four E2 conformations were classified into three distinct classes **A**, **B**, and **C**, with respect to the position of the E2 helical segment Among these conformations, conformer **a1**, whose E2 helical segment is parallel to the membrane surface, was selected as the best E2 conformation in consideration of the degree of the molecular interactions not only between the residues within E2 but also between E2 and other parts of the receptor. It is shown that E2 of the $CB_1$ receptor, without having the covalently bonded disulfide linkage tied to the transmembrane (TM) helix 3 (H3) but with the presence of the intra-loop $H_{E2}$ in amphipathic alignment, maintains domain coupling to the helical bundle through an extensive salt bridge network as well as a network of aromatic stacking. Significantly reduced binding affinity of CP55940 by the D184A mutation (Murphy and Kendall, *Biochem. Pharmacol*. **2003**, 65:1623-1631) is successfully demonstrated by the MD simulation showing that interference of E2 by the D184A mutation likely to be remote to the ligand binding pocket affects ligand binding without direct contact to the ligand. In summary, the results obtained from the present study provide insight into the roles of E2 of the $CB_1$ receptor in stabilizing the receptor structure and the ligand binding pocket, presumably in a sequence-specific manner (Ulmschneider et al., *Protein Eng. Des. Sel*. **2005**, 18:563–570).

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# CONFORMATIONAL ANALYSIS AND MOLECULAR DYNAMICS STUDY OF 2-ARACHIDONOYL-sn-GLYCERO-3-PHOSPHOINOSITOL (2-AGPI) AND 1-STEAROYL-sn-GLYCERO-3-PHOSPHOINOSITOL (LPI) IN A POPC BILAYER

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GPR55 is a Class A G protein-coupled receptor that has been shown to be activated by cannabinoids (Henstridge et al. FASEB J. 2008). The receptor is expressed in several mammalian tissues including several regions of the brain. The Sugiura group has reported that GPR55 is also activated by LPI found in rat brain (Oka et al. BBRC, 2007), with 2-AGPI having the highest activity (Oka et al. *J Biochem* **2009,** 145, 13-20). Since 2-AGPI and LPI are found in lipid and are shown to interact with the membrane-embedded GPR55 receptor, we undertook a study of the location and conformations that 2-AGPI and LPI can adopt in a phospholipid bilayer.

Monte Carlo/simulated annealing (Conformational Memories) studies of 2-AGPI and LPI in distance dependent dielectric, chloroform and in water were undertaken producing 105 conformers each. Then, 2-AGPI and LPI were added to a fully hydrated, pre-equilibrated POPC bilayer (28 waters/lipid; 72 lipid molecules with 36 in each leaflet) and the behavior of each in POPC was studied using the NAMD2 molecular dynamics software package (NPAT ensemble; P = 1atm, T=310K) with the CHARMM27 parameter set including data for polyunsaturated lipids, and the TIP3P model for water.

The 2-AGPI distance dependent dielectric calculation yielded conformers with mainly a tight helical shape because the acyl chain tries to shield the charged headgroup. Their shape was affected by hydrogen bonds present between the inositol, phosphate and glycerol moieties. The chloroform calculation yielded more extended conformations than the distance dependent dielectric ones. Hydrogen bonding was not as prevalent and did not affect their shape. For the water calculation, few conformers had internal hydrogen bonds and the acyl chain shape was mainly extended with some L shaped chains. The LPI calculations yielded similar results as the 2-AGPI only the acyl chains were straighter in chloroform and water.

MD simulations showed that the 2-AGPI and LPI headgroups are in the lipid-water interface with either the inositol moiety buried in the headgroup region because of intramolecular hydrogen bonds or extended into water and solvated. Both the 2-AGPI and LPI acyl chains prefer more extended conformations in lipid but sample many other conformations.[Support: NIH DA023204 (M.E.A.) and DA021358 (P.H.R.)]

# IDENTIFICATION OF NOVEL HUMAN AND RODENT CB2 CANNABINOID RECEPTOR ISOFORMS AND THEIR DIFFERENTIAL REGULATION IN BRAIN

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Cannabinoids, endocannabinoids and marijuana activate two well-characterized cannabinoid receptors (CBRs), CB1-Rs and CB2-Rs. The expression of CB1 cannabinoid receptors (CB1-Rs) in the brain and periphery has been well studied but CB2-Rs have received much less attention than have CB1-Rs. The functional neuronal expression of CB2-Rs in the brain had therefore been ambiguous and controversial. Therefore, many features of CB2-R gene structure, regulation, and variation remain poorly characterized.

Isoform-specific TaqMan probes were designed to quantify human, mouse and rat CB2 isoforms in different tissues and brain regions as well as mice that were treated chronically with cannabinoid receptor agonists and antagonists. We found profound species differences of CB2 isoform tissue expression patterns and gene structures. Here, we report on the discovery of a novel human hCB2 gene promoter transcribing testis (hCB2A) isoform with the starting exon located ca 45 kb upstream from the previously identified promoter encoding the spleen isoform (hCB2B). The 5' exons of both hCB2 isoforms are untranslated 5'UTRs and alternatively spliced to the major protein coding exon of the hCB2 gene. hCB2A is expressed higher in testis and brain than hCB2B that is expressed predominantly in spleen and generally higher in other peripheral tissues than hCB2A except in testis. Species comparison found that the CB2 gene of human, mouse and rat genomes deviated in their gene structures and isoform expression patterns. Mouse mCB2A expression was increased significantly in the cerebellum of mice treated with the CB-R mixed agonist, WIN55212-2. This increase was also observed in the cerebellum of BTBR mouse strain that serves as an animal model for autism spectrum disorder. The promoter activities of mouse mCB2A and mCB2B were enhanced in the brain stem of mCB2 knock out mouse with the C-terminal 130 amino acid deletion. Rat rCB2 contains two different C-terminal intracellular domains as a result of alternative splicing that produces $rCB2_{410}$ and $rCB2_{360}$ isoforms. The 3' untranslated regions of mCB2 and rCB2 contain retroviral insertion sites that might change the expression of rodent CB2-R. These results provide much improved information about CB2 gene structure and its human and rodent variants that should be considered in developing CB2-R-based therapeutic agents.

# CANNABINOID RECEPTOR INTERACTING PROTEIN ($CRIP_{1a}$) ATTENUATES CONSTITUTIVE $CB_1$ RECEPTOR SIGNALING AND $CB_1$ DOWNREGULATION

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A recently discovered protein, Cannabinoid Receptor Interacting Protein ($CRIP_{1a}$) interacts with the C-terminal tail of the $CB_1$ receptor (aa 464-472) (Niehaus et. al. Molecular Pharmacology (2007) 72:1557-1566). To examine the effects of $CRIP_{1a}$ on $CB_1$ receptor function, this laboratory characterized HEK cells stably transfected with $CB_1$ ($hCB_1$-HEK) to cells with a stable co-transfection of $CRIP_{1a}$ ($hCB$-HEK-$CRIP_{1a}$). Co-transfection of $CRIP_{1a}$ had no effect on $CB_1$ expression levels in HEK cells stably expressing $CB_1$ receptor (1.87± 0.26 pmol/mg without $CRIP_{1a}$, 2.01 ± 0.29 pmol/mg with). Stoichiometric analysis revealed a $CRIP_{1a}/CB_1$ molar ratio of 0.28 ± 0.06 in $hCB_1$-HEK cells and 4.10 ± 0.32 in $hCB_1$-HEK-$CRIP_{1a}$ cells. In [$^{35}$S]GTPγS binding studies, $CRIP_{1a}$ co-expression significantly lowered $E_{max}$ values for the full agonist WIN 55,212-2 (WIN) (79 ± 2.4% with $CRIP_{1a}$, 111 ± 6.6% without). The $E_{max}$ value of methanandamide (MethA) was unaffected (80 ± 8.0% with $CRIP_{1a}$, 85 ± 8.4% without). Importantly, the inverse antagonism by SR141716A (SR1) was significantly reduced in the presence of $CRIP_{1a}$ (-7.3 ± 1.2% with $CRIP_{1a}$ versus -13.3 ± 1.6% without), in agreement with observances by Neihaus et al. $CB_1$ receptor desensitization and downregulation in response to agonist occupancy were measured by pre-incubating $hCB_1$-HEK cells (± $CRIP_{1a}$ co-transfection) with WIN, $\Delta^9$-tetrahyrdocannabinol (THC) or vehicle for 4 hours, followed by MethA-stimulated [$^{35}$S]GTPγS binding to assess receptor desensitization or [$^3$H]SR-141716A saturation analysis to quantify changes in receptor number. In $hCB_1$-HEK cells, both WIN and THC caused significant $CB_1$ receptor desensitization, but $CRIP_{1a}$ had no effect on this desensitization. Both ligands also produced significant downregulation of the $CB_1$ receptor. However, $CRIP_{1a}$ co-transfection attenuated receptor downregulation in the presence of WIN but not THC. Following WIN pre-treatment, $CB_1$ receptor $B_{max}$ values were 85.9 ± 22% of vehicle control in $hCB_1$-HEK-$CRIP_{1a}$ cells, compared to 48.2 ± 7.9% of vehicle control in $hCB_1$-HEK. [$^3$H]SR-141716A $K_D$ values were unaffected (data not shown), suggesting effective drug removal prior to assay. Because $CRIP_{1a}$ did not affect $CB_1$ receptor desensitization, but did affect downregulation, these results suggest that $CRIP_{1a}$ probably does not affect GRK-mediated phosphorylation or β-arrestin binding, but may sterically hinder GASP1 binding to the phosphorylated receptor. Future studies will address these potential actions of CRIP1a

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# LYSOPHOSPHATIDYLINOSITOL INDUCES RAPID PHOSPHORYLATION OF P38 MITOGEN-ACTIVATED PROTEIN KINASE IN HEK293 CELLS EXPRESSING GPR55

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GPR55 is a seven transmembrane, G protein-coupled receptor. Recently, we explored the endogenous ligand for GPR55 using HEK293 cells which express GPR55 and obtained evidence that lysophosphatidylinositol (LPI) is a possible endogenous ligand for GPR55 (*Biochem. Biophys. Res. Commun*., 362, 928-934, 2007). LPI induced rapid phosphorylation of ERK and a $Ca^{2+}$ transient in GPR55-expressing cells, but not in vector-transfected cells. In the present study, we examined the effects of LPI on p38 mitogen-activated protein kinase (p38 MAP kinase) in HEK293 cells expressing GPR55. LPI induced rapid phosphorylation of p38 MAP kinase in GPR55-expressing cells. The response was detectable at 2.5 min following stimulation and persisted at least for 20 min. The $EC_{50}$ was around 300 nM. No apparent effect was observed with vector-transfected cells. Lysophosphatidylcholine (LPC) and nonionic detergent SM-1200 did not affect the phosphorylation of p38 MAP kinase in these cells, suggesting that the effect of LPI is not attributed to a nonspecific detergent effect. The exposure of the GPR55-expressing cells to LPI also triggered the phosphorylation of activating transcription factor-2 (ATF-2), a downstream of p38 MAP kinase. The effect of LPI on the phosphorylation of ATF-2 in vector-transfected cells was negligible. Treatment of the cells with Y-27632 blocked the LPI-induced phosphorylation of both p38 MAP kinase and ATF-2, suggesting that the Rho-ROCK pathway is involved in both reactions. These results strongly suggest that GPR55 and its endogenous ligand LPI play some essential role in the homeostatic responses to stress signals in certain types of mammalian cells.

# THE COUPLING OF $CB_1$ RECEPTORS WITH BETA-ARRESTIN1 AND 2

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Previous studies in our laboratory have demonstrated that beta-arrestin2-/- mice are more sensitive than wild-type mice to the physiological and behavioral effects THC, while there appeared to be no difference between these genotypes in the effects other cannabinoid agonists like CP5540 or methanandamide. The purposes of the present studies were to find evidence for the biochemical basis for the agonist selective effects of beta-arrestin2 by: 1) characterizing relative amounts of beta-arrestin1 and beta-arrestin2 in various mouse brain regions, 2) determining if knocking out beta-arrestin2 affects beta-arrestin1 levels the same brain regions, and 3) assessing $CB_1$ receptor association with beta-arrestin1 and beta-arrestin2 in response to THC and CP55940.

Western blotting was employed to quantify the relative amounts of beta-arrestin1 and beta-arrestin2 protein within each of six (cerebellum, hippocampus, basal ganglia, brain stem, midbrain, and cortex) mouse brain areas. This is the first demonstration of relative levels of beta-arrestin1 and 2 in mouse brain. We found that beta-arrestin1 was highest in cortex and hippocampus and beta-arrestin2 was highest in cortex. Thus, subsequent co-immunoprecipitation experiments were carried out using mouse brain cortex. Comparisons were made within the same brain regions between wild type and beta-arrestin2 knockout samples to determine whether absence of beta-arrestin2 influenced levels of beta-arrestin1. There did not appear to be any physiological compensatory production of beta-arrestin1 in beta-arrestin2-/- mice.

Finally, immunoprecipitation of $CB_1$ from CHAPS-solubilized cortex membrane homogenates was used to determine association of beta-arrestin1 and 2 with $CB_1$ receptors from mice treated with vehicle, THC or CP55940. Experiments are ongoing, but so far it has been observed that beta-arrestin1 does associate with $CB_1$. The data are too preliminary at this point to determine the association of beta-arrestin2 with $CB_1$ whether this association is affect by agonist treatments.

# CANNABINOID RECEPTOR INTERACTING PROTEIN (CRIP) 1A: DEVELOPMENT OF OVEREXPRESSING NEURONAL CELLS AND ADENO-ASSOCIATED VIRAL DELIVERY OF CRIP1A TRANSCRIPTS IN VIVO

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$CB_1$ cannabinoid receptor are coupled to $G_{i/o}$ proteins, and activation leads to inhibition of adenylyl cyclase, regulation of the ERK/MAPK pathway, inhibition of specific $Ca^{2+}$ channels, and activation of G protein linked $K^+$ channels. The Cannabinoid Receptor Interacting Protein (CRIP1a) has provided a new avenue for modulation of the endocannabinoid system. CRIP1a binds to the distal portion of the C-terminal tail of $CB_1$, and has been shown to suppress the tonic inhibition of voltage-gated $Ca^{2+}$ channels induced by $CB_1$ [Niehaus et al., 2007 Mol. Pharmacol. 72:1557–1566]. Further studies need to be conducted in order to elucidate the mechanisms involved in CRIP1a modulation of $CB_1$ receptors. The focus of our work was to create plasmids and a viral vector that could overexpress and knock-down CRIP1a protein levels in vitro and in vivo.

Total mRNA from mouse cortex was isolated and converted to cDNA. CRIP1a primers were used for polymerase chain reaction (PCR) amplification of the CRIP1a cDNA from mouse brain. The coding region amino acid sequence from mouse brain was found to be similar to that reported for rat and human by Niehaus et al., 2007. In order to investigate the impact of CRIP1a overexpression in vitro, N18TG2 cells were transiently transfected with CRIP1a cDNA ligated into pACP-BHI or stably transfected with CRIP1a ligated into pcDNA3.1 (+). Tetrahydrolipstatin-treated (to block 2-arachidonoyl glycerol production) N18TG2 over-expressing CRIP1a were challenged with 1 μM WIN55212-2 or CP55940 for 5 minutes, resulting in an increase in ERK tyr-phosphorylation over basal. Decrease in ERK phosphorylation by rimonabant was used to quantitate the effects of transient expression of CRIP1a on $CB_1$-mediated constitutive activation of ERK phosphorylation in 96-well format in-cell-Westerns.

In order to investigate the impact of in vivo overexpression, CRIP1a cDNA was ligated into pACP-BHI, an adeno-associated virus (AAV) expression plasmid. The ligation products were transformed into E. Coli and positive colonies isolated using ampicillin selection, expanded and the resulting plasmid DNA purified. Cloning of CRIP1a-pACP plasmid was verified with restriction enzyme digestion, followed by separation on an agarose gel, as well as sequencing analysis. The CRIP1a-pACP plasmid was then used to package AAV10 viruses containing the CRIP1a overexpression transgene. The CRIP1a-AAV10 viral vector was unilaterally injected into rat striatum stereotaxically. Animals were sacrificed after 5 or 10 days and 1mm brain slices were taken at the site of injection. RNA was isolated and purified, converted to cDNA, and quantitative real-time PCR was performed for the following genes: 18s ribosomal RNA, Enolase 2, $CB_1$, CRIP1a, and $D_2$ dopamine receptors. In vivo, the AAV-CRIP1a virus increased levels of CRIP1a, but not $CB_1$ receptors. These studies confirm the feasibility of modulation of $CB_1$ receptors by CRIP1a both in vitro and in vivo.

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# EVIDENCE FOR A BIMATOPROST-SENSITIVE PROSTAMIDE RECEPTOR IN HUMAN OCULAR CELLS ASSOCIATED WITH CONVENTIONAL OUTFLOW

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Bimatoprost is widely used as an ocular hypotensive to treat glaucoma and lowers intraocular pressure in humans by increasing both uveoscleral outflow and conventional, pressure-dependent outflow. The clinical effects of bimatoprost on conventional outflow have been confirmed in organ culture utilizing the isolated human anterior segment preparation. The present studies extend previous investigations by studying those cells that control conventional, pressure-dependent aqueous humor outflow; namely trabecular meshwork (TM cells) and endothelial cells of Schlemm's canal (ECSC).

Studies involving Cellular Dielectric Spectroscopy (CDS) revealed that bimatoprost produced a concentration-dependent change in cell monolayer impedance for both TM and ECSC cells. Bimatoprost potency in TM cells was similar to that previously reported for human ciliary smooth muscle cells, isolated feline iris and lung parenchymal cells, and rabbit myometrium (i.e.: EC50=7.9nM). In ECSC cells, bimatoprost was even more potent ($EC_{50}$=2.4nM). The effects of bimatoprost in both cell types was completely inhibited by the prostamide antagonist AGN-211334. Bimatoprost effects were insensitive to cholera toxin and pertussis toxin but were abolished by phorbol 12-myristate 13-acetate; suggesting Gq-involvement. A cellular mechanism underlying bimatoprost effects on conventional outflow was confirmed in a model for the juxtacanalicular TM where bimatoprost significantly increased hydraulic conductivity by 75%. In summary, these studies provide evidence for prostamide receptor-mediation of bimatoprost effects on conventional outflow facility.

# SIGNALLING INTERACTIONS BETWEEN $CB_1$ RECEPTORS AND $\beta_2$ ADRENERGIC RECEPTORS IN HUMAN TRABECULAR MESHWORK CELLS

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Heterodimerization of G protein coupled receptors (GPCRs) affects many of the functional properties of these receptors. The $CB_1$ receptor forms these heterodimers with several other GPCRs including: $D_2$ dopamine receptors, μ, κ, and δ opioid receptors, orexin-1 receptors and $A_{2A}$ adenosine receptors. Recently, we have reported that when expressed in human embryonic kidney 293H cells, $CB_1$ will also heterodimerize with the $\beta_2$ adrenergic receptor ($\beta_2$AR), and that this interaction alters the trafficking and signalling of both $CB_1$ and $\beta_2$AR (Hudson and Kelly 2007 and 2008; 17th and 18th Annual Symposium on the Cannabinoids).

The present study examines possible functional consequences of $CB_1/\beta_2$AR heterodimerization in ocular trabecular meshwork cells. In the eye intraocular pressure (IOP) is maintained by the balance of aqueous humor production from the ciliary body and aqueous humor outflow through the trabecular meshwork. Increased IOP is the primary risk factor for glaucoma, a blinding eye disease. Pharmacological treatment of glaucoma involves the reduction of IOP, either by decreasing aqueous humor production or by increasing outflow. Trabecular meshwork cells endogenously express both $CB_1$ and $\beta_2$AR and drugs targeting both $CB_1$ (agonists) and $\beta_2$AR (antagonists) are known to reduce IOP *in vivo*.

Interactions between the ERK signalling pathways of $CB_1$ and $\beta_2$AR were assessed in primary human trabecular meshwork cells (HTMCs). Treatment of HTMCs with the cannabinoid agonist WIN 55,212-2 (WIN) produced an initial increase in ERK phosphorylation (pERK) (5-15 min treatment) that was blocked by the $CB_1$ inverse agonist AM251 and by PTx. The initial WIN-induced increase in pERK was followed by a prolonged reduction in pERK (45-60 min treatment) that was unaffected by AM251. In contrast, treatment with the non-selective β-AR agonist isoproternol (ISO) resulted in an immediate and prolonged decrease in pERK (5-60 min treatment), which was blocked by the $\beta_2$AR selective antagonist ICI 118,551. Co-application of WIN and ISO produced an inhibitory effect on the initial WIN mediated increase in pERK, but had no additive effect on the prolonged WIN mediated decrease in pERK. AM251 enhanced the ability of ISO to inhibit pERK in HTMCs, while the $CB_1$ neutral antagonist O-2050 did not.

These results demonstrate that HTMCs endogenously express both $CB_1$ and $\beta_2$AR and that activation of these receptors results in pERK signalling responses unique to these cells. In addition, interactions between $CB_1$ and $\beta_2$AR pERK responses were observed, suggesting a potential role for $CB_1/\beta_2$AR heterodimerization in HTMCs that may be relevant to the use of cannabinoid and adrenergic drugs in the treatment of glaucoma.

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# EXPLORING THE ROLE OF NOVEL CANNABINOID RECEPTORS IN BREAST CANCER METASTASIS

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We have conducted preliminary investigations into expression levels of various putative novel cannabinoid receptors in human breast cancer cell lines. Using quantitative RT-PCR, we have shown that these receptors are expressed in the highly metastatic MDA-MB-231 cell line, with lower levels of expression in the non-invasive MCF-7 cell line. Further investigations into the role of these receptors in migration and invasion have also been conducted. Agonists and antagonists of these receptors both enhance and inhibit migration, respectively. Furthermore, over-expression studies in MCF-7 cells have shown these receptors to be involved in the invasive potential of breast cancer cells. Taken together, our data suggest that certain orphan receptors, which are activated by various cannabinoid ligands, may be involved in human breast tumour migration and invasion.

# NEUROPROTECTIVE EFFECTS OF PALMITOYLETHANOLAMIDE (PEA) TREATMENT ARE MEDIATED BY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR)-ALPHA

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Endocannabinoids like 2-arachidonoylglycerol strongly modulate the complex processes of neuronal damage and are shown to improve neuronal survival after excitotoxic lesion. Palmitoylethanolamide (PEA), the naturally occurring fatty acid amide of ethanolamine and palmitic acid, is an endogenous lipid known to mimic several effects of endocannabinoids even without binding to classical cannabinoid receptors CB1 and CB2. Here we administered PEA (0.001µM-1µM) and the synthetic peroxisome proliferator-activated receptor (PPAR)-alpha agonist 4-chloro-6-(2, 3-xylidino)-2-pyrimidinylthio acetic acid (Wy-14,643; 0.1µM-1µM) to excitotoxically lesioned organotypic hippocampal slice cultures (OHSCs) and determined the number of degenerating neurons and microglial cells in the dentate gyrus. Both substances reduced number of microglial cells and degenerated neurons indicating that PEA as well as Wy-14,643 protected granule cells from excitotoxic lesion. Treatment with the PPAR-alpha antagonist N-((2S)-2-(((1Z)-1-Methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl)propanamide (GW6471; 0.05µM-5µM) blocked PEA-mediated neuroprotection and reduction of microglial cell numbers. In contrast, the PPAR-gamma antagonist 2-chloro-5-nitro-N-phenyl-benzamide (GW9662; 0.01µM-1µM) did not reverse PEA mediated neuroprotective effects. In conclusion our data provide evidence that 1) PEA counteracted excitotoxically induced secondary neuronal damage and 2) PPAR-alpha but not PPAR-gamma is the endogenous target for PEA-mediated neuroprotection.

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# ALTERATIONS IN CB1 RECEPTOR NEURONAL POLARITY AND AGONIST-INDUCED INTERNALIZATION DURING MATURATION *IN VITRO*

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The CNS actions of cannabis and related compounds (cannabinoids) are thought to be primarily mediated by the CB1 subtype of cannabinoid receptor (CB1R). As one of the main physiological actions of CB1 receptors is the presynaptic regulation of neurotransmitter release, the correct trafficking of CB1 receptors to the axonal surface is a critical cellular event. Recently we have demonstrated that this process reflects constitutive, domain-specific endocytosis (McDonald et al., Mol. Pharm., 71, 1-9, 2007), with somatodendritic receptors being internalized more rapidly than those on the axonal plasma membrane leading to a net accumulation of cell surface CB1Rs on the axon. In this study we have used immunocytochemical methods and confocal imaging to study in more detail developmental changes agonist-induced trafficking and the cellular localization of native CB1Rs in cultured hippocampal neurons. We find that, as expected, surface receptor expression in mature neurons (1-2 weeks in culture) is highly polarized towards the axon. However in immature neurons (1-3 days in culture) receptor expression is much less polarized, with pronounced surface labelling observed within the somatodendritic domain. In addition, agonist-induced CB1R internalization is evident in the somata of younger neurons, with marked effects observed following 30 min treatment with the cannabinoid agonist O2545 (100 nM). In mature cultures, agonist-induced endocytosis was less pronounced and primarily localised to axonal structures at 30-60 min. Our data therefore suggests that there is a clear change in CB1R polarity and in the characteristics of agonist-induced endocytosis during maturation in culture. Given that CB1R activation can affect a variety of cellular process, including neurite outgrowth and transmitter release, alterations in CB1R surface expression during development are likely to be an important factor in defining the cellular response to CB1R activation in neurons.

# DISCRIMINATIVE STIMULUS FUNCTIONS OF METHANANDAMIDE AND $\Delta^9$-THC IN RATS: TESTS WITH AMINOALKYLINDOLES (WIN55,212-2 AND AM678) AND ETHANOL

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*Introduction*: To characterize *in vivo* the aminoalkylindoles (AAI) WIN55,212-2 and AM678 [(naphthalene-1-yl(1-pentyl-1*H*-indol-3-yl)methanone] as cannabinoid receptor ($CB_1R$) ligands using drug discrimination (DD). Tests also involved $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and R-(+)-methanandamide (mAEA), a metabolically stable analog of anandamide (AEA), as well as the $CB_1R$ antagonist/inverse agonist rimonabant; ethanol was examined to assess pharmacological specificity.

*Methods:* There were three concurrently trained groups of rats. One group was trained to discriminate between ip injected vehicle and 10 mg/kg mAEA. The other groups discriminated between vehicle and $\Delta^9$-THC (1.8 mg/kg and 3 mg/kg).

*Results:* Dose generalization curves for AM678, WIN55,212-2, $\Delta^9$-THC and mAEA suggested the following rank order of potency: AM678 > WIN55,212-2 ≈ $\Delta^9$-THC > mAEA (the latter ligand only examined in the mAEA trained rats) in all three DD groups. Challenge by 1 mg/kg rimonabant, a selective $CB_1R$ antagonist, resulted in shifts to the right of the dose generalization curves for WIN55,212-2 and AM678, suggesting surmountable antagonism. Ethanol did not generalize in all DD groups tested, suggesting pharmacological specificity.

*Conclusion:* Data are congruent with the general observation that there is substantial overlap in the discriminative stimulus effects of $CB_1R$ ligands across different chemical classes. However, differential quantitative interactions between the two AAI's and rimonabant in the DD groups might point to subtle variations in the ligand-receptor activation(s).

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# THE PUTATIVE ENDOTHELIAL CANNABINOID RECEPTOR ANTAGONIST, O-1918 INHIBITS VASCULAR LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS

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Evidence suggests that a novel cannabinoid receptor ($CB_x$) in the endothelium plays a role in the relaxant effects of endocannabinoids in some vascular regions, including mesenteric arteries. Whilst the molecular identity of $CB_x$ is yet to be defined, it has been partially characterised in pharmacological studies. In particular, O-1918 is thought to be a $CB_x$ antagonist. $CB_x$ has also been linked to endothelial release of nitric oxide and activation of large conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) in the vascular smooth muscle by nitric oxide-dependent and -independent mechanisms. This study aimed to examine the effects of O-1918 on relaxation induced by nitric oxide and activation of $BK_{Ca}$ in mesenteric arteries.

Male Wistar rats (200-350g) were killed by cervical dislocation. The third-order branches of the superior mesenteric artery (2mm long) were mounted in a wire myograph and maintained at 37$^o$C in gassed (95% $O_2$/5% $CO_2$) Krebs-Henseleit solution. The endothelium was removed by rubbing the intima with a human hair and ≤10% carbachol-induced relaxation indicated successful removal. Relaxations to cumulative addition of a $BK_{Ca}$ activator to vessels precontracted with methoxamine (3-10μM) were expressed as mean±s.e.m (n≥4 rats) and analysed by two-way analysis of variance of the whole data set. Inhibitors were incubated with vessels for 30min before determination of a concentration-response curve. $P<0.05$ was considered statistically significant.

In endothelium-denuded vessels, concentration-dependent relaxation to the nitric oxide donor, sodium nitroprusside (SNP) was attenuated by O-1918 (control: $pEC_{50}$=7.0±0.2, $E_{max}$=90±3%; + 3μM O-1918: $pEC_{50}$=6.3±0.3, $E_{max}$=82±6%). SNP responses were similarly attenuated by the selective $BK_{Ca}$ inhibitor, iberiotoxin (+ 50nM iberiotoxin: $pEC_{50}$=6.0±0.5, $E_{max}$=63±7%) and the combined addition of O-1918 and iberiotoxin did not cause further inhibition (+ O-1918 + iberiotoxin: $pEC_{50}$=6.0±0.3, $E_{max}$=71±7%). Precontracting the vessels with 60mM KCl, which inhibits efflux through $K^+$ channels, also inhibited relaxations to SNP ($pEC_{50}$=5.8±0.4, $E_{max}$=72±6%). On the other hand, O-1918 had small or no effect on relaxations induced by the synthetic $BK_{Ca}$ activators, NS1619 or NS11021 (data not shown). It was noted that treatment with O-1918 or iberiotoxin, or both, potentiated contractions to methoxamine, consistent with O-1918 acting as an inhibitor of $K^+$ channels.

We conclude that O-1918 inhibits nitric oxide-induced activation of $BK_{Ca}$ in the smooth muscle of rat mesenteric arteries and that the inhibitory effect of O-1918 might depend on the mechanisms of activation of the $K^+$ channels. Thus, cautious interpretation is warranted where cannabinoid responses are sensitive to O-1918, especially if signalling through nitric oxide and/or $BK_{Ca}$ is implicated.

# FORCED SWIM EXPOSURE ELICITS RAPID ALTERATIONS IN CORTICOLIMBIC ENDOCANNABINOID CONTENT

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The endocannabinoid system has recently emerged as a vital component of the stress response in the brain and represents an appealing target for treatment of mood and anxiety disorders. Our laboratory has shown that global activation of the $CB_1$ receptor ($CB_1R$) elicits antidepressant-like behavior in the forced swim test, a preclinical tool used to assess the efficacy of novel antidepressant compounds. Moreover, microinfusion studies have revealed an antidepressant effect in this paradigm following infusions of a $CB_1R$ agonist directly into the hippocampus or the prefrontal cortex (PFC). These data suggest that $CB_1R$ activation in these corticolimbic regions promotes active coping responses to stressful stimuli such as forced swimming; however, the degree to which endocannabinoid content and $CB_1R$ binding are altered in response to forced swim exposure has yet to be elucidated. The forced swim test typically consists of two swim sessions; a 15-min pre-exposure session followed by a 5-min test session occurring 24 hours later. For the present study, four separate cohorts of rats were compared; a D1 basal group (no forced swim exposure), a D1 FST group (sacrificed immediately following the first 15-min exposure session), a D2 basal group (sacrificed just prior to the day 2 test session), and a D2 FST group (sacrificed immediately following the day 2 5-min test session). Endocannabinoid content was analyzed in the PFC, hippocampus, and amygdala and compared across all four groups, while $CB_1R$ binding and $CB_1R$-mediated GTPγS binding were compared between D1 and D2 basal groups only. In the PFC, forced swim exposure elicited a rapid decrease in anandamide (AEA) on both day 1 and day 2 relative to D1 basal controls. Furthermore, the D2 FST group exhibited significantly lower anandamide levels in the PFC compared to the D2 basal group, and there were no significant changes in 2-arachidonyl glycerol (2-AG) levels across any group in this region. Both $CB_1R$ binding and $CB_1R$-mediated GTPγS binding were significantly upregulated in the PFC 24 hours following forced swim exposure. In the hippocampus, a similar effect was found, such that forced swim exposure significantly reduced AEA content (with no change in 2-AG) on both day 1 and day 2, but relative to the basal D1 group only. $CB_1R$ binding was also increased in the hippocampus 24 hours after day 1 exposure, but with no significant changes in $CB_1R$-mediated GTPγS binding. In the amygdala, AEA was reduced only in the D2 FST group relative to the D2 basal group, and there were no significant differences in 2-AG levels or $CB_1$ receptor binding across these groups. Collectively, these data indicate that forced swim exposure induces a rapid decrease in AEA content in the PFC and hippocampus, coupled to a reduction in $CB_1R$ binding in these same regions.

# THE ROLE OF THE ENDOGENOUS CANNABINOID SYSTEM IN PERIPHERAL ANALGESIA

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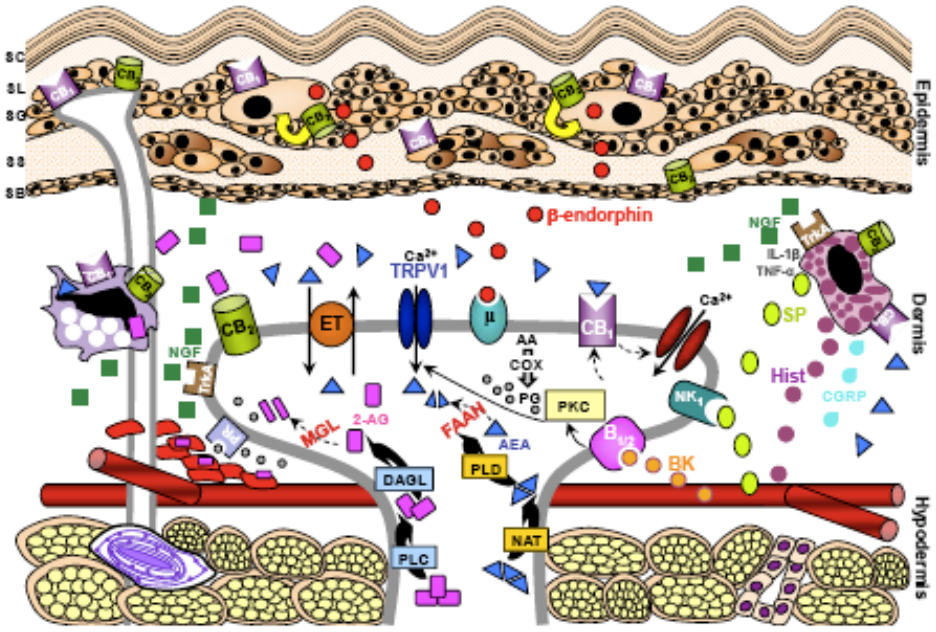
The therapeutic potential of cannabinoids has been studied and investigated through centuries, although many interesting discoveries have emerged from this field in the past decades. Indeed, peripheral analgesic effects of cannabinoids are a new avenue of treatment since they are avoiding the deleterious central side effects of systemic administration. Recently, it has been demonstrated that cannabinoid receptors (more specifically $CB_1$ and $CB_2$ receptors) and their endogenous ligands are present at the peripheral level, especially in the different layers of the skin, and mostly, in the epidermis and dermis. Those findings are reinforcing and confirming the efficacy of peripheral administration of cannabinoids used to alleviate pain in many different animal models. However, many studies have shown that the endocannabinoid system interacts with other receptors and pathways to modulate pain at the peripheral level. Thereof, the main goal of this review is to explain, in a better way, the different interactions regarding the cannabinoid system with other cellular components of its environment, its involvement in the modulation of pain at the peripheral level and, more precisely, in the different layers of the skin.



# VASOACTIVE ACTIONS OF ABNORMAL CANNABIDIOL AND 3-(5-DIMETHYLCARBAMOYL-PENT-1-ENYL)-N-(2-HYDROXY-1-METHYL-ETHYL)BENZAMIDE IN RETINAL ARTERIOLES

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**Purpose:** Cannabinoids have vasoactive actions in the eye, however, their therapeutic use is limited by behavioral side effects. Abnormal cannabidiol (Abn-CBD) and 3-(5-dimethylcarbamoyl-pent-1-enyl)-N-(2-hydroxy-1-methyl-ethyl)benzamide (VSN16) are non-psychotropic, atypical cannabinoids that produce vasodilation in some vascular beds and have many therapeutic benefits in treating eye diseases. The actions of atypical cannabinoids in the retinal vasculature are currently unknown; therefore we examined the pharmacology of Abn-CBD and VSN16 in isolated retinal arterioles.

**Methods:** Retinal arterioles were isolated from male Fisher-344 rats. Following enucleation, the retinas were removed in a low $Ca^{2+}$/high $Mg^{2+}$ solution (0.1 $CaCl_2$ and 8 $MgCl_2$) and triturated with a fire-polished Pasteur pipette (300 μm internal diameter). The isolated vessel fragments (25-30 μm) were allowed to adhere to a laminin-coated bath dish. The dish was superfused with Ringer's composed of (in mM): 145 NaCl, 5 KCl, 10 HEPES, 5 D-Glucose, 2 CaCl, 1 $MgCl_2$, pH 7.30 at 37°C. All drugs were diluted from stocks in Ringer's solution and applied to the isolated vessels via a 6-channel rapid switcher or included in the superfusate. Changes in arteriole diameter were measured with a video edge detector.

**Results:** Both Abn-CBD and VSN16 caused relaxation of endothelin-1 (ET-1) vasoconstricted arterioles. Abn-CBD treatment produced 43% vasorelaxation at 10 μM (n=10, p=0.0004). VSN16 treatment produced 42% vasorelaxation (n=7, p=0.002) at 10 μM, and 61% vasorelaxation at 20 μM (n=4, p=0.002). Both Abn-CBD and VSN16 mediated vasorelaxations were not inhibited by antagonists of cannabinoid 1 (CB1) or cannabinoid 2 (CB2) receptors. Abn-CBD-mediated vasorelaxation was completely blocked by O-1918 (108% inhibition, n=5, p=0.0003), an antagonist of the novel endothelial cannabinoid (CBx) receptor, and was reduced by L-NAME (31% inhibition, n=6, 100 μM, p=0.022), a nitric oxide synthase (NOS) blocker.

**Conclusions:** Abn-CBD and VSN16 produced vasorelaxation of ET-1 contracted retinal arterioles via activation of a vascular cannabinoid receptor(s) distinct from CB1 and CB2. The vasorelaxant properties of the behaviorally inactive cannabinoids, Abn-CBD and VSN16, may indicate therapeutic potential for treatment of retinopathies.

**Support:** CIHR

# BLOCKADE OF 2-AG HYDROLYSIS BY SELECTIVE MONOACYLGLYCEROL LIPASE INHIBITOR JZL184 ENHANCES RETROGRADE ENDOCANNABINOID SIGNALING

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Depolarization-induced suppression of excitation (DSE) and inhibition (DSI) are prominent forms of retrograde synaptic transmission. Although DSE and DSI require type I cannabinoid (CB1) receptor activation, the identity of the endocannabinoid (eCB) involved in DSE and DSI has not been firmly established. The eCB system has at least two endogenous ligands, *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). AEA and 2-AG are degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. Previous studies have shown that selective FAAH inhibitor URB597 does not affect DSE/DSI, suggesting that AEA is not the eCB mediator for DSE/DSI. However, evidence supporting the role of 2-AG in DSE/DSI has been indirect because selective MAGL inhibitors were not available and blocking a 2-AG biosynthetic pathway did not consistently block DSE/DSI. A selective MAGL inhibitor is, therefore, critical for determining the role of 2-AG in DSE/DSI.

JZL184 is a recently developed, highly selective and potent MAGL inhibitor that increases 2-AG but not AEA levels in mouse brain. If 2-AG is the mediator of DSE/DSI, blockade of 2-AG degradation with JZL184 should enhance DSE/DSI. Using whole-cell patch clamp recordings from brain slices, we examined the effects of blockade of MAGL and FAAH on DSE in mouse cerebellar Purkinje neurons and DSI in mouse hippocampal CA1 pyramidal neurons. Bath application of JZL184 prolonged the mean decay time constant ($\tau$) of DSE in a dose-dependent manner, and the half-maximal potentiation of $\tau$ was produced at 36.1 ± 6.2 nM. JZL184 also significantly prolonged $\tau$ of DSI in hippocampal neurons. Nonselective MAGL inhibitor MAFP prolonged $\tau$ of DSE and DSI as well. In contrast, selective FAAH inhibitor URB597 had no significant effect on DSE. DSE and DSI in slices prepared from FAAH knockout mice were not significantly different from those in wild-type littermates. Thus, disruption of the degradation of 2-AG, but not AEA, enhances DSE and DSI. These results provide further evidence that 2-AG is the eCB mediator for DSE and DSI.

Acknowledgements. This work was supported by National Institutes of Health Grants DA09155 (C.J.H.), DA017259 (B.F.C) and DA024741 (Q.S.L).

# MORPHOLOGICAL CHANGES IN ZEBRA FINCH TELENCEPHALON FOLLOWING DEVELOPMENTAL CANNABINOID EXPOSURE

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We have found that when zebra finches are exposed to modest dosages of the cannabinoid agonist WIN55212-2 (WIN) during song learning, vocal patterns are altered. This altered song persists through adulthood, and isnt produced in adults that have already learned song. Now we are working to identify neurophysiological changes potentially responsible for WIN-altered song. Normal vocal development is known to include a decrease in dendritic spine densities within distinct telencephalic regions necessary for song learning (lMAN and Area X of striatum), perception (auditory Field L and NCM) and motor production (HVC and RA). This raised the possibility that cannabinoid exposure may alter normal synaptic pruning processes. To test this we measured dendritic spine densities as a function of developmental WIN exposure. Zebra finches were tutored by an adult male until 50 days of age and assigned to treatment (1 mg/kg WIN) or vehicle control groups (n = 8 animals each). Treatments were given by IM injection of 50 µl once daily from 50-75 days of age (corresponding to the sensory motor stage of song learning). After 100 days songs were recorded and brains prepared for Golgi-Cox staining. Dendritic spines were counted in Area X, HVC, RA, and lMAN. WIN treatment resulted in significantly elevated dendritic spine densities within Area X (from 0.61 to 0.77 spines/µ, $p < 0.05$ SNK), and HVC (from 0.35 to 0.46 spines/µ, $p < 0.05$ SNK). Spine density differences were not observed in other regions (lMAN, RA).

Recent evidence suggests a non-apoptotic role for caspase-3 in resorption of dendritic spines. In zebra finch telencephalon, events associated with learning and memory such as hearing a novel song, trigger brief increases in levels of activated caspase-3. Combined, these findings led to the hypothesis that WIN-induced changes in dendritic spine densities may involve inhibition of caspase-3 activation. To begin to test this possibility adults were assigned to WIN (3 mg/kg), vehicle or untreated control groups (n=4). Prior to experiments, birds were isolated in a quiet room and treatments given 30 min before exposure to a novel song recording. Following 10 min of song exposure, birds were anesthetized, perfused with paraform and brains prepared for immunohistochemistry with an antibody against cleaved (the activated form of) caspase-3. We found that treatment with WIN suppressed normally robust changes in the levels of activated caspase-3 in Field L2, a primary sensory area for auditory stimuli in the avian telencephalon after 10 minutes of novel song exposure.

In summary, our results demonstrate that cannabinoid-altered vocal development is associated with inappropriately high dendritic spine densities within Area X of striatum, a brain region known to be critical for song learning, and HVC, a region essential for the learning and production of song. In addition, we have shown that WIN alters normal activation of caspase-3 following auditory perception of song, raising the possibility that elevated Area X spine densities may due to similar caspase-3 inhibition during song learning. We are currently testing this possibility.

# INFLUENCE OF ENDOCANNABINOIDS ON FLIGHT REACTION INDUCED BY A NITRIC OXIDE DONOR (SIN-1) ADMINISTRATION INTO THE DORSOLATERAL PERIAQUEDUCTAL GRAY

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Administration of nitric oxide (NO) donors into the dorsolateral periaqueductal gray (dlPAG) causes flight reactions probably by facilitating glutamate release. Endocannabinoids (eCB) such as anandamide (AEA) have also been reported to modulate glutamate release, either inhibiting or facilitating it by activating CB1 or Transient Receptor Potential Vanilloid Type 1 (TRPV1) receptors, respectively. Facilitation of eCB-mediated neurotransmission in the dlPAG produces anxiolytic effects. However, although the eCB and nitrergic systems have been suggested to interact in the central nervous system, it is still unknown if this interaction occurs in dlPAG. The aims of present work, therefore, are 1. to verify if pretreatment with AEA or URB597 (inhibitor of FAAH enzyme) would attenuate flight reactions induced by SIN-1 (a NO donor) administration into dlPAG and 2. to investigate the involvement of CB1 receptors in these effects. To these aims, male Wistar rats (n=118; 6 to 9/group) with cannulae aimed at the dlPAG received a first microinjection of vehicle (200 nL) or AM251, an antagonist of CB1 receptors (100 pmol) followed, 5 minutes later, by a second injection of vehicle, AEA (5 pmol) or URB597 (0.01 nmol). Five min after this, they received a third injection of vehicle or SIN-1 (150 nmol). The rats were then immediately placed in a circular arena for recording of flight responses (characterized by episodes of wild running and jumps) and total distance travelled during 10 minutes. SIN-1 induced flight reactions (percentage of animals showing flight responses: control 0% vs SIN-1 86.7 to 100%) and increased total distance traveled in the open arena (control: 6.12 to 17.79 m, SIN-1: 10 to 69.15 m). Although AEA was not able to abolish the flight responses caused by SIN-1 (Chi-square: 29.9, $p<0.0001$), this drug significantly attenuated the increased in total distance moved induced by the NO donor ($F_{(3,27)}=3.37$, $P=0.035$). This effect was prevented by AM251. Moreover, pretreatment with the CB1 receptor antagonist not only prevented AEA attenuation of SIN-1 effect but potentiated it 5 min after the injection ($F_{(3,30)}=2,9$ $p=0.053$). URB597 decreased the number of animals that presented flight reactions (URB597 vs URB597+SIN-1, $p=0.04$, Fisher) and the total distance moved ($F_{(3,32)}=4.39$ $p=0.012$). These results suggest that activation of CB1 receptors by AEA can attenuate flight reactions induced by a NO donor in the dlPAG. In addition, in the presence of CB1 receptor blockade AEA effects on TRPV1 receptors could prevail, further facilitating glutamate release and potentiating the effects of NO. URB597 also attenuated SIN-1 effects, but AM251 did not interfere in this result, maybe because SIN-1 did not work. Together, they suggest that eCB and nitrergic systems could interact to control aversive responses mediated by the dlPAG.

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# CHANGES IN SENSITIVITY TO THC AFTER REPEATED ADMINISTRATION IN MALE AND FEMALE ADOLESCENT AND ADULT RATS

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Well-characterized effects of delta-9-tetrahydrocannabinol (THC), the primary psychoactive compound in *Cannabis sativa*, include robust hypothermia, antinociception, catalepsy, and hypomotility. The observed tolerance to these effects that occurs upon repeated administration has been attributed to changes in the number and function of the brain cannabinoid ($CB_1$) receptor, which is both ubiquitous in the mammalian brain and known to exhibit differential expression throughout development.

Considering that adaptation to chronic THC exposure and age-related changes both result in decreased $CB_1$ receptor expression, the present study sought to determine whether repeated administration of THC would have different effects depending on the developmental stage during which dosing occurred. Adolescent and adult rats of both sexes were administered either 10 mg/kg THC or vehicle (VEH) twice daily for 38 days, with dosing initiated on postnatal day (PN) 30 for adolescents and >PN68 for adults. Over the course of this period, the rats were tested five times (days 0, 7, 14, 31 and 38) in a cumulative THC dosing paradigm (cumulative doses = 0, 3, 10, 30, 100 mg/kg). Following administration of each dose, spontaneous activity, catalepsy and body temperature were assessed. Control groups received chronic VEH injections and cumulative VEH dosing for the first four assessments with cumulative THC dosing on the final test (day 38). These groups were included to examine acute effects of THC in rats that had received previous exposure to the testing procedure, but no active drug.

Initial cumulative dose-effect curves revealed an effect of age on spontaneous activity for both males and females, with adolescents of both sexes exhibiting greater sensitivity to THC's hypomotility effects; however, adult males and females were more sensitive to THC's effects in the bar test of catalepsy. Increased sensitivity to hypothermic effects was also found in adolescent (vs. adult) males, but not in female adolescents (vs. adults). Final cumulative dose-effect curves, conducted when adolescents had reached adulthood (PN68), revealed dose-dependent decreases in spontaneous activity regardless of age, sex, or treatment condition. Hypothermia occurred only in adolescent and adult rats of both sexes that received THC acutely (i.e., chronic and cumulative VEH injections during previous assessments). However, in both sexes, dose-dependent increases in catalepsy were observed in adolescent and adult rats in all treatment conditions, with adults attending the bar significantly longer than adolescents. Hence, sensitivity to the acute effects of THC differed across age and sex. Further, administration of THC during development altered this level of sensitivity, regardless of whether exposure is chronic or intermittent. Nevertheless, age and sex differences in sensitivity to THC's effects in the bar test persisted, suggesting the possibility that age and sex differences in THC's effects may be task-specific.

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# EXPOSURE TO ESCALATING DOSES OF A CANNABINOID $CB_1$ RECEPTOR AGONIST DURING ADOLESCENCE SUPPRESSES ADULT NEUROGENESIS

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Adolescence is a critical period in neurodevelopment in which specific, yet subtle structural and functional changes occur, such as pruning of unnecessary neural connections and further myelination of those that are used, with the purpose of maximizing cognitive efficiency. During this period, there is extensive evidence demonstrating that the endocannabinoid system serves a crucial role in different stages of neurodevelopment, influencing the release of and action of various neurotransmitters. Adolescence also represents an age group in which cannabis use commonly begins. Thus, heavy cannabis consumption in a period that is characterized by involvement of the endocannabinoid system in brain development may leave an individual especially susceptible to long term neural alterations resulting in emotional and cognitive impairments (Rubino et. al., 2009). The hippocampus is known to play an integral role in cognitive processes and is one of only a few brain structures that possess the potential for its cells to proliferate, differentiate and survive as functional neurons in adulthood. With this in mind, we sought to investigate how chronic administration of HU-210 during adolescence would affect neurogenesis in the adult dentate gyrus of the hippocampus in male and female Sprague-Dawley rats. Escalating doses of cannabinoid $CB_1$ receptor agonist, HU-210, were administered interperitoneally (i.p.; doses: 25, 50, and 100 μg/kg) or vehicle over 12 days from post-natal day 35 to 46. Animals were left undisturbed until adulthood (post-natal day 70), at which point they were injected i.p. with bromodeoxyuridine (BrdU; 200 μg/kg) to label one population of dividing cells. Also, lavage smears were conducted to assess the stage of the estrous cycle female rats were in at the time of BrdU administration. Following BrdU administration animals were left undisturbed for 21 days to allow time for newly born cells to reach maturity. Results demonstrated that, regardless of sex, administration of HU-210 during adolescence suppressed neurogenesis in adulthood. Additionally, there was also a sex difference, such that males exhibited higher levels of neurogenesis than females. This data is in agreement with previous research suggesting that high cannabis consumption during adolescence may result in long term changes vital to neural processes, such as adult neurogenesis, possibly resulting in cognitive or emotional impairment.

# BLOCKADE OF THE CANNABINOID CB1 RECEPTOR IMPAIRS MATERNAL BEHAVIOR AND OFFSPRING DEVELOPMENT AND PERFORMANCE AT ADULTHOOD IN MICE

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Maternal care is the first socially interactive experience of the newborn which will affect development and his/her social competence throughout life. The endocannabinoid system (ECS) has been shown to be important for development and growth. The first postnatal week is critical for the formation of maternal behavior. Therefore the aims of this study were 1) to examine a putative role for the ECS in mother- pup interactions and 2) to investigate repercussions of interference with the maternal ECS postpartum on adult offspring behavior.

Methods: Eleven Dams received Vehicle or 10 mg/kg of the CB1 receptor antagonist SR141716 daily during postnatal days 1-8. Spontaneous maternal behavior (sniffing, licking, crouching over pups and distance from pups in nest) and pup-retrieval were measured on days 3, 5 and 7. Ultrasonic vocalizations (USVs) (at 10-80 KHz) and pup development (body weight, milk bands and body temperature) were recorded on days 4, 6 and 8. At 10 weeks of age, body weight and temperature, pain perception on a hot plate, motor activity, 'anxiety-like-behavior' in a plus maze and 'depressive-like behavior' in forced swim test were investigated.

Results: Pups of SR141716-treated dams had lower body weights throughout the first week of life. Milk bands (reflecting milk intake) were smaller than those in controls on day 3. SR141716 moms crouched less over their pups, perhaps explaining the pups' impaired growth and milk intake, and displayed delayed pup retrieval which may be explained by the fewer USVs emitted by the pups. At 2.5 months of age, body weights were still lower in the experimental vs. control offspring [this difference reached significance for males but not females]. Males displayed less motor activity and pain sensitivity than controls. Female behavior only tended toward hypoactivity. Both sexes displayed depressive-like behavior.

Conclusions: Overall, this study shows that during the first week after birth, the maternal ECS plays an important role in maternal behavior and in development of the offpsring. However, direct effects on the pups, by SR141716 transferred through maternal milk cannot be excluded. Irrespective of the mode of transmission, SR141716-treatment in lactating dams also affected the offspring at adulthood as expressed mainly in persistent low body weight, hypoactivity and depressive-like behavior. We postulate that hypothalamic oxytonergic functioning and/or dopaminergic transmission in the ventral tegmental area and nucleus accumbens indirectly impaired by ECS dysfunction, underlie the current observations.

# ACUTE AND CHRONIC HU210 TREATMENT DIFFERENTIALLY AFFECTS SPATIAL LEARNING AND MEMORY

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It is well documented that cannabinoids impair learning and memory processes. Previous work from our group has revealed that acute administration of the $CB_1$ agonist HU210 induces impairments in spatial learning and memory. However, to date very few studies have investigated the effects of long-term chronic cannabinoid exposure on learning and memory. The aim of this present study was to determine the effect of different treatment regimes of HU210 on spatial learning and memory processes using the water maze.

Male Lister Hooded rats (Harlan, UK) weighing approximately 300-400g at the start of testing were used. Animals were assigned to different treatment groups (N=7 or 8 per group) and pre-treated in the following way before testing: 1) no pre-treatment, 2) daily injections of HU210 (50µg/kg) or Tween 80 for 10 days (5 days/week) and 3) daily injections of HU210 (50µg/kg) or Tween 80 for 25 days (5 days/week). Animals were weighed daily and drugs were administered at the same time each day via intraperitoneal injection; all groups were injected during behavioural testing. A reference memory task was employed using the water maze with 4 days of acquisition (4 trials per day) and two probe trials (no platform) 1 and 4 days following the end of acquisition. Reversal learning was performed 24 hours after the second probe trial. During reversal (4 trials) the platform was located in the opposite quadrant of the pool. A further probe trial was performed 24 hours after reversal.

A significant reduction in body weight was observed following both acute and long-term treatment with HU210.In addition all HU210 treatment regimes impaired spatial acquisition learning; however, the effect was most prominent following acute treatment. Eventually, all groups acquired the target location, but memory was still impaired in the probe trial in the ‘acute’ HU210 group compared to controls. In contrast, the groups exposed to HU210 for longer periods of time performed no different to controls. No deficits in reversal learning were observed, although probe trial performance revealed that all HU210 groups were impaired in retention for the reversal platform quadrant.

These results corroborate previous findings that acute treatment with HU210 impairs spatial learning and memory (Robinson et al. 2007, British Journal of Pharmacology; 151, 688-700). Long-term exposure to HU210 prior to testing however induces differential effects with learning being initially impaired whilst retention remains intact. This may be due to tolerance that has developed over the treatment period, and may be co-incident with receptor desensitization.

# MICROANALYSIS OF FOOD-MAINTAINED LEVER PRESSING DISTINGUISHES EFFECTS OF THE CB1 ANTAGONIST AM 6527 FROM THOSE OF PREFEEDING

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Food-maintained operant responding is a reliable and sensitive assay of cannabinoid antagonist function. However, it can be difficult to distinguish between the effects of different response-reducing manipulations by simple examination of changes in response rate. As the effects of CB1 antagonists on responding have been assumed to be due to attenuated food motivation, it is important to compare these effects to satiation via prefeeding. In the present study, two groups of rats were trained on a fixed-ratio 5 (FR5) schedule, in which every fifth lever press response is reinforced with a palatable food pellet. FR5 sessions occurred for 30 min per day. Following training, the AM 6527 group was given i.p. doses of 0.5, 1.0, 2.0, 4.0, and 8.0 mg/kg, plus vehicle, one dose per week in pseudorandomized, counterbalanced fashion. The prefeeding group was given 3 h access to 0, 4.5, 9.0, 18.0, or 36.0 g of the palatable operant pellets, also in a pseudorandomized, counterbalanced design with two test sessions per week. As expected, both manipulations significantly suppressed responding for food, with significant differences from control conditions at the 1.0, 2.0, 4.0, and 8.0 mg/kg doses of AM 6527, and the 18.0 and 36.0 g prefeeding quantities. The operant response record was further analyzed by measuring the duration of session events (i.e., responses and food tray entries), and of time between events (i.e., interresponse time, pellet retrieval time, and postreinforcement pause time). Both AM 6527 and prefeeding significantly slowed many aspects of responding, particularly at high doses and food amounts. Both manipulations slowed the local rate of response (i.e., rate during response bursts), and lengthened response durations, as well as pauses between responses and following reinforcement. AM 6527, but not prefeeding, also lengthened pellet retrieval time and duration spent in the food tray. To determine whether the overall patterns of responding could be distinguished between the two variables, a discriminant function analysis was performed on all cases in the data set that exhibited a level of overall responding that was below the 95% confidence interval of the control condition. A stepwise procedure was applied to identify variables that best predicted group membership. One function was identified that separated the effects of AM 6527 from those of prefeeding on the basis of the frequency of longer postreinforcement pauses, the local rate of response, and the length of pauses in responding. It is concluded that the CB1 antagonist used in this study does not produce a pattern of reductions in food-maintained responding that is identical to satiation via prefeeding; however, other rate-reducing manipulations (e.g., CB1 agonists and psychostimulant appetite suppressants) should be submitted to an identical analysis to determine which pharmacological treatments are least differentiated from satiation. It is also concluded that discriminant function analysis of the operant response record is a useful technique for such analyses.

# INHIBITION OF FAAH ANTAGONIZES COCAINE AND NICOTINE EFFECT ON NEURONS IN THE NUCLEUS ACCUMBENS SHELL IN THE RAT

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**Rationale:** The Nucleus accumbens (NAc) is a brain area crucially involved in the modulation of goal-directed behavior. Within NAc sub-regions, the shell (ShNAc), responds to natural stimuli and primary reinforcing properties of drugs of abuse. In fact, it has been demonstrated that different addictive substances, such as nicotine and cocaine, elevates dopamine (DA) levels in the ShNAc (Di Chiara and Imperato, Procl.Nat.Acad.Sci. USA **85**:5274, 1988). Moreover, the endocannabinoid (ECb) system has been hypothesized as an important modulator of the effects of drugs of abuse in the NAc. Hence, it has been shown that the cannabinoid receptors type-1 (CB-1) antagonist SR141716-A (SR, rimonabant) inhibits the efflux of DA induced by nicotine, cocaine and ethanol in the ShNAc (Cheer et al., J.neurosci **27**:791, 2007). In addition, an increase of anandamide levels has been reported in the striatum after cocaine exposure (Centonze et al., Neuropsychopharmacology, **29**:1488, 2004). **Methods:** we studied whether the enhancement of ECb levels induced by FAAH inhibitor URB597 modulated the effects of nicotine and cocaine on the medium spiny neurons (MSNs) of the ShNAc by using electrophysiological single unit recordings in anaesthetized rats. Since these neurons do not fire spontaneously in anaesthetized animals, they were identified by stimulation of the basolateral amigdala (BLA), which is the major excitatory projecting area to the NAc. **Results:** both nicotine (0.2 mg/kg i.v.) and cocaine (1 mg/kg i.v.) depress the excitability of MSNs of the ShNAc (n=6, one-way ANOVA, $P<0.05$, for nicotine; n=7, one-way ANOVA, $P<0.01$, for cocaine) as measured by their response to threshold BLA stimulation. Interestingly, pretreatment with URB597 (0.1 mg/kg i.v., 1-2 hours before recordings) fully reverts nicotine effect, which become excitatory instead (n=6, two-way ANOVA, $P<0.01$). Moreover, contrary to the VTA DA neurons (Luchicchi et. al., 18$^{th}$ ICRS symposium **18**:P86, 2008), URB597 fully prevents the inhibitory action of cocaine on the MSNs of the ShNAc (n=5, two-way ANOVA, $P>0.01$), suggesting a specific modulatory effect of FAAH inhibition in this area. **Conclusions:** our results suggest that the increase of ECbs tone fully antagonizes nicotine and cocaine inhibitory effects in the ShNAc. This may occur through a blockade of drug-induced release of DA to the terminal areas, and contributes to modulate drug-induced seeking-behavior.

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# DISCRIMINATIVE STIMULUS EFFECTS OF THC IN FAAH KNOCKOUT AND WILD-TYPE MICE

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$\Delta^9$-Tetrahydrocannabinol (THC) drug discrimination, a preclinical assay that models cannabis intoxication in humans, has been established in non-human primates, pigeons, rats, and most recently in mice. The purpose of the present study was to evaluate whether inactivation of fatty acid amide hydrolase (FAAH) in mice alters the discriminative stimulus effects of THC. To this end, male FAAH knockout (KO) and wild-type (WT) littermate mice bred on a C57BL/6 background were trained to discriminate 5.6 mg/kg THC (s.c.) from vehicle in an operant conditioning task.

FAAH KO and WT mice readily acquired the discrimination in a similar number of trials (mean $\pm$ SEM = 29.2 $\pm$ 4.7; 28.6 $\pm$ 3.2, respectively). THC fully and dose-dependently substituted for itself in both genotypes without influencing response rate, yielding $ED_{50}$ values of 1.31 mg/kg (1.07-1.61) in KO mice and 1.51 mg/kg (1.06-2.13) in WT mice. These effects were attenuated by pretreatment with the $CB_1$ receptor antagonist, rimonabant, with similar $AD_{50}$ values of 0.53 mg/kg (0.36-0.79) and 0.39 mg/kg (0.27-0.57) for FAAH KO and WT mice, respectively. Anandamide (AEA) fully substituted for THC in FAAH KO mice ($ED_{50}$=4.03 mg/kg; 3.34-4.87), but not in WT mice. The discriminative stimulus effects of THC did not generalize to exogenous 2-arachidonylglycerol (2-AG) in FAAH KO or WT mice, likely due to the rapid degradation of 2-AG by monoacylglycerol lipase (MAGL).
Surprisingly, the recently developed MAGL inhibitor JZL184 fully substituted for THC in FAAH KO mice 90.7% drug-lever responding (DLR), but produced only a partial degree of THC-like responding in WT mice (55.7% DLR). Rimonabant (3.0 mg/kg) fully blocked responding on the THC lever in both genotypes. Overall, these results suggest that absence of FAAH does not appreciably alter the acquisition of THC discrimination or the potency of THC. In contrast, however, differences were noted with AEA and JZL184, with greater substitution seen in FAAH KO mice than in WT mice. These results suggest that endocannabinoid substitution for THC is facilitated when metabolic enzymes are inhibited. Further testing, including evaluating a full $CB_1$ agonist (e.g., WIN55,212-2) and methanandamide in this task, will help elucidate the role endocannabinoids play in THC's discriminative stimulus effects.

Acknowledgements: This work was supported by NIH grants DA-03672 and DA-09789.

# EFFECTS OF ACUTE $\Delta^9$-THC ON PERCEPTION AND MEMORY IN HEALTHY VOLUNTEERS – RELATION TO VALENCE, MOOD

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Introduction: Drugs of abuse, including $\Delta^9$-THC, produce positive mood effects like euphoria, which likely contribute to their use. In addition, however, drugs may also alter perception of, and/or memory for, emotional stimuli in ways that affect future use of the drug. For example, $\Delta^9$-THC may modulate the positive or negative valence of affective stimuli, or it may affect memory for affective stimuli. While there is ample evidence that $\Delta^9$-THC impairs memory, it is not known if these impairments are specific to the emotional valence of the stimuli. Emotional (particularly negative) valence enhances memory for a stimulus, and studies from our lab suggest that $\Delta^9$-THC can selectively reduce reactivity to threatening vs. non-threatening stimuli. Thus, if $\Delta^9$-THC selectively impairs memory for negative stimuli, this could lead to continued use of the drug. In this study we investigate how mood-altering effects of acute oral $\Delta^9$-THC relate to perceptual alterations and memory impairments for picture and word stimuli of varying valence.

Methods: Healthy young adult volunteers received $\Delta^9$-THC (7.5 and 15mg; p.o.) in a within-subject, double-blind, placebo-controlled study, and mood was assessed using subjective scales (feel drug, like drug, dislike drug, high, want more drug, anxious, sedated, confused, elated, angry, positive affect negative affect schedule, addiction research center inventory, and hallucinogen rating scale). Following capsule administration, subjects rated 20 positive, neutral, and negative IAPS images, 10 positive, neutral, and negative personality trait words, 30 noun words, and were asked to identify emotions of Ekman faces. Two days following each drug session, the subjects returned to the lab to view the previously-presented stimuli (excluding Ekman faces) interspersed with equal numbers of novel stimuli. No drug was administered on memory test days. We assessed their recognition of each image, and asked them to rate each again based on current perceptions.

Results: Initial data (13 out of 24 subjects) suggest that $\Delta^9$-THC selectively impairs memory for negative personality trait words. Picture memory for negative images was also impaired, as well as memory for neutral and positive images. Neither ratings of $\Delta^9$-THC preference nor effects of $\Delta^9$-THC on mood were related to memory for, or ratings of, word stimuli or IAPS images. $\Delta^9$-THC also did not affect erroneous memory ratings for novel stimuli during memory sessions, and it did not change recognition of emotion in faces.

Conclusions: $\Delta^9$-THC impairs recognition memory for stimuli encountered while drug-affected, and these effects appear to be more pronounced with negatively-valenced stimuli. The effects on memory appear to be independent of effects on perception of stimuli during the initial presentation, or to effects on mood. The drug does not affect false memory, regardless of stimulus valence.

# INDUCTION OF PHYSICAL WITHDRAWAL THROUGH PROLONGED ELEVATIONS OF ENDOCANNABINOIDS

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The two major endocannabinoids (ECs) are anandamide (AEA) and 2-arachidonyl glycerol (2-AG), which have distinct biosynthetic and inactivation pathways. AEA and 2-AG are inactivated by the enzymes fatty amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL), respectively. Previous studies have shown that inhibition of either of these enzymes leads to elevations in the appropriate endocannabinoid but only MAGL blockade can lead to a subset of $CB_1$ receptor mediated behavioral effects, such as analgesia. The purpose of the present study was to investigate whether elevating (EC) levels with pharmacological FAAH and MAGL inhibitors as well as FAAH (-/-) mice would result in a cannabinoid withdrawal syndrome when precipitated with the $CB_1$ antagonist, rimonabant. We previously found that rimonabant did not precipitate withdrawal in either mice treated subchronically with the FAAH inhibitor, URB597, or FAAH (-/-) mice that have constitutively elevated AEA levels. In the present study, we evaluated the intensity of somatic withdrawal by measuring the frequency of paw tremors and head shakes. Mice treated subchronically with the FAAH inhibitor, PF3845, did not show precipitated withdrawal signs. However, rimonabant precipitated mild withdrawal signs ($p < 0.05$ for paw tremors) in mice treated subchronically with the MAGL inhibitor, JZL184. Interestingly, subchronic administration of JZL184 led to a more robust precipitated withdrawal syndrome ($p < 0.001$ for paw tremors) in FAAH (-/-) mice than in wildtype mice. The magnitude of withdrawal was similar to that seen with a mild $\Delta^9$-tetrahydrocannabinol (THC) subchronic dosing regimen. These results suggest that, while prolonged elevation of 2-AG leads to mild dependence, dual blockade of FAAH and MAGL leads to elevated levels of both AEA and 2-AG, eliciting strong cannabinoid dependence. Thus, the endocannabinoid system is a tightly regulated homeostatic system, which, when the catabolic enzymes are inactivated for a prolonged period of time, will elicit effects similar to that of THC.

# PHARMACOLOGICAL ELEVATION OF ANANDAMIDE IMPAIRS SHORT-TERM MEMORY BY ALTERING HIPPOCAMPAL NEUROPHYSIOLOGY

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Exogenous cannabinoid agonists ($\Delta^9$-THC, WIN 55,212-2, HU-210) produce impairments in short-term memory (STM) by altering hippocampal neural activity in rats (Hampson & Deadwyler, *J. Neurosci*, 2003; Robinson et al., *Bri. J. Pharm*., 2007). However, the role of endocannabinoids such as anandamide and 2-arachidonylglycerol (2-AG) in modification of short-term memory is yet fully understood. In the current study, we determine effects of one aspect of the endocannabinoid system on performance of a Delayed Nonmatch to Sample (DNMS) task and concomitant hippocampal ensemble activity by manipulating anandamide metabolism using the fatty-acid amide (FAAH) inhibitor URB-597, the anandamide membrane transporter inhibitor AM404, the stable analog *R*-methanandamide.

Adult, male, Long Evans rats (n=14) were implanted with multi-electrode arrays targeting CA3/CA1 subfields of hippocampus and pre-trained to perform the DNMS task with delay (1-30s). Following isolation of hippocampal principal cells (firing rate-0.5-6Hz) all animals were administered Tween-80 (vehicle), URB-597 (0.3 or 3.0 mg/kg), AM-404 (1.5 or 10.0 mg/kg) or *R*-methanandamide (3.0 or 10.0 mg/kg) on alternate test days. The neuronal activity was 'tracked' throughout DNMS performance following all treatments.

Results demonstrate that prolonging the action of anandamide via inhibition of FAAH (URB-597) or competition with the stable analog *R*-methanandamide produced both dose- and delay- dependent impairments in DNMS performance. In contrast, blocking anandamide reuptake with AM-404 failed to produce significant deficits in DNMS performance at any delay intervals or dose. Furthermore, URB-597 and *R*-methanandamide suppressed hippocampal ensemble activity during the sample (encoding) but not the nonmatch (retrieval) phase of the task, at doses which produced deficits in DNMS performance.

These results suggest that anandamide disrupts short-term memory in a delay-dependent manner when its presence in the brain is pharmacologically enhanced beyond normal physiological levels. As has been previously proposed for exogenously applied cannabinoids (Hampson & Deadwyler, J. Neurosci 20:8932-8942, 2000), concurrently recorded electrophysiological data show that these deficits are most likely produced by impairment of the encoding, but not necessarily retrieval of task-related information by the hippocampus.

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# MEDICINAL CANNABIS: HOW CANNABINOIDS CAN HELP TREATING ATTENTION DEFICIT/HYPERACTIVITY DISORDER (ADHD)?

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This work is a review of the Endocannabinoid System (ECS) implication in ADHD. It reports two European cases, sample of the growing number of Medicinal Cannabis (MC) recommended/prescribed for the treatment of ADHD cases.

Scientific studies have demonstrated a relation between cannabinoïd receptors (CB1, CB2), endocannabinoids (Anandamide, 2-Arachidonylglycerol), the Central Nervous System and the Neuro-immune System. CB1 receptors, abundant in the brain, interact with the Dopaminergic System[2]. Genetic studies found a correlation between Cannabinoïds Receptors Gene and ADHD[3]. Regarding behaviour, CB1 are possible targets to reduce hyper-impulsivity[4], Tourette Syndrome tics, fears, anxiety[5] and improve emotional learning (synaptic plasticity[6]) and distractibility[7]. The neuro-chemical mechanism of action is Retrograde Signaling Inhibition[8]. This is dopamine mediated. An increase in unbound cannabinoid levels leads to cannabinoïds replacing dopamine bound to dopamine transporters binding sites. With more dopamine available to slow down the speed of neurotransmission there are fewer, slower moving neural inputs and the cerebral cortex has a better opportunity to focus and attend to the neural stimulation.

Cannabinoïds are accepted by many cannabinoid medicine specialists for treating ADHD. Two patients, from Luxembourg (52, retired policeman) and France (35, engineer), had the opportunity to experiment MC therapy with Dutch products, available in pharmacies since 2005. Both report an improvement of their condition and chronic symptoms (distractibility, agitation, alcoholism, depression, anxiety, obsessive/suicide thoughts), without unacceptable side effects. CBD, no-psychoactive cannabidiol, was found necessary to reduce anxiety and adverse dronabinol effects (dronabinol/CBD ratio: 1/1-3/1). MC therapy was considered complementary to psychotherapy.

Both clinical experience, particularly in California, and scientific data suggest that targeting ECS with exocannabinoïds is an exciting new alternative to treat ADHD. Many doctors, who have experience with patients, recommend cannabinoïds for ADHD. Further clinical studies are required to investigate this new field.

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[1] Patients Members of the International Association for Cannabis as Medicine (IACM)

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# A PATIENT SURVEY OF CANADA'S MEDICAL CANNABIS PROGRAM AND PRODUCT

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## Introduction

Cannabis has a long medical history, and anecdotal evidence from contemporary users points to an array of therapeutic uses. Although cannabis remains a controlled substance in Canada, court decisions have upheld the right to "compassionate access" for serious medical conditions, and in 1999 the federal government initiated one of the world's first medical cannabis programs. At the time of this survey, there were approximately 1300 patients authorized to use cannabis through this program, but no effort had ever been made to survey these stakeholders to assess their satisfaction with the federal program and cannabis supply. Systematic compilation of this information could inform the evolution of service delivery in the program, and could inform efforts to establish centrally administered production and distribution models in other countries.

## Methods

A self-administered 50 question survey was designed to gather basic socio-demographic data, specific symptoms and conditions for which cannabis is used, preferred strains/methods of ingestion, general use patterns, and open-ended information about their experience with the program. Recruitment was conducted through online medical cannabis patient groups, and access to the anonymous online survey was password protected. The 100 responses received represent roughly 7.5% of the patients enrolled in the program at the time of the survey.

## Results

The results of this survey shed light on many of the preferences and patterns of use of this patient population. While 88.6% report smoking cannabis, 71.6% eat or orally ingest their medicine, and over half (52.3%) vaporize. 90.9% report that not all strains are equally effective at relieving their symptoms. 97.6% state a preference for purchasing cannabis from a source that offers multiple strains, and 90.2% would prefer to have access to multiple methods of ingestion (baked goods, tinctures, oils, hashish, etc.). 57% had tried pharmaceutical cannabinoids, although only 18.5% used them on a regular basis. 68.2% report cultivating their own cannabis, and 48.8% had tried the cannabis distributed by Health Canada, with 75.6% of these ranking it as either 1 or 2 on a scale of 1-10 with 1 being "Very Poor" and 10 being "Excellent". 72.1% stated that they were "somewhat" or "totally unsatisfied" with the federal program.

## Conclusion

Canada was one of the first nations to implement a medical cannabis program, but many patients report a low level of satisfaction with the federal policies and cannabis product. Most patients cultivate their own medicine, and the majority stated a preference for multiple strains of organically-cultivated cannabis and for different methods of ingestion. These findings may explain the low uptake with both the Health Canada program and the single-strain medical cannabis supply, and could point the way to more patient-centered medical cannabis policies.

# CANNABINOID CB1 RECEPTOR ANTAGONIST BLOCKADE AT BIRTH MAY BE ASSOCIATED WITH ADHD

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Attention Deficit Hyperactivity Disorder (ADHD) is a condition that becomes apparent in some children in the preschool and early school years, but also appears at adulthood. It is estimated that between 3-5 percent of children have ADHD in USA, and 5-10 percent in the Israel. ADHD is characterized by inattention, impulsivity and/or hyperactivity. A well-known feature of ADHD is the positive response to psychostimulants such as methylphenidate (Ritalin) and D-amphetamine (Adderall), expressed as a reduction in excess motor activity and improved concentration. Although ADHD has been known for over 80 years, the etiology and risk factors for ADHD are still unclear. Importantly, low birth weight may be one of the most important predictive factors of ADHD (Chadapim et al. 2005). Non organic failure-to-thrive (NOFTT) in infants is defined as an abnormally low weight and/or height for age without a known organic cause. In a series of studies performed in neonatal mice we have demonstrated that the cannabinoid CB1 receptor is critically important for feeding and weight gain, apparently caused by an oral-motor dysfunction, similarly to that which characterizes infants suffering from NOFTT (Fride et al., 2001; 2003; 2007). Children who suffered from NOFTT are thought to display behavioral and cognitive dysfunctions in later years. Here we propose that a deficient ECS (endocannabinoid system) at birth may comprise a risk factor for ADHD.

**Methods**: Male and female pups were administered a single injection of SR141617 (rimonabant, 5, 10 or 20 mg/kg), within 24 hours of birth. At two months of age, mice were tested in an assay for pre-pulse inhibition (PPI) of the acoustic startle response (ASR). At the age of 16 weeks the same mice were examined for motor activity in an open field, immobility (catalepsy) on an elevated ring, for anxiety-like behavior in an 'elevated plus-maze' and in the Porsolt forced swimming test for depressive-like symptoms.

**Results**: Pups treated with 10 or 20 mg/kg rimonabant showed a reduction in body weight during the first 2 weeks of life. However as adults, all (surviving) mice were of normal weight. Behaviorally, both male and females displayed significant hyperactivity in the open field and on the 'ring'. We also observed a decreased performance in the PPI assay at both doses for the females but only at the 5 mg/kg dose for males. We observed a lower level of anxiety in the plus maze, again primarily in the males which had received 10 mg/kg SR141716. Compatible with this observation, a trend towards a reduced startle response was seen, again mainly in the males. In the forced swimming test, no differences were observed between rimonabant-treated and control mice. Preliminary observations suggest that DL-amphetamine administration (4 mg/kg) normalizes neonatal rimonabant-induced hyperactivity and PPI.

**Conclusions**: Our observations suggest that brief neonatal blockade of the cannabinoid CB1 receptor at birth precipitates symptoms of ADHD at adulthood as apparent in hyperactivity, impaired sensorimotor gating a tendency toward reduced 'anxiety' and the reversibility of these symptoms by amphetamine. In addition, similarly to observations in humans treated for ADHD (Pinckhardt et al., 2009), primary mood regulation was not affected in mice with blocked CB1 receptors at birth. We conclude that at least a subgroup of ADHD may be caused by a developmental deficiency of the endocannabinoid system.

# THE AMERICAN ACADEMY OF CANNABINOID MEDICINE

Jeffrey Hergenrather, M.D. General VP
David Bearman, M. D. VP for Credentials and Quality Assurance

**Founding of AACM -** At the upcoming meeting we wish to make the members of the International Cannabinoid Research Society aware of the recent founding of the American Academy of Cannabinoid Medicine (AACM),it's goals and mission. The AACM is the professional medical organization dedicated to the clinical and scientific understanding of the endocannabinoid system, the therapeutic application of cannabis and cannabinoids and establishing high standards for the practice of cannabinoids medicine.
We will go over the material on our website **www.aacmsite.org .**

**Goals – 1)** A principal goal of the AACM is disseminating accurate, scientific data regarding the clinical utility of cannabis; **2)** Promote and support exemplary ethical and practice standards in the asseesment, approval and/or recommendation of the medicinal use of cannabis and cannabinoids; **3)** Toward that end we have promulgated certifying **practice standards and guidelines** for practicing physicians who, acting within relevant state law, recommend and approve the medicinal use of cannabis. (Note: There are 14 states in the U.S. which specifically approve of the legal use of cannabis for medicinal purposes.); **4)** Provide education with accurate data to the medical community and lay public on the endocannabionoid system and the medical utility of cannabis. And cannabinoids; and **5)** Return cannabis to the United States Pharmacopeia.

**Research base -** Modern research augments anecdotal knowledge gained from thousands of years of therapeutic use of cannabis preparations. The AACM is dedicated to applying and promoting the understanding of credible review reports like the U.S. government commissioned and funded 1999 Institute of Medicine Report and the 1997 House of Lords Science and Technology Committee report on Medicinal Cannabis.Since these reports were released, additional research has expanded our knowledge of the therapeutic value of cannabis and cannabinoids and the mechanism of action of these therapeutic agents. The AACM is a resource for a better understanding of the clinical implications of this modern research.

**Clinical Expertise -** Much has been written and said about medicinal cannabis, but very little effort has been made to utilize the expertise of clinicians and researchers in the field. The AACM is an organization designed to fill that need. Our membership has experience treating thousands of patients with cannabis and cannabinoids. We have both a clinical and research knowledge base.

**Medical Information Resource -** The Academy will serve as an authoritative information source for doctors, state medical boards and the media on the medical application and research related to the clinical use of cannabis and cannabinoids. We are dedicated to educating physicians about the clinical, therapeutic usefulness of cannabis. There are a myriad of diseases whose symptoms respond to this class of medications.

**Membership –** Presently our bylaws provide for several categories of membership: Fellow, Member, Associate Member, Resident/Student, Supporting and Honorary. We encourage all practitioners, researchers, graduate students and others with a serious interest in the cannabinoid medicine field to join.

**Background -** The physicians in governance positions for AACM represent a diversity of experience across a broad range of settings in clinical practice, clinical research, and policy-making positions. Specialties represented include Rehabilitative Medicine, Neurology, Internal Medicine, Psychiatry, Public Health, and Family Practice, plus expertise in addiction medicine, pain management, ALS and medical geography. The members of the AACM have the medical training, clinical experience and familiarity with the scientific literature to speak knowledgeably about the current medical utility and future therapeutic potential for cannabis and cannabinoids. We are excited about the prospects of gaining a deeper understanding of the endocannabinoid system and what that will mean for better understanding of brain functioning and disease treatment.

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# CHRONIC COCAINE TREATMENT, WITHDRAWAL AND CHALLENGE ELICIT BRAIN REGIONAL ALTERATIONS IN ANANDAMIDE (AEA) CONCENTRATION AND FATTY ACID AMIDE HYDROLASE (FAAH) ACTIVITY

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Presynaptic CB1 cannabinoid receptors (CB1R) are activated when endocannabinoid (eCB) concentrations rise and inhibit the release of glutamate and GABA in many brain regions. There is considerable evidence that postsynaptic depolarization and activation of metabotropic receptors increase the synthesis of 2-arachidonoylglycerol, resulting in short term changes in CB1R activation. However, AEA concentrations also regulate CB1R activation and accumulating data suggest that AEA concentrations are sensitive to environmental and chemical changes, such as stress. Cocaine directly and indirectly leads to shifts in dopamine and glutamate signaling in the mesocorticolimbic reward system; these changes are thought to underlie the development of cocaine dependence. Since eCBs modulate neurotransmission, we hypothesized that changes in AEA are induced by cocaine treatment and contribute to the development of cocaine dependence.

Male C57Bl/6 mice received cocaine (15 mg/kg i.p.) or saline control injections once per day for 10 days. Subjects then underwent 7 days of withdrawal, followed by a challenge injection of cocaine or saline 1 day later. Brains were harvested at day 1 (acute), day 10 (chronic), day 17 (withdrawal) and day 18 (challenge). AEA content was quantified in mesocorticolimbic brain regions using isotope dilution LC/MS. The activity of fatty acid amide hydrolase (FAAH), a catalytic enzyme for AEA, was measured in membrane fractions by hydrolysis of [$^3$H]AEA.

Significant decreases in AEA were found in the hippocampus with chronic cocaine (8.3±0.5 vs 6.3±0.4 pmol/g, P=0.012, N=8) and the motor cortex following withdrawal (4.0 ±0.2 vs 3.2±0.2 pmol/g, P=0.016, N=8) compared to the same regions and time points in saline treated mice. Amygdalar AEA was decreased when cocaine-treated mice were challenged with saline on day 18 (5.6±0.6 vs 3.6±0.2 pmol/g, P<0.01, N=8). In the nucleus accumbens, AEA was elevated in both the cocaine and saline groups at the withdrawal phase compared to the other treatment phases. While no changes in the maximal activity of FAAH were found, the $K_m$ of FAAH for AEA was altered in 2 regions: acute cocaine increased the $K_m$ in the hippocampus, (0.32±0.02 vs 0.45±0.05 μM, P=0.030, N=6), while in the amygdala, the $K_m$ was increased when cocaine-treated mice were challenged with saline (0.20±0.01 vs 0.26±0.03 μM AEA, P=0.049, N=8).

In summary, these data suggest that cocaine could influence the regional concentrations of AEA in a treatment phase-specific manner; in some cases, through the regulation of the $K_m$ of FAAH for its AEA substrate.

Supported by NIDA grants RO1 DA09155 and F30 DA19754.

# SELECTIVE ALTERATIONS IN THE CB1 RECEPTOR AND THE FATTY ACID AMIDE HYDROLASE IN THE VENTRAL STRIATUM OF ALCOHOLIC AND SUICIDE VICTIMS

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Recent studies have suggested a role for the central endocannabinoid system in the regulation of mood and alcohol related behavior. Alcohol abuse disorder is often associated with suicidal behavior. In the present study, we examined whether abnormalities in the endocannabinoid system exist in the ventral striatum of alcoholics and alcoholic suicides. The levels of CB1 receptors, receptor-mediated G-protein signaling and activity of the fatty acid amide hydrolase (FAAH) were analyzed postmortem in the ventral striatum of chronic alcoholics (CA, n=9), alcoholic suicides (AS, n=9) and normal controls (n=9). The results revealed lower levels of the CB1 receptors and the CB1 receptor-mediated G-protein signaling in the ventral striatum of CA compared to control group. However, these parameters were elevated in AS than CA group. The activity of FAAH was lower in CA compared to normal controls whereas it was found to be significantly higher in AS than CA group. Alteration in the activity of FAAH enzyme that regulates the anandamide content may in turn explain differences in the ventral striatal CB1 receptor function in alcohol dependence and suicide. These findings might have etiological and therapeutic implications for the treatment of alcohol addiction and prevention of suicidal behavior.

## BRAIN CANNABINOID CB2 RECEPTOR AND SCHIZOPHRENIA

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Neural endocannabinoid function appears to be involved in schizophrenia, according to some previous studies. Cannabis use has also been associated with a slightly increased risk of schizophrenia, and may also worsen its symptoms in some patients. A decrease in gray matter density in the right posterior cingulate cortex was found in first-episode schizophrenia with cannabis-use when compared with to those without cannabis use. Cerebrospinal fluid (CSF) from first-episode schizophrenics contains significantly higher levels of the endolipid, Anandamide, than does CSF from healthy volunteers. While many of these phenomena have been interpreted to be produced via CB1 receptors because CB1 receptors were thought to be a specific brain-type cannabinoid receptor, we recently identified a role of CB2 receptors in the brain. To evaluate its involvement in the vulnerability for schizophrenia, we examined associations between tag SNPs in the *CNR2* gene and schizophrenia in approximately 4000 Japanese subjects and identified R63Q and rs12744386 that were associated with schizophrenia. We observed differences in cyclic AMP levels between R63 and Q63 in CB2 receptor-expressed cultured cells and associations between rs12744386 and gene expression in human postmortem brain tissues. In addition, CB2 antagonist worsen MK-801 induced disturbance of prepulse inhibition in C57/B6 mice. Our data indicate that low function of CB2 receptor associated with R63Q and rs12744386 may increase the risk for schizophrenia.

# CANNABIS SATIVA USE IN FOOD CULTURE OF NEPAL

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Cannabis is one of the revered and commonly found plants in Nepal. It has been in use since pre-historic times and even today it is in a state of holy plants of Nepal that is worshiped and offered to the famous Hindu deity Lord Shiva. The sage and mystic saints drink the decoction or puff the pipe in search of divine bliss. Some festivals like Holi, Shivaratri are incomplete without the use of the plant in its various preparations, mostly in sweet dishes and milk shakes. The seed of the cannabis plant is used for culinary purpose and in preparing pickles. The medicinal use of cannabis plant in various forms is a prevalent practice for various ailments of the human and veterinary ethno medicine. Presence of cannabis plant in the kitchen garden is a common sight in both urban and rural areas of Nepal. An emerging tolerance and inclusion of few shoots of the green cannabis plant in the mixed vegetable from kitchen garden is a new food culture in Nepal. People using such mixed vegetable including the cannabis plant, cite better sleep quality, increased zeal and vigor the next day and express no remorse for its inclusion in food. Avoiding excess inclusion and for use to adult members of the family is the trend here and suggestions of those who are using it. In conclusion, this food culture trend using one of the best gifts of nature controversies for human misuse will surely spread far and wide. The benefits of the inclusion of cannabis in the food culture in the nutrient requirements and the concerns that it may pose need to be evaluated in a scientific line. This paper intends to present the experience from Nepal and the perception of the community members using it for wider communication.

# CB1-ANTAGONIST SURINABANT INHIBITS THC-EFFECTS IN HUMAN THC-CHALLENGE TEST

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Cannabinoid receptor type 1 ($CB_1$) antagonists are being developed for the treatment of metabolic disorders and substance abuse. Surinabant is a high affinity $CB_1$ receptor blocker in vitro. The aim of this study was to assess the magnitude of inhibition by surinabant of CNS effects and heart rate induced by $\Delta^9$-tetrahydrocannabinol (THC) in humans.

This was a double blind, placebo-controlled, randomized, six-treatment four-period six sequence incomplete balanced cross-over study. Thirty healthy young male occasional cannabis users (less than once per week) were included. A single oral dose of surinabant (5, 20 or 60 mg) or matching placebo was administered followed 1.5 hours later by four increasing, intrapulmonary doses of THC (2, 4, 6 and 6 mg) or THC vehicle, administered at 1 h intervals and prepared using a Volcano® vaporiser. Wash-out period between each THC-challenge test was between 14 and 21 days. Pharmacodynamic (PD) measurements were: body sway, the alertness factor from Bond and Lader visual analogue scales (VAS), all items from the modified-Bowdle VAS including "feeling high" and derived composite factors ("internal perception" and "external perception"), and heart rate. PD parameters were analysed using a linear mixed effect model with treatment, period, time and treatment by time as fixed effects, subjects and subject by treatment as random effects and with baseline value as covariate.

Surinabant 60 mg by itself did not show significant effects on any studied PD parameters. Single doses of surinabant 20 and 60 mg were able to inhibit all CNS and cardio-vascular effects induced by THC in a similar range for both doses (e.g., inhibition rates on external perception, composite factor from Bowdle VAS were of 87.1% [95% CI 45.6;128.6] and 87.9% [95% CI 46.1;129.6] , respectively for 20 and 60 mg doses), whereas the 5 mg dose was not shown to be significantly active.

The dose-related reversal of all THC-effects by surinabant, without any effect of its own at a high dose, suggests that this compound behaves as a neutral CB-1 receptor antagonist in humans.

# THE RELATIONSHIP BETWEEN SCHIZOTYPAL PERSONALITY TRAITS AND CHRONIC CANNABIS USE

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Approximately 5.1 million Americans use cannabis on 20 or more days per month, making it the most popular illicit substance in the United States. Research during the past 20 years strongly supports the notion that cannabis (CB) use is an independent risk factor for the development of schizophrenia (SZ) spectrum disorders. This association may be due in part to the fact that SZ itself is linked with abnormalities of the endocannabinoid system. As predicted by these data, it has recently been found that increasing CB use rates may be a contributing factor in the higher incidence of SZ found in several populations. The purpose of the present study was to further the understanding of psychosis-proneness in CB users by quantifying and assessing dimensions of schizotypal personality disorder (SPD). To accomplish this goal, we contrasted CB-naïve controls with chronic CB users on measures of schizotypy and perceptual aberration. Our findings demonstrated that compared to non-users, CB users scored higher on traits associated with positive-syndrome schizotypy (e.g., suspiciousness) and disorganized-syndrome schizotypy (e.g. odd or eccentric speech or behavior). CB users did not, however, differ from non-users on traits associated with negative-syndrome schizotypy (e.g. social isolation, affective blunting). Importantly, total years of CB use positively correlated with scores on both the SPQ and the PAS. That is, the longer an individual consumed CB, the greater the number of traits within the schizotypy spectrum. Furthermore, there was no relationship between the number of alcoholic drinks consumed per week and PAS scores, SPQ total score, or SPQ dimensional scores. These data provide continuing support for the role of CB as an independent risk factor in schizophrenia spectrum disorders. Although it is unclear whether elevated schizotypy is a causal influence or consequence of heavy CB use, ongoing quantification and psychometric assessment may lead to a better understanding of the abnormalities arising from CB use, thereby providing stronger evidence for the specific role of the cannabinoid system in at-risk populations.

**DISRUPTION OF THE GAMMA-BAND AUDITORY STEADY-STATE EEG RESPONSE BY CANNABINOIDS IN HUMANS**

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Recent animal and cellular studies have shown that cannabinoid receptors (CB1) mediate GABAergic network oscillations in the gamma-range (~ 40 Hz), which may play an important role in the linking of neurons into cell assemblies during perceptual and cognitive processing. In order to test this in humans, the current study used electroencephalography (EEG) to examine the effect of chronic cannabis consumption on neural synchronization utilizing broadband (5-50 Hz) auditory steady-state responses (ASSRs). ASSRs were assessed employing varying rates of binaural stimulation (auditory click-trains at frequencies of 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 Hz). Chronic cannabis users (24 hours abstinence prior to study; positive THC urine drug test) and cannabis naïve controls were evaluated. Time-frequency analyses were performed using event-related spectral perturbation (ERSP) and inter-trial coherence (ITC) measures. ERSP analysis revealed a selective decrease in spectral power during 40 Hz stimulation in the cannabis group. No differences were observed between the groups in ITC. This frequency-specific deficit in the generation and maintenance of gamma-range neural synchrony is in concurrence with recent work in animals, further demonstrating a general role of the cannabinoid system in mediating GABAergic network oscillations.

# EFFECTS OF CANNABIGEROL (CBG) ON CONDITIONED AVOIDANCE RESPONDING IN RATS

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**Introduction**: Phytocannabinoids, which are extracted from *Cannabis sativa*, are thought to convey various benefits for human health. Recently, some reports indicated that cannabinoids have antipsychotic effects on animal models like apomorphine-induced stereotyped behaviour. However, the effects on conditioned avoidance response (CAR) paradigm remain poorly understood. This paradigm has traditionally been viewed as an animal model with high predictive validity for the screening of antipsychotic candidate compounds, since all clinically effective antipsychotics are known to specifically suppress the CAR.

**Materials and Methods***:* Male Wistar Han rats were used in this study. Animals were trained over several days to attain criterion (3 days with 75% avoidance responses). Well-trained rats were administrated with CBG (0.1-100 mg/kg) or vehicle and tested for conditioned avoidance. As well as CAR, inter-trial interval (ITI) crossing and avoidance latency (AL), which indicated possibility of motor abnormality and lowered responding, were measured.

**Results***:* Intraperitoneal treatment with CBG did not show any significant suppression on CAR at any dose tested. Moreover, no differences between vehicle- and drug-treated groups on ITI crossings and AL were found.

**Discussion**: This is the first report to analyse whether CBG suppressed CAR in rats. Our result indicates that CBG, even at high doses, is devoid of an effect. Given that antipsychotic drugs that show suppression of CAR are active through the dopaminergic system, it appears that CBG has no such activity and conveys only weak cannabinoid receptor activity that possibly leads to the modulation of other transmitters.

# POSTNATAL NMDA-INHIBITION INDUCES ALTERATIONS IN CB1 RECEPTOR EXPRESSION LEADING TO SCHIZOPHRENIC-LIKE BEHAVIOUR IN ADULTHOOD

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Schizophrenia affects 1% of the population and erupts mostly at late adolescence or early adulthood. This disease is associated with impairments of emotional and cognitive functions, which can be mimicked by phencyclidine (PCP), an NMDA receptor blocker. As the endocannabinoid system is intimately involved in brain wiring during CNS development, we hypothesised that early postnatal treatment of mice with PCP will induce changes in the endocannabinoid system and lead to "schizophrenia-like" behaviour. The effects of administering PCP (20 mg/kg) every other day between postnatal days 3 to 15 was evaluated with a variety of animal behaviour paradigms relevant to anxiety and symptoms associated with schizophrenia when animals had reached adulthood. We have also characterised the biochemical expression of CB1 receptors in different brain regions. CB1 receptor expression was evaluated by Western blot analysis by the end of the treatment. Fluorescent labelling of CB1 receptor antibody revealed two major bands at 42 kDa and 65 kDa, and a less bright band at 135 kDa that is thought to represent CB1 receptor dimers.

Alterations in CB1 receptor expression were observed in the cortex and brain stem. In the cortex, CB1 receptor expression was reduced by 17% while in the brain stem it was reduced by 28%. Two months after the PCP treatment, a significant increase in the acoustic startle reflex was observed in the treated animals compared to saline controls. Additionally, we observed a significant inhibition of the pre-pulse inhibition of the startle reflex (PPI) in the PCP-treated animals. Three months after PCP exposure, animals were tested in an 'open field' test for motor activity and in the 'plus maze' for 'anxiety-like' behaviour. The PCP-treated animals were significantly more hyperactive and displayed increased anxiety measures. Taken together, these results suggest that NMDA-induced alterations in the endocannabinoid system during postnatal development of the mouse brain contribute to the development of "schizophrenic-like" behaviour in adulthood.

# SEXUAL DIMORPHISM IN CB1R-MEDIATED RECOVERY FROM STRESS-INDUCED HPA AXIS ACTIVATION

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**INTRODUCTION**: The endocannabinoids (eCBs) play a modulatory role in circadian and stress-induced changes to the hypothalamic-anterior pituitary-adrenocortical (HPA) axis. Actions of eCBs have been confirmed in the paraventricular nucleus (PVN) of the hypothalamus in male rodents [Patel, S. Endocrinology, 2004. **145**: 5431.; Di, S. J Neurosci, 2003. **23**: 4850.] and the anterior pituitary in female rodents [Cota, D. Endocrinology, 2007. **148**: 574.]. However, attention has not been given to the possibility that demonstrated sexual dimorphism in CB1R hypothalamic expression or limbic forebrain affinity [Rodriguez de Fonseca, F. Life Sci, 1994. **54**: 159.] could contribute to the well known differences in stress responsivity between males and females.

**METHODS**: Male and female ICR mice (>7 weeks of age) were used in these studies. Wild-type (WT) and CB1R knock-out (KO) mice were littermates from in house breeding. WT mice for acute pharmacological studies were purchased from Harlan (Madison, WI). The CB1R antagonist, rimonabant (5 mg/kg i.p.) or vehicle were administered 30 minutes before restraint stress. Mice were restrained by tail taping to a table-top for 30 minutes. Blood was collected by trunk or tail blood, which were determined to have identical contents of corticosterone (CORT). Serum CORT content was measured by radioimmunoassay available from MP Biomedicals (Solon, OH).

**RESULTS**: Rimonabant significantly increased serum CORT concentrations in blood obtained 30 minutes after the end of the acute restraint stress period in both male and female mice. Rimonabant had no effect on CORT concentrations in blood obtained immediately after restraint in either sex. Male CB1R KO mice had significantly increased CORT levels after 30 and 60 minutes of recovery from restraint stress compared to WT. However, there were no significant differences in serum CORT concentrations following restraint stress between female WT and CB1R KO littermates.

**CONCLUSIONS**: Our data suggest that eCB signaling is required for appropriate recovery of CORT to basal levels following an acute psychological stress. This suggests a role for eCBs in long loop negative feedback rather than a role during initial activation of the PVN and anterior pituitary. Candidate sites of action for this robust physiological change are the prefrontal cortex, hippocampus, and amygdala. Our data also suggest that there are sex differences related to CB1R signaling that affect the ability to turn off the HPA axis. Specifically, it appears that female mice developmentally compensate for a genetically deleted CB1R; while male mice do not, despite significant changes in CORT. Further investigation into these sexually dimorphic differences is warranted due to a possible sex difference in psychiatric side effects when people are treated for obesity with rimonabant [Van Gaal, L. Diabetes Care, 2008. **31 Suppl 2**: S229.].

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# CLINICAL APPLICATION OF BASIC RESEARCH:CANNABIS AND CANNABINOIDS IN THE TREATMENT OF ADD/ADHD

David Bearman

Cannabis and cannabinoids effectively treat ADHD with fewer side effects than traditional sympathomimetic treatments(e.g. amphetamines , Ritalin®, Adderal, Strattera). With today's skyrocketing heath costs, here is a treatment that does not require pharmacies and health insurance. This paper will discuss the experience of doctors in California in treating ADD/ADHD with cannabis and cannabinoids. It will look at the work of Alger, Piomelli and others in discussing the role of retrograde inhibition and the endocannabinoid system in ADD/ADHD. More importantly it will suggest how cannabis and cannabinoids affect dopamine and impacts retrograde inhibition. The presentation will discuss a practical clinical application of some of what scientists have learned the endocannabinoid system .It will address clinicians treating patients with cannabis, cannabinoids and the prospect for using metabolic anandamide blockers for treating ADHD.

ADHD is a costly problem that strains medical/mental health, educational, and legal institutions[9]. ADHD afflicts 3-5% of Americans [10]. It is one of the most common psychiatric disorders of childhood[11] and in adults frequently results in tragic consequences in professional and personal lives[12]. ADHD is characterized by persistent impairments in attention (or concentration) and/or symptoms of hyperactivity and impulsivity[13]. The preponderance of studies show marijuana use is overwhelmingly prevalent with ADHD sufferers, either as a self-medicament or for recreation[14]. While some apply preconceptions that marijuana exacerbates ADHD almost all California cannabinologists believe camnabis and cannabinoids have substantially improved the lives of ADHD sufferers, and with less negative side effects than common stimulant drug ADHD treatments. As we have come to understand more about the brain and the role of dopamine and the endocannabinoid system we are starting to unravel how cannabis, anandamide and the synthetic delta 9 thc,dranabinol, act to free up dopamine and decrease the overstimulation of the midbrain

It turns out for many with ADD/ADHD cannabis or cannabinoids are a better choice than the traditional sympathomimetic drugs. For many years stimulants (sympathomimetic drugs) have been the mainstay for treating ADHD. They are thought to work by tying up dopamine transporter thus freeing up dopamine, previously bound to the dopamine transporter, thus having more free dopamine to engage in retrograde inhibition. Dopamine is essentially acting as a damper on neurotransmission by depolarizing the neuron that just released it. The stimulants main draw back is that they come with a host of unacceptable side effects-jitteriness, anxiety, sleep difficulty, appetite suppression and a propensity to be quick to anger.

It turns out that cannabis also frees up dopamine but it has a very benign side effect profile. Noting cannabis' vastly superior side effect profile DEA Administrative Law Judge, Francis L. Young, after a two-year hearing to reschedule cannabis in 1998 said:

> "Nearly all medicines have toxic, potentially lethal effects. But marijuana is not such a substance. There is no record in the extensive medical literature describing a proven, documented cannabis-induced fatality.... In strict medical terms marijuana is far safer than many foods we commonly consume ... Marijuana, in its natural form, is one of the safest therapeutically active substances known to man."

Clinicians have found that the results in treating ADHD with cannabis can be quite impressive .Patients report grades going from Cs and Ds to As and Bs. One of my patients said "I graduated from the Maritime Academy because I smoked marijuana", and another "I got my Ph. D because of smoking marijuana." Almost universally ADHD patients who therapeutically used cannabis reported it helped them pay attention in lecture, focus their attention instead of thinking of several ideas almost at the same time, helped them to stay on task and do their homework.

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# LACK OF BEHAVIOURAL AND NEUROMOLECULAR EFFECTS OF CHRONIC PUBERTAL WIN 55,512-2 TREATMENT IN ADULT RATS

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Puberty is a crucial ontogenetic period important for the development of normal behaviour in adults and is particularly vulnerable to the consequences of psychoactive drug exposure. In a previous study in rats in which food availability was restricted to maintain all rats within a defined weight range it was shown that pubertal exposure to cannabinoids (CB) results in psychotic-like symptoms, such as deficits in sensorimotor gating [as measured by disruption of pre-pulse inhibition (PPI)]. The present study investigated the effects of chronic peripubertal administration of the cannabinoid receptor agonist WIN 55,212-2 (WIN) and less restrictive food limitation than used previously on locomotor activity (open field), anxiety (elevated plus maze) and sensorimotor gating in adult rats. In addition, the effect of chronic pubescent WIN treatment and food limitation on expression of transcription factors pCREB and ΔFosB in discrete brain regions using immunohistochemistry assays was investigated.

Following weaning at 21 days of age (pd 21) male Sprague-Dawley rats were housed in groups of 5. Food and drinking water were available *ad libitum*. From pd 28 to pd 34, the rats were tested in the open field, on the elevated plus maze and in startle chambers. From pd 40 to pd 65 rats were injected i.p. with 1.2 mg/kg WIN or vehicle (10 days of one injection, 5 days of two injections and 10 days of no injection) administered in a pseudorandom manner. Half the rats in each treatment group had their food limited to 85% of the free-feeding quantity. From pd 77 to pd 83, rats were again tested in the open field, on the elevated plus maze and in startle chambers. The number of pCREB and ΔFosB positive cells was determined on pd 83. No variation in locomotor activity, anxiety or sensorimotor gating was detected in any of the experimental groups. These results suggest that experimental procedure of WIN treatment and food limitation during puberty does not induce any behavioural changes later in life. Consistent with the lack of behavioural changes, there was no significant change in pCREB or ΔFos B expression in any of the examined brain regions of adult rats following pubertal cannabinoid treatment and food limitation.

It is suggested from these results that more restrictive food availability is required for pubertal WIN administration to induce behavioural changes later in life.

# EFFECTS OF AM281 AND CANNABIGEROL PURIFIED FROM CANNABIS EXTRACTS ON STEREOTYPED BEHAVIOUR INDUCED BY APOMORPHINE

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**Introduction**: The cannabis plant has acquired much interest in recent years, not only because of the problems associated with its abuse, but also because of the therapeutic potential of many of its constituents. Some of these ingredients appear to have the potential as treatment against psychiatric disorders such as schizophrenia, anxiety, depression etc. It is well known that apomorphine hydrochloride (APO), which is a dopamine receptor agonist, induces stereotyped behaviour in rodents. This test has predictive value in terms of antipsychotics acting through postsynaptic $D_2$ receptors and was set up to determine the effects of the two putative cannabinoid receptor antagonists pure cannabigerol (pure CBG) and AM281.

**Materials and Methods**: Male Wistar Han rats (Harlan, UK) were used in this study. Apomorphine was injected at a dose of 6.4 mg/kg subcutaneously. At the same time, pure cannabigerol (pure CBG) at 0.1, 1, 10 and 100 mg/kg and the CB1 inverse agonist AM281 at 1, 3, 10 and 30 mg/kg were administered intraperitoneally. Stereotyped behavior was classified according to severity for 120 minutes and we evaluated inhibitory effects of pure CBG and AM281 on this animal behavior.

**Results**: Neither pure CBG nor AM281 suppressed stereotyped behaviour induced by apomorphine.

**Conclusion**: This is the first report which analysed pure CBG and AM281 for suppression of apomorphine-induced stereotypy in rats. While it is known that cannabidiol (CBD) can suppress 6.4 mg/kg apomorphine-induced stereotypy in rats, our results do not reinforce the notion of a CB1 mediated action of the CBD effect. However, we cannot exclude the possibility that CB1 receptor antagonists that are devoid of inverse agonist activity (see recently published compounds from the AM range) may be effective.

This work was supported by a collaboration between GW Pharmaceuticals and Otsuka Pharmaceuticals.

# CANNABINOID-INDUCED BEHAVIORAL EFFECTS IN A MOUSE MODEL OF AUTISM SPECTRUM DISORDERS

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The goal of this research is to determine the role that the endocannabinoid system plays in the development of behavioral changes associated with autism spectrum disorders (ASD) in a mouse model. The working hypothesis is that the ubiquitous endocannabinoid system (eCBs) may be involved in the biological processes involved in the development of behavioral and biochemical changes associated with autism. Much progress in cannabinoid research has revealed an endocannabinoid system in animals and humans. The eCBs consists of genes encoding cannabinoid receptors (CB1-Rs and CB2-Rs), their endogenous ligands called endocannabinoids and proteins that synthesize and degrade these endogenous cannabinoid ligands. Both CB1-Rs and CB2-Rs are distributed in the brain and peripheral tissues and are activated by endocannabinoids, and also the active ingredient in cannabis, $\Delta^9$-THC. We recently observed that the basal level of CB2A gene expression in the BTBR T+tf/J mice was upregulated in the cerebellum compared to control mice. In this study we used the BTBR T+tf/J mice that have been shown to exhibit autism-like behavioral phenotypes to evaluate cannabinoid-induced behavioral changes. The forced swim test (FST) that is a reliable model with predictive validity for screening anti-depressants and the wheel running activity that measured the basal locomotor activity of the animals were used to study the cannabinoid-induced behavioral effects. We first determined the basal behavioral effects of the BTBR mice in the forced swim test (FST) and spontaneous wheel running activity in comparison to those of C57BL/6Js and 129S1/SvImJ that are the background control mice. Then we evaluated the effects of $\Delta^9$-THC (1 and 10 mg/kg ip) following 20 mins of administration. For the FST, all animals were pre-exposed for 15 mins on day one prior to the 5 min- swim test on day 2. On test days animals were treated acutely for 20 mins and tested on the FST for 5 mins or 10 mins for the spontaneous wheel running behavior. Our preliminary results indicate that the BTBR mice exhibited an enhanced basal spontaneous locomotor behavior in the spontaneous wheel running test and a reduced depressogenic profile in comparison to the control animals. The responses appeared to be enhanced by $\Delta^9$-THC. In conclusion, these preliminary data provides a basis for further studies in evaluating the role of the various components of the endocannabinoid system in the etiology of autism spectrum disorders.

# ACUTE RESTRAINT STRESS ENHANCES HIPPOCAMPAL ENDOCANNABINOID FUNCTION VIA GLUCOCORTICOID RECEPTOR ACTIVATION

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**INTRODUCTION**: Exposure to stress normally triggers a complex, multi-level response from the activation of the hypothalamic-pituitary-adrenal (HPA) axis that serves to maintain homeostatic balance. Although the endocannabinoid system (ECS) is reported to be regulated by stress, few studies have directly addressed the changes caused by acute stress. Previous investigations have found that chronic unpredictable stress down-regulates the ECS, both the cannabinoid receptor (CB1R) and the endocannabinoid (eCB) levels are reduced. This work provides important insight into the consequences of prolonged stress, but it is a counter-intuitive response if the ECS is normally part of an endogenous anxiolytic system. If this is the case, then acute stress might up-regulate the ECS. We have tested this hypothesis using an acute restraint stress model in rats. **METHODS**: Male Sprague/Dawley rats, 4-5 weeks old, were restrained for 30 min in a clear Plexiglas chamber within which they could not turn around. After removal of the rat from the chamber, conventional 400-μm thick hippocampal slices were made from the animals and stored in a humidified atmosphere for >1 hr prior to use. CB1Rs are present on interneurons that express N-type $Ca^{2+}$ channels. The irreversible P/Q type $Ca^{2+}$-channel blocker, agatoxin, was present in the incubation medium to eliminate responses from non-CB1R expressing interneurons. Whole-cell recordings were made from CA1 pyramidal cells in standard, bicarbonate-buffered artificial CSF. Ionotropic glutamate receptor antagonists, AP5 and NBQX, were present to block excitatory synaptic responses and isolate inhibitory post-synaptic currents (IPSCs). Under these conditions the IPSCs evoked by stimulation in stratum radiatum are mainly large, basket-cell responses that are suppressed by cannabinoids. Endocannabinoids released from a pyramidal cell suppress the eIPSCs through a retrograde signaling process called DSI, which therefore serves as a bioassay for the ECS. DSI was induced by +70 mV voltage-steps (from the holding potential of -70 mV) that lasted 0.5 – 3 sec. **RESULTS**: DSI is enhanced in slices made >30 min after removal of the animal from the restraint chamber, but not if the slices were made immediately upon removal. The enhancement is dependent on the activation of glucocorticoid receptors, because the glucocorticoid receptor antagonist, RU 38486, given *in vivo* prior to the stressful restraint, prevented it. *In vitro* bath application of corticosterone increased DSI, thus mimicking the effects of stress. In contrast to the enhancement of DSI, which reflects $Ca^{2+}$-dependent mobilization of eCB, the peak effect of eCBs mobilized by $Ca^{2+}$-insensitive, muscarinic receptor activation was not altered, although its duration was prolonged, by stress. A possible target of stress, the inducible enzyme, cyclooxygenase-2 (COX-2), can degrade eCBs. We found that COX-2 is not involved in the regulation of the stress-induced up-regulation of DSI, since the prolongation of DSI caused by meloxicam, a selective COX-2 inhibitor, was not occluded by prior behavioral stress. COX-2 inhibition increased DSI in slices from stressed animals as much as it did in control slices. **CONCLUSIONS**: Our results indicate that acute stress up-regulates the $Ca^{2+}$-dependent hippocampal ECS, and suggest that the onset of the negative consequences of maintained stress may correlate with the inability of the ECS to continue functioning at a high level.

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# REMOVAL OF ADRENAL STEROIDS MODULATES CORTICOLIMBIC ENDOCANNABINOID SIGNALING

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Previous research has demonstrated that chronic stress and glucocorticoid administration decreases endocannabinoid signalling; however, the magnitude and direction of this effect appears to depend on the structure of interest. To examine the extent to which adrenal steroids regulate the endocannabinoid system we performed adrenalectomy surgeries, followed by replacement with a corticosterone pellet which produced basal levels of corticosterone, or a blank cholesterol pellet. Within the hippocampus, adrenalectomy resulted in an up-regulation of the $CB_1$ receptor and a downregulation of anandamide content; both of these effects were normalized following corticosterone replacement. Similarly, adrenalectomy up-regulated $CB_1$ receptor binding in the prefrontal cortex, but this response was only partially reversed by corticosterone replacement. Anandamide content in the prefrontal cortex was unaffected by adrenalectomy, however 2-AG content was significantly increased following adrenalectomy, which again was only partially reversed following corticosterone replacement. Within the hypothalamus, $CB_1$ receptor binding site density, anandamide content and 2-AG content were all reduced by adrenalectomy. Corticosterone replacement normalized anandamide and 2-AG content, but did not reverse the effects of adrenalectomy on $CB_1$ receptor binding. There was no effect of adrenalectomy on $CB_1$ receptor binding within the amygdala; however, the removal of adrenal steroids did result in a reduction in anandamide content, which was only partially reversed by corticosterone replacement. Taken together, these data demonstrate that adrenal steroids exert region specific regulation of endocannabinoid signalling. The relevance of these changes for processes such as neuroplasticity, neuroendocrine regulation and cognitive/emotional behaviour has yet to be determined.

# STUDIES OF POST-TRANSLATIONAL MODIFICATIONS OF NEURONAL MONOACYLGLYCEROL LIPASE

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Monoacylglycerol lipase (MAGL) is a serine hydrolase that hydrolyzes endocannabinoid, 2-arachidonoyl glycerol (2-AG), into arachidonic acid, and glycerol. Activity based protein profiling (ABPP) assay has shown that MAGL contributes to ~85% of 2AG hydrolysis in mouse brain [Blankman et. al. 2007, Chemistry and Biology 14(12): 1347]. Northern blot and in situ hybridization analyses revealed that MAGL mRNA is heterogeneously expressed in the rat brain with highest amounts in regions where CB1 cannabinoid receptors are also present (hippocampus, cortex, anterior thalamus, and cerebellum).

MAGL-mediated inactivation of 2AG is one of the major mechanisms to regulate (turn off) cannabinoid receptor type 1(CB1R) signaling; however, very little is known about the mechanisms by which MAGL activity is regulated. Western blot analyses of mouse and rat brain exhibit two MAGL immunoreactive bands, one migrating about 33kDa, its expected molecular weight, and another with a slightly larger size (~35kDa). The difference in migration on Western blot of MAGL-like immunoreactive proteins could be evidence of MAGL splice variants, isoforms, or post-translational processing. Primary sequence analysis of rat MAGL revealed two consensus sequences for N-glycosylation sites and several consensus sequences for phosphorylation by various protein kinases, including $Ca^{2+}$ /calmodulin kinase II and protein kinases A and C.

We carried out biochemical assays to characterize the post-translational modification of neuronal MAGL. Briefly, whole cell lysate was prepared by lysing cerebellar granule neurons in MPER buffer containing protease inhibitor. Lysates were centrifuged to eliminate unbroken cells and nuclei. Peptide N glycosidase F (PNGase F) was used in N-glycosylation experiments, and two-dimensional gel electrophoresis analysis (pH 3-10 linear immobilized pH gradient gel strips) followed by western blot analyses was performed to determine MAGL phosphorylation.

Our results indicate that neuronal MAGL is not N-glycosylated. Two-dimensional gel electrophoresis coupled with western blot analysis suggests that MAGL is phosphorylated. Interestingly, the patterns of phosphorylation between two MAGL immunoreactive bands are different. Identification of the specific phosphorylation sites and their role in the regulation of MAGL activity will help us better understand regulation of MAGL-mediated 2AG inactivation and CB1 receptor signaling.

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# LIPIDOMIC ANALYSIS OF ANANDAMIDE METABOLITES REVEALS NOVEL BIOTRANSFORMATION PATHWAYS

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Several enzymes are known to be involved with the metabolism and biotransformation of the endogenous cannabinoid anandamide. Our laboratory has previously demonstrated that the arachidonic acid backbone derived from anandamide can be recycled by cells to form new endocannabinoid molecules. We hypothesize that cellular pathways exist that direct the anandamide-derived arachidonate chain into a specific set of metabolites. This metabolic pool of arachidonate would be distinct from that which is derived from exogenously administered arachidonic acid. Using a stable isotope encoding strategy and liquid chromatography-mass spectrometry, we identified distinct pools of lipid metabolites derived from exogenous anandamide or arachidonic acid in RBL-2H3 cells. We discovered that arachidonic acid-derived metabolites were comprised of one main lipid class—eicosanoids, whereas anandamide-derived arachidonic acid, in addition to eicosanoids, was metabolized into diradylglycerols, fatty acid amides, sterols, and glycerophospholipids. The identification of differing metabolic fates of anandamide and arachidonic acid suggests that specific pathways exist for intracellular transport of each substrate and their associated metabolites. From the list of lipids that we identified as potential anandamide metabolites, one molecule in particular was of great interest to us because of its structural similarity to the known anandamide precursor N-acyl phosphatidylethanolamine (NAPE). The lipid was identified as 1-*O*-arachidonyl-*sn*-glycero-3-phosphocholine using the Lipid Metabolites And Pathways Strategy database (Lipid MAPS, http://www.lipidmaps.org) and verified via mass spectrometry of a synthesized standard. We determined that the levels of 1-*O*-arachidonyl-*sn*-glycero-3-phosphocholine were increased in RBL-2H3 cells after treatment with ionomycin. Furthermore, we determined that 1-*O*-arachidonyl-*sn*-glycero-3-phosphocholine was abundant in mouse brain tissue. Overall, our results provide a novel approach to study the metabolic fate of endocannabinoids and fatty acid-derived signaling molecules.

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# 1-METHOXY ANALOGS OF CP-47,497

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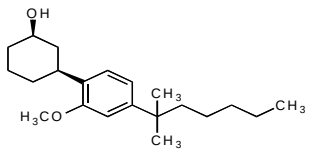
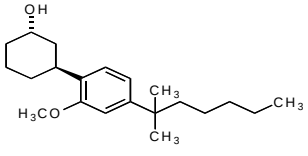
The Pfizer group used the potent synthetic cannabinoid (–)-9-nor-9β-hydroxy-hexahydrocannabinol as a template to develop new analgesics. This initiated the synthesis of nontraditional cannabinoids from which the Pfizer bicyclic cannabinoid CP-47,497 was synthesized. In view of the high $CB_2$ receptor affinities of several 1-deoxy-$\Delta^8$-THCs, a series of 1-deoxy analogs of CP-47,497 was synthesized (Huffman *et al Bioorg. Med. Chem.* **2008**, 16, 322). The 1,1-dimethylalkyl side chains of these compounds ranged from two to nine carbon atoms. The published data indicate that none of these compounds has better than modest affinity for either the $CB_1$ or $CB_2$ receptor. Of these compounds (1*R**,3*R**)-3-[4-(1,1-dimethyloctyl)phenylcyclohexanol (JWH-405) has the highest affinity for the $CB_2$ and $CB_1$ receptors with $K_i$ = 154 ± 8 nM and $K_i$ = 193 ± 3 nM, respectively. One conclusion from that study was that an oxygen substituent appended to the aromatic ring may be necessary for $CB_1$ or $CB_2$ receptor binding. To investigate this hypothesis, a series of 1-methoxy analogs of CP-47,497 was synthesized.

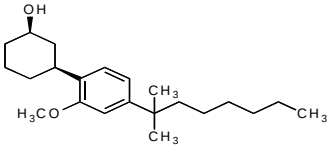
Three analogs, (1*R**,3*R**)-3-(2-methoxy-4-[1,1-dimethylhexyl])phenylcyclohexanol (JWH-442), its (1*R**,3*S**) isomer (JWH-440) and (1*R**,3*S**)-3-(2-methoxy-4-[1,1-dimethylheptyl])phenylcyclohexanol (JWH-441) were synthesized and their $CB_1$ and $CB_2$ receptor affinities were determined. Both JWH-442 and JWH-440 have little affinity for the $CB_2$ receptor ($K_i$ = 693 ± 80 and 553 ± 32 nM respectively) and poor affinity for the $CB_1$ receptor ($K_i$ = 4123 ± 1319 and 4414 ± 693 nM respectively). JWH-441 also has weak affinity for the $CB_2$ receptor ($K_i$ = 808 ± 32 nM) and no affinity for the $CB_1$ receptor ($K_i$ > 10,000 nM). It is apparent that the hypothesis that the addition of an aromatic oxygen functionality would improve $CB_1$/$CB_2$ binding in 1-methoxy CP-47,497 analogs (as it does for the 1-deoxy-$\Delta^8$-THCs) is incorrect. $CB_2$ receptor docking studies show that the total conformational cost for JWH-441 to dock at the receptor site is too high (10.4 kcal/mol) and negates any interaction that results from binding at the CB2 receptor site.



JWH-440



JWH-442



JWH-441

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# EFFECTS OF ACUTE VERSUS REPEATED INHIBITION OF FAAH ON ENDOCANNABINOID SYNTHESIS AND CATABOLISM IN THE MIDBRAIN AND LIVER

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Fatty acid ethanolamides (FAEs) including anandamide (AEA) and *N*-palmitoylethanolamine (PEA) are primarily produced by the action of *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD). Fatty acid amide hydrolase (FAAH) hydrolyses FAEs including AEA and PEA) and is widely distributed in the brain and peripheral tissues. Acute inhibition of FAAH by the irreversible inhibitor URB597 increases levels of AEA and produces a number of physiological effects, including analgesia (Fegley, et. al, 2005, Jhaveri *et. al*., 2008). The use of FAAH inhibitors for the treatment of clinical treatment of pain will require a repeated dosing regime, the cellular and molecular impact of which is unknown.

Here, the effects of acute and repeated administration of the FAAH inhibitor URB597 on levels of FAEs and 2AG, FAAH mRNA, protein expression and activity were determined. The effects of URB597 on the expression of NAPE-PLD, N- acylethanolamine acid hydrolysing amidase, (NAAA, another FAE catabolic enzyme) and monoacylglycerol lipase (MAGL, which hydrolyses 2-AG) were also studied.

Adult male Sprague-Dawley rats (200-250g) received intraperitonial injection of URB597 (0.3 mg kg-$^{1}$) or vehicle for one day (acute) or four days (repeated dosing). Rats were killed on day 1 or day 4 and midbrain and liver samples were collected and snap frozen in liquid nitrogen. FAAH, NAPE-PLD, NAAA and MAGL mRNA in each tissue was determined, relative to 18s RNA, using Taqman-based real-time PCR, while protein levels were assayed using immunoblotting. FAAH activity (measured as the hydrolysis of 100 μM oleamide) and tissue levels of AEA, PEA, OEA and 2AG were measured using a modified LC-MS-MS method based on previous work (Richardson et al, 2007). Statistical analysis on data from 4-6 rats was conducted using 1-way ANOVA with Bonferonni's multiple comparison test (for FAAH activity and mRNA data) unpaired t-test for protein data) or Mann-Whitney U test (for endocannabinoid data).

Acute and repeated dosing with URB597 significantly reduced FAAH activity in the midbrain (Acute ~ 50%, Chronic ~80%) and liver (Acute~50%, Chronic~80%). Acute URB597 significantly elevated levels of PEA and OEA in the midbrain, whereas AEA and 2AG were unaltered.There were no changes in levels of FAEs or 2AG in the liver. Repeated URB597 dosing significantly increased levels of AEA, PEA and OEA in the midbrain and PEA and OEA in the liver. Repeated, but not acute, dosing with URB597 significantly reduced FAAH and NAPE-PLD mRNA expression in the midbrain, but not the liver. Note, however, FAAH protein levels in the midbrain were unaltered by repeated URB597 treatment. MAGL and NAAA mRNA were unaltered by any treatments in either tissue.

Compared to the acute URB597 treatment, repeated dosing increased the degree of inhibition of FAAH activity in the midbrain and liver, which is reflected by more consistent elevations of FAEs in both midbrain and in the liver. These effects were associated with decreases in expression of FAAH and NAPE-PLD mRNA in the midbrain which suggests there may be longer-term adaptive changes in synthesis and catabolism of FAEs following sustained pharmacological inhibition of FAAH.

# AMINO ACID RESIDUES IMPORTANT FOR PH DEPENDENCY AND PROTEOLYTIC MATURATION OF *N*-ACYLETHANOLAMINE-HYDROLYZING ACID AMIDASE (NAAA)

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*N*-Acylethanolamine-hydrolyzing acid amidase (NAAA) is a lysosomal enzyme which hydrolyzes various *N*-acylethanolamines, including anandamide and *N*-palmitoylethanolamine (NPE), with optimal pH around 4.5. NAAA is activated by limited proteolysis, which also shows acidic pH optimum. In the present studies, we attempted to identify amino acid residues responsible for the characteristic pH dependency and proteolytic activation of human NAAA. We first constructed two single mutants of Glu-195 (E195A and E195Q) and overexpressed them in human embryonic kidney 293 cells. In the pH-dependent profile of NPE-hydrolyzing activity, the purified Glu-195 mutants did not exhibit a sharp peak around pH 4.5, which was typical of the wild type. Moreover, treatment of NAAA-expressing cells with concanamycin A, a reagent raising lysosomal pH, inhibited intracellular proteolysis of the wild type, but not of the mutants. The proteolysis of the purified mutant precursors was observed not only at pH 4.5 but also at pH 7.4, while that of the wild-type precursor occurred only at pH 4.5. Mutation of other seven glutamic acid residues of NAAA did not cause such an abnormal pH dependency. These results suggested an important role of Glu-195 in the pH dependency of NPE-hydrolyzing activity and proteolytic maturation. Furthermore, mutation of Cys-126, Arg-142, Asp-145, and Asn-287, all of which were presumed to be catalytically important, resulted in failure of the limited proteolysis. These results strongly suggested that the pH-dependent proteolysis occurs through an autocatalytic mechanism.

# NEXT GENERATION MOUSE MODELS FOR FATTY ACID AMIDE HYDROLASE

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Fatty acid amide hydrolase (FAAH) regulates the endocannabinoid anandamide and related *N*-acyl ethanolamines (NAEs) as well as the newly discovered class of substrates, the *N*-acyl taurines (NATs) (Saghatelian et al., 2004 *Biochemistry*, 43, 14332-9). Using a structure-guided approach, we designed a FAAH mutant with reduced NAT activity by introducing a negatively charged residue into the cytosolic access channel at sites predicted to produce charge-charge repulsion with the NAT sulfonate group (McKinney and Cravatt, 2006 *Biochemistry,* 45, 9016-22). This serine-268 to aspartate (S268D) mutation resulted in an enzyme that displayed wild-type kinetic parameters with NAEs, but severely reduced activity with NATs *in vitro*. Because the S268D-FAAH mutant would provide a valuable tool to selectively perturb the endogenous levels of NATs *in vivo*, we created S268D-FAAH knockin (KI) mice. S268D-FAAH-KI mice are viable, fertile, and have normal cage behavior. Initial biochemical characterization reveals that S268D-FAAH-KI mice have wild-type levels of protein expression and have elevated NAT levels while maintaining wild-type NAE levels. These S268D-FAAH-KI mice provide the first *in vivo* model to specifically examine the physiological functions of FAAH-regulated NATs by functionally uncoupling NAE and NAT hydrolysis.

In addition to the engineered mutation described above, we are also interested in the naturally occurring functional polymorphism in the human *FAAH* gene, C385A, which converts a conserved proline residue to threonine (P129T). This residue is located on the surface of the predicted cytoplasmic face of FAAH. In homozygous form, the P129T-FAAH variation has been associated with problem drug use and obesity in certain human populations (Sipe et al., 2002 *Proc. Nat. Acad. Sci.* 99, 8394-9). The P129T-FAAH variant also shows reduced expression and activity, suggesting that endocannabinoid tone could be altered in humans possessing this variation. To further investigate this premise, we have created a knockin mouse model that expresses the P129T-FAAH variant to study the physiological effects associated with this human polymorphism. Full characterization of this mutation is currently in progress.

In summary, we have identified structure-guided and natural mutations that alter the expression and function of FAAH and have generated mouse models to study these mutations *in vivo*. These next generation mouse models will be valuable tools to further characterize the FAAH degradative pathway.

# CHARACTERIZATION OF NOVEL MOUSE BRAIN 2-AG HYDROLASES

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Endocannabinoid signaling is terminated by enzymatic hydrolysis and endocannabinoid hydrolases are thought to be promising therapeutic targets for treating conditions such as anxiety, depression and pain. Inactivation of anandamide by fatty acid amide hydrolase (FAAH) has been well characterized through the use of recombinant FAAH protein, selective FAAH inhibitors and FAAH-deficient mice. Degradation of 2-arachidonoylglycerol (2-AG) has been less thoroughly characterized, though the recent development of the selective monoacylglycerol lipase (MAGL) inhibitor JZL184 has confirmed that MAGL is the primary brain 2-AG hydrolase responsible for ~85% of the total 2-AG hydrolase activity in the mouse brain [Long et. al. 2009, Nat Chem Biol 5(1):37-44]. This result agreed well with the findings of our previous survey of mouse brain serine hydrolases that ascribed ~85% of the 2-AG hydrolase activity in mouse brain membranes to MAGL and identified two novel 2-AG hydrolases, ABHD6 and ABHD12, which are responsible for the remaining ~15% activity [Blankman et. al. 2007, Chem Biol 14:1347-1356]. In order to begin to elucidate the biologically relevant functions of ABHD6 and ABHD12 and their roles in endocannabinoid mediated signaling, we have generated ABHD12-deficient mice (ABHD12 KO) and recombinant ABHD6 protein.

In order to study the function of ABHD12 *in vivo*, we have generated a mouse model bearing a targeted disruption of the abhd12 gene in the C57BL/6 background. ABHD12 KO mice lack active ABHD12 expression and are viable, fertile, and largely indistinguishable from wild-type littermates. Preliminary results indicate that ABHD12 KO brain membranes display ~40% less 2-AG hydrolase activity after MAGL inhibition following JZL184 treatment than wild-type membranes. To characterize the biochemical properties of ABHD6, we have generated a soluble, transmembrane-deleted ABHD6 isoform (ΔTM-ABHD6) in *E. coli*. ΔTM-ABHD6, but not the catalytically dead mutant ΔTM-ABHD6 S148A, can hydrolyze 2-AG *in vitro* and is sensitive to the ABHD6 inhibitor WWL70. Both ABHD12 KO mice and recombinant ΔTM-ABHD6 protein should prove to be valuable tools for the further characterization of these novel 2-AG hydrolases.

# CIRCADIAN PATTERNS IN mRNA EXPRESSION AND ACTIVITY OF FATTY ACID AMIDE HYDROLASE

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**Introduction**: All mammals display changes in gene activity, biochemistry, physiology, and behavior that wax and wane through the cycle of days and nights. Even when the organism is placed in constant conditions (such as continuous darkness), these rhythms persist – giving rise to the name circadian rhythms. However, without environmental cues, the period of the rhythms tend to be somewhat longer or somewhat shorter than 24 hours. Circadian rhythms are entrained to the light: dark cycle by visual information coming through the retina to the suprachiasmatic nucleus of the hypothalamus, which is the site of the master circadian clock in mammals. Many physiological systems are known to vary in expression and/or activity in a circadian manner; several studies suggest that endocannabinoid signaling is among these. In particular, Di Marzo and colleagues have shown that brain concentrations of the endocannabinoid, N-arachidonoylethanolamine (AEA) are different in brain tissue harvested in the day and night (*Cell Mol Life Sci*, 2004 Apr;61(7-8):945-50). We examined the hypothesis that the concentration of AEA changes between day and night because the expression of its catabolic enzyme, fatty acid amide hydrolase (FAAH) is circadian.

**Methods**: Wild-type male ICR mice 8 weeks old were maintained on a 12-hour light: dark cycle with lights on at 6:00AM and lights off at 6:00PM for at least two weeks. Mice were sacrificed at 6 time points throughout the day (3:00 AM, 7:00 AM, 11:00 AM, 3:00 PM, 7:00 PM, and 11:00 PM) and brains were harvested and dissected for biochemical assays. Quantitative PCR) was used to measure the amount of FAAH mRNA present. GAPDH mRNA was used as a control. The average changes in cycle threshold between FAAH and GAPDH mRNAs were compared at each time point. Enzyme activity assays for FAAH were performed in membranes harvested at each time point. Vmax and Km were determined using a one-site binding model. Data were analyzed by one-way ANOVA followed by t-tests if appropriate.

**Results**: In the hypothalamus, there was a significant difference in FAAH mRNA expression in brains harvested at 3:00AM and 7:00AM. The cycle threshold for mRNA detection was lower at 3:00AM than at 7:00AM, indicating that significantly more FAAH mRNA was present at 3:00AM. FAAH activity is significantly greater in membranes from mice sacrificed at 7:00AM than 3:00AM.

**Conclusions**: The studies done here indicate that the expression of FAAH is indeed circadian, with a significant change in mRNA expression and activity at the dark-to-light transition.

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# THE TUMOR SUPPRESSOR H-REV107 FUNCTIONS AS PHOSPHOLIPASE $A_{1/2}$ WITH A LOW *N*-ACYLTRANSFERASE ACTIVITY

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Recently, we identified $Ca^{2+}$-independent *N*-acyltransferase (iNAT) which transfers an acyl chain from phosphatidylcholine (PC) to the nitrogen atom of phosphatidylethanolamine (PE), generating *N*-acyl-PEs (NAPEs) known as precursors of anandamide and other *N*-acylethanolamines. During this study, we found H-rev107 as a protein structurally homologous to iNAT. H-rev107 was previously cloned as a negative regulator of proto-oncogene Ras and classified as a class II tumor suppressor. However, its molecular mechanism remained unclear. Based on the structural similarity between H-rev107 and iNAT, we examined a possible *N*-acyltransferase activity of H-rev107. We cloned cDNAs of H-rev107s from rat, mouse, and human, and transiently overexpressed the recombinant proteins in COS-7 cells. The cell homogenates could form [$^{14}$C]NAPE from [$^{14}$C]PC and non-radiolabeled PE. However, further examination with the purified recombinant rat H-rev107 revealed that the protein possesses a much more potent phospholipase (PL) $A_{1/2}$ activity for PC, releasing free fatty acid and lyso PC. The *N*-acylation and $PLA_{1/2}$-type hydrolysis were hardly affected by $Ca^{2+}$. Dithiothreitol was required for full activity, while iodoacetic acid, an SH-blocker, abolished the activity. Histidine-21 and cysteine-111 of H-rev107 were presumed to form a catalytic dyad based on database analysis and mutagenesis study. These results demonstrated that H-rev107 has capability of functioning as $Ca^{2+}$-independent $PLA_{1/2}$ of the thiol-type with a low *N*-acyltransferase activity.

# NAM INHIBITION OF MAGL: COMPARISON OF A COLORIMETRIC AND RADIOMETRIC ASSAY

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

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

Incubation of cell lysate protein (5μg) with varying concentrations of arachidonoyl-1-thio-glycerol yielded a $K_m$= 67.96 ± 26.09 μM and $V_{max}$= 659.5 ± 81.76 nmol/min/mg (mean± SEM; n=3). This $K_m$ is similar to the $K_m$ of 67.8 ± 4.0 μM for hydrolysis of 2-AG by MAGL in rat cerebellar membranes (Saario et al., Biochem. Pharmacol. (2004) 67:1381-7). Membrane and cytosolic fractions contributed about equally to hydrolysis with 49.6±2% and 50.4±2% activity respectively (n=3). The rate of MAGL hydrolysis increased linearly with up to 11 μg COS7 cell lysate protein. Mutation of the catalytic serine (Ser122) to an alanine residue reduced MAGL activity by 95.3±2% (n=3; P<0.0001). This loss of enzyme activity was seen in membrane and cytosolic fractions.

Saario et al. (Chem. Biol. (2005) 12:649-656) tested the potency of several *N*-ethylmaleimide analogs and discovered that *N*-arachidonylmaleimide (NAM) was the most efficient inhibitor ($IC_{50}$=140 nM), but inhibition of wild-type MAGL by NAM was only detectable at high NAM concentrations (≥ 10 μM) using the colorimetric assay. Cys208 and Cys242 were proposed as potential residues near the catalytic site that react with NAM. Cys208Ala and Cys242Ala mutants were indistinguishable from wild-type MAGL when tested in the absence of inhibitor, but were inhibited by NAM (10 μM). These colorimetric assay data suggested that neither of these cysteine residues were essential for NAM inhibition of MAGL. To confirm these results NAM inhibition of cys-to-ala mutants was examined using a conventional radiometric method. Inhibition of MAGL hydrolysis of $^3$H-2-oleoylglycerol (100 μM) by NAM was detectable at lower inhibitor concentrations (300-750 nM). Percent NAM inhibition of Cys208Ala decreased whereas inhibition of Cys242Ala increased. Percent inhibition of a double cys-to-ala mutant was similar to inhibition of Cys242Ala. This reveals that Cys208 is the more essential residue for NAM inhibition as demonstrated by recent studies using human MAGL (Zvonok et al. Chem. & Bio. (2008)15:854-862). Although the colorimetric assay employed here is useful as a means to measure MAGL hydrolytic activity due to fast reaction times and low protein quantities needed, comparison with a radiometric assay reveals that it is not sensitive enough to detect inhibition at low concentrations. This colorimetric assay may be used as a tool for initial high throughput screening of inhibitors, but subsequent sensitive measurements should be carried out using other means.

# 4-NITROPHENYLACETATE AS A POTENTIAL SUBSTRATE FOR MAG LIPASE: INVESTIGATION OF AN ASSAY SUITABLE FOR HIGH-THROUGHPUT SCREENING USING RAT TISSUES

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A recent report identified that 4-nitrophenylacetate (NPA) was a useful substrate for MAG lipase in recombinant preparations, although hydrolysis by esterases other than MAG lipase in rat tissues limited its usefulness (Muccioli et al., ICRS2008). Here we have explored whether NPA could be utilised in a high-throughput screen for MAG lipase from rat liver.

Compounds at 100 μM were pre-incubated in 96-well microtitre plates with rat liver cytosolic fractions in the absence (total NPA hydrolase activity) and presence of 1 μM MAFP for 15 min at 37°C, prior to addition of NPA. Hydrolysis of 250 μM NPA was measured by monitoring absorbance of 4-nitrophenol product at 405 nm, following 15 min incubation. Our underlying hypothesis was that compounds which inhibited total NPA hydrolase activity, but not hydrolysis in the presence of MAFP were likely to be MAG lipase inhibitors.

In one day, 137 compounds were screened at 100 μM as single replicates using four preparations of rat liver cytosol. Absorbance in the absence of inhibitors was typically 1.6 AU, reduced to 0.5 AU in the presence of MAFP. The majority of compounds tested failed to inhibit total NPA hydrolase activity, without altering activity in the presence of MAFP. Examples of inactive compounds included NSAIDs (e.g. salicylate, diclofenac, diflunisal, nimesulide, ibuprofen and indometacin), as well as flavonoids (e.g genistein, daidzein, flavone and flavanone) and endogenous ethanolamides (OEA, PEA, SEA). A number of PPAR ligands (e.g. gemfibrozil, bezafibrate, fenofibrate, clofibrate, WY14643, GW6471, rosiglitazone), cannabinoid receptor ligand (WIN55212-2) and TRPV1 ligands (NADA, capsaicin) were also inactive. Many flavonoid compounds appear to elevate background hydrolysis (e.g. quercetin, morin, myricetin, rutin, kaempferol, curcumin, fisetin), as did dopamine.

GW0742 (90 %), PPAHV (90 %), AEA (83 %), CBD (85 %), all-*trans*-retinoic acid (77 %), barbiturate (85 %), carbenoxolone (86 %) and 7-hydroxyflavanone (70 %) evoked small, but statistically significant reductions of MAFP-inhibitable NPA hydrolysis.

We conclude that NPA is a useful substrate for screening native tissue MAG lipase activity for potential inhibitors.

We thank the MRC for funding.

# TARGETED STABLE-ISOTOPE DILUTION GC-MS/MS DETERMINATION OF THE ENDOCANNABINOID ANANDAMIDE AND OTHER FATTY ACID ETHANOL AMIDES IN HUMAN PLASMA

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Ongoing clinical research projects in our group required a sensitive and specific assay for anandamide (AEA) and other fatty acid ethanol amides (FAEAs) that could cope with small sample volumes. Most currently available data on FAEAs have been generated by using the LC-MS/MS methodology. We developed a stable-isotope dilution GC-MS/MS method for the quantitative determination of AEA, oleic acid ethanol amide (OEA) and palmitic acid ethanol amide (PEA) in human plasma.

We applied single solvent extraction of FAEAs and their internal standards from plasma (50 – 1000 μl) with toluene and a two-step derivatization to the pentafluorobenzamide pentafluoropropionyl derivatives (FAEA-PFBz-PFP) with pentafluorobenzoyl chloride and pentafluoropropionic anhydride. Then, we simultaneously quantified FAEAs by selected reaction monitoring of the respective fatty acid carboxylate anions. The anions were produced by collision-induced dissociation (CID) of the parent ions $[M - PFBz]^-$.

The present method was fully validated for anandamide. Accuracy and precision of the method were within the range of 100 ± 20% and less than 20%, respectively, in the concentration range 0 - 4 nM. Mean overall recovery was 90 ± 3%. The LOQ and LOD values of the method were 0.25 nM of added AEA in plasma samples and 400 amol of injected AEA-PFBz-PFP derivative, respectively. In plasma of 16 healthy individuals plasma AEA concentration was 1.35 ± 0.32 nM. Among the 16 subjects, we observed no correlation between plasma AEA concentrations and gender or body mass index. The underlying mechanism during CID was similar for OEA and PEA and their plasma concentration were approximately 10 nM. We also identified stearic acid ethanol amide (SEA), linolenic acid ethanolamide, and linoleic acid ethanol amide in human plasma. A considerable amount of OEA, PEA and SEA may have been released from laboratory materials and was reduced when we applied glass ware.

We developed a stable-isotope dilution GC-MS/MS method for the quantitative determination of AEA, oleic acid ethanol amide (OEA) and palmitic acid ethanol amide (PEA) in human plasma using their stable-isotope labeled analogs as internal standards. The methodology is straightforward, accurate, precise and highly specific for FAEA.

# MONOACYL GLYCEROL LIPASE (MGL) LIMITS THE DURATION OF ENDOCANNABINOID-MEDIATED DEPOLARIZATION-INDUCED SUPPRESSION OF EXCITATION (DSE) IN AUTAPTIC HIPPOCAMPAL NEURONS

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The endocannabinoid signaling system is the target of the psychoactive components of marijuana and hashish. This system is active throughout much of the CNS where it plays a key role in modulating neurotransmission. Depolarization-induced suppression of excitation (DSE) is a major form of short-term retrograde neuronal plasticity and is found in numerous brain regions. Autaptically cultured murine hippocampal neurons are an architecturally simple model for the study of cannabinoid signaling, including DSE. In autaptic cultures DSE is mediated by the endocannabinoid 2-arachidonoyl glycerol (2-AG). 2-AG is made post-synaptically and travels across the synapse in a retrograde fashion to activate presynaptic cannabinoid $CB_1$ receptors. The transient nature of DSE – tens of seconds – is likely determined by the regulated hydrolysis of 2-AG. No less than five candidate enzymes have been considered to serve this role: fatty acid amide hydrolase (FAAH), cyclooxygenase-2 (COX-2), monoacyl glycerol lipase (MGL), α/β-hydrolase domain 6 and 12 (ABHD6 and ABHD12). We previously found that FAAH and COX-2 do not have a role in determining the duration of autaptic DSE.

We now report that while an inhibitor of ABHD6 (WWL70) does not alter the time course of DSE, two structurally distinct inhibitors of MGL (NAM (*N*-arachidonoyl maleimide) and JZL184) markedly prolong DSE in autaptic hippocampal neurons. This represents the first evidence for a role of MGL in modulating DSE duration. In addition, we developed antibodies against MGL and ABHD6 and determined the expression of these enzymes in autaptic cultures. MGL is chiefly expressed at presynaptic terminals, and is therefore optimally positioned to break down 2-AG that has engaged presynaptic $CB_1$ receptors. ABHD6 is also expressed in two distinct locations on autaptic islands, including a prominent localization in some dendrites.

In summary, we provide strong anatomical and functional evidence that MGL regulates the duration of DSE in autaptic hippocampal neurons.

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# THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ LIGAND TROGLITAZONE IS A POTENT INHIBITOR OF MONOACYLGLYCEROL LIPASE

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Monoacylglycerol lipase (MGL) is primarily responsible for the metabolism of the endocannabinoid 2-arachidonoylglycerol in the brain. Until recently, potent selective inhibitors of the enzyme were not available. Long et al. (Nature Chem Biol 5 [2009] 37-44) have reported the characterisation of JZL184 as a selective irreversible inhibitor of the enzyme and demonstrated that the behavioural consequences of MGL inhibition are different from those seen following inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for the metabolism of anandamide. In the present study, we have conducted a screen of a small chemical library of compounds available at the Department with the aim of identifying reversible MGL inhibitors that can serve as lead compounds for synthetic programmes.

MGL activity was measured at room temperature using the spectrophotometric assay of Muccioli et al. (ChemBioChem 9 [2008] 2704-10), 4-nitrophenyl acetate (0.25 mM unless otherwise stated) and commercially available recombinant human MGL as enzyme source.

A series of 96 compounds were tested for MGL inhibitory activity at concentrations of 3 and 10 μM. The major groups of compounds used were NSAIDs, neuroleptic drugs, anti-depressives, benzodiazepines, cannabinoids and natural products. At the lower concentration, there were three "hits" (defined as causing >60% inhibition), namely arachidonoyldopamine, CP 55,940 and the peroxisome proliferator-activated receptor γ (PPARγ) ligand troglitazone. At the higher concentration AM404, JWH015 and WIN 55,212-2 were also "hits". Subsequent experiments indicated that troglitazone inhibited MGL with an $IC_{50}$ value of 1.2 μM. In comparison, the PPARγ ligands ciglitazone, rosiglitazone and CAY10415 had $IC_{50}$ values of 2.5, 29 and 49 μM, respectively, whilst CAY10514 produced <50% inhibition at the highest concentration tested (100 μM). The inhibition produced by troglitazone was not affected by preincubation, in contrast to that for JZL184, where $IC_{50}$ values of 180 and 17 nM were found following preincubation times of 0 and 60 min, respectively. Using a substrate concentration range from 0.08 to 0.64 mM, troglitazone was found to inhibit MGL in a competitive manner with a $K_i$ value of ~0.4 μM. Using a different assay with rat brain cytosolic preparations and 2-oleoylglycerol (0.5 μM) as MGL substrate, troglitazone was found to be active, albeit with a lower potency than seen in the recombinant MGL assays. The compound did not inhibit FAAH, measured in rat brain membranes with 0.5 μM anandamide as substrate.

It is concluded that troglitazone can be described as a dual-action MGL inhibitor - PPARγ agonist. Given that rosiglitazone is less potent than troglitazone as an MGL inhibitor, whilst the reverse is true for their interaction with PPARγ, it may be possible to use troglitazone as a template for the design of novel reversible MGL inhibitors.

# SIMULTANEOUS ANALYSIS OF 2-ARACHIDONOYLGLYCEROL, ARACHIDONIC ACID AND SELECTED DIACYLGLYCEROLS BY LC-ESI-MS/MS

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**Introduction**: The endocannabinoid 2-arachidonoylglycerol (2-AG) has been implicated in several physiological and pathophysiological processes. 2-AG is thought to be biosynthesized by the action of diacylglycerol lipase (DAGL) on various diacylglycerols (DAGs) that contain an arachidonate moiety at the *sn*-2 position. This DAGL-mediated hydrolysis produces 2-AG and arachidonic acid (AA). Here we report a quantitative analytical method for the DAGs *1-stearoyl-2-arachidonoylglycerol* (SAG) and *1-oleoyl-2-arachidonoylglycerol* (OAG), as well as 2-AG and AA from murine brain tissue. This method is LC-MS/MS-based and utilizes the ability of the silver cation to coordinate with unsaturated lipids as an ionization strategy for the neutral 2-AG and DAGs.

**Methods**: Lipids were purchased from Cayman Chemicals except for OAG, which was synthesized in-house. LC-MS/MS analysis was accomplished using a Thermo Surveyor pump and autosampler in-line with a Quantum triple quadrupole mass spectrometer. Electrospray ionization (positive ion mode) was used. Chromatography occurred in the reverse phase with gradient elution. The mobile phase contained 80 μM silver acetate.

The analytes were detected via selected reaction monitoring (SRM) as $[M+Ag]^+$ complexes (except AA, which formed a $[(M-H)+2Ag]^+$ complex under the described conditions). We used the following SRM transitions (the mass in parentheses represents the mass of the deuterated internal standard): AA (*m/z* 519(527)∧409(417)); 2-AG (*m/z* 485(493)∧411(419)); SAG (*m/z* 751(759)∧411(419)) and OAG (*m/z* 749∧411). Quantitation was achieved via stable isotope dilution against the deuterated analogs of AA, 2-AG and SAG while OAG was quantitated relative to SAG-d8.

Micropunches collected from murine brain tissue (mass: ~5mg) were prepared by homogenizing the sample in acetonitrile followed by sonication for 30 min in an ice bath. Samples were then centrifuged and the supernatant removed, dried and reconstituted in Methanol-$H_2O$, 9:1 (v/v) prior to LC-MS/MS analysis.

**Results and Conclusions**: Upon MS/MS analysis, the $[M+Ag]^+$ complexes of 2-AG and the DAGs fragment to produce an intense ion corresponding to $[AA+Ag]^+$. This fragmentation pattern is the basis for the SRM transitions used to detect 2-AG, SAG and DAG. These compounds, in addition to AA, were detected with abundant signal:noise ratios in micropunches taken from murine amygdala. Thus, the described method allows sensitive and accurate quantification of the endocannabinoid 2-AG, its metabolic products and DAG biological precursors from small, homogenous brain regions. This methodology may be modified for the analysis of other DAGs and monoacylglycerols.

# CANNABIGEROL BEHAVES AS A POTENT ALPHA-2-ADRENOCEPTOR PARTIAL AGONIST

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We have reported previously that the little-investigated plant cannabinoid, cannabigerol (CBG), can stimulate binding of [$^{35}$S]GTPγS to brain membranes with high potency ($EC_{50}$ = 0.2 nM) but with an efficacy ($E_{max}$ = 16%) well below that of CP55940 ($E_{max}$ = 79%) (Gauson et al., 2008). It is unlikely that CBG produces such stimulation by activating $CB_1$ receptors as it displaces [$^3$H]CP55940 from specific binding sites on brain membranes with much lower potency ($K_i$ = 440 nM) and as it behaves as a $CB_1$ receptor antagonist/inverse agonist with an apparent $K_B$ value of 736 nM for antagonism of CP55940 (Gauson et al., 2008). The present investigation was directed at identifying the mechanism underlying CBG-induced stimulation of [$^{35}$S]GTPγS binding to mouse brain membranes.

Experiments were carried out with adult male MF1 mouse or adult C57BL/6J $CB_1$ knockout mouse whole brain membranes using [$^{35}$S]GTPγS binding assays (Thomas et al. 2007) or with adult MF1 mouse isolated vasa deferentia in which the measured response is inhibition of electrically-evoked contractions (Thomas et al., 2005). CBG was obtained from GW Pharmaceuticals. Yohimbine was dissolved in water and all other compounds in DMSO.

We first obtained confirmatory evidence that CBG is not a $CB_1$ receptor agonist. More specifically, we showed that CBG stimulates binding of [$^{35}$S]GTPγS to C57BL/6J $CB_1$ knockout mouse whole brain membranes ($EC_{50}$ = 0.2 nM; $E_{max}$ = 10.3%) and also that it produces a concentration-related inhibition of electrically-evoked contractions of the vas deferens ($EC_{50}$ = 78 nM) in a manner that is not attenuated by a concentration of rimonabant (100 nM) that does antagonize CP55940. Next, we showed that this inhibitory effect of CBG in the vas deferens was blocked by yohimbine, a selective $\alpha_2$-adrenoceptor antagonist, at both 100 and 316 nM. The apparent $K_B$ value of yohimbine for this antagonism (12.5 nM) did not differ significantly from its apparent $K_B$ value for antagonism of clonidine (19.9 nM), suggesting that CBG is an $\alpha_2$-adrenoceptor agonist. In support of this hypothesis, we also found that yohimbine can antagonize CBG-induced stimulation of [$^{35}$S]GTPγS binding to MF1 mouse brain membranes with an apparent $K_B$ (0.6 nM) similar to that for its antagonism of two selective $\alpha_2$-adrenoceptor agonists, clonidine (0.7 nM) and dexmedetomidine (3.9 nM) in the [$^{35}$S]GTPγS binding assay.

Further experiments are required to establish whether CBG also behaves as a $\alpha_2$-adrenoceptor agonist in vivo and, if so, whether it shares the ability of established $\alpha_2$-adrenoceptor agonists to relieve pain either by itself or in combination with other compounds.

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# BINDING AFFINITIES OF MINOR CANNABIS CONSTITUENTS TO CB1 AND CB2 RECEPTORS

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Cannabis is very complex in its chemistry due to the vast number of its constituents and their possible interaction with one another. These compounds represent almost all of the chemical classes, e.g. mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others. The best-known and the most specific class of cannabis constituents is the $C_{21}$ terpenophenolics, the cannabinoids, with (-)-$\Delta^9$-*trans*-(6a*R*,10a*R*)-tetrahydrocannabinol (**$\Delta^9$-THC**) being the most psychologically active constituent.

The availability of high potency marijuana on the illicit market with unprecedented **$\Delta^9$-THC** concentrations (>20% by dry weight), has renewed our interest in the discovery of new constituents from *C. sativa* L. As a part of our program aimed at the discovery of psychoactive constituents from *C. sativa* L., we herein report the binding affinities of minor isolated constituents to CB1 and CB2 receptors.

Fifty two isolated compounds were evaluated in CB1 and CB2 receptor binding assays in Sf9 membrane preparations obtained from Perkin Elmer using [$^3$H]CP55,940 as the radioligand. The CB1 receptor binding assays showed that two compounds have significant binding affinities ( 0.8 and 5.5 nM, respectively), much greater than that of $\Delta^9$-THC (100 and 20 fold, respectively). Seven compounds have affinities (46.2-141.6 nM), comparable with that of $\Delta^9$-THC. On the other hand, four compounds showed lower affinities for CB1 receptors (224.1-552.9 nM) and other four compounds were essentially inactive (725-2,263 nM). Five compounds showed significant binding affinities ( 5.9–116.0 nM) for CB2 receptors. Two of them were very selective since they showed no affinities for CB1 receptors. One Compound showed the highest binding affinity for both CB1 and CB2 receptors, exceeding the synthetic positive control, CP55,940.

# CHARACTERIZATION AND IN VIVO EVALUATION OF A CONTROLLED RELEASE DELIVERY SYSTEM FOR $\Delta^9$-TETRAHYDROCANNABINOL ADMINISTRATION

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## Introduction

Delta-9-tetrahydrocannabinol (THC) has been shown to be clinically useful for a large variety of indications, including nausea and vomiting associated with chemotherapy and AIDS-related anorexia (which are FDA-approved), spasms in multiple sclerosis, chronic neuropathic pain, cancer and glaucoma, among others.

Despite this promising clinical potential, an optimal dosage form has not been marketed yet. This may be due to its viscous tar-like nature and its low aqueous solubility, which difficult its handling and therefore, its formulation. The only marketed formulation is a soft gelatin capsule for oral administration (Marinol®). However, cannabinoid oral absorption is slow and erratic, due to its high lipophilicity and its high first-pass metabolism.

Therefore, the aim of the present study was to develop a controlled-release delivery system for THC parenteral administration, which would facilitate its handling and dosing.

## Materials and methods

The controlled drug delivery systems were prepared by combining the drug with biocompatible and biodegradable polymers. The obtained formulations were characterized in terms of drug-excipient compatibility and miscibility, and *in vitro* drug release, simulating *in vivo* conditions. Furthermore, their *in vivo* efficacy in a subcutaneous glioblastoma multiforme xenograft model in nude mice was also evaluated.

## Results

A sustained-release delivery system suitable for parenteral administration was obtained. The differential scanning calorimetry (DSC) studies showed a drug-excipient miscibility (the drug was dispersed at molecular state into the polymeric matrix) and compatibility. *In vitro* release studies revealed a sustained release of THC for a 10-day period.

The *in vivo* experiment on GBM xenograft model demonstrated the efficacy of these systems. Gliomas´ size decreased considerably in the THC treated animals versus the mice treated with the placebo preparation.

In conclusion, the present investigation suggests the potential efficacy of these systems as an alternative formulation for THC administration.

## Acknowledgments

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# EFFECTS OF TETRAHYDROCANNABIVARIN (THCV) AND RISPERIDONE ON MK-801-INDUCED SOCIAL RECOGNITION DEFICITS IN RATS

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**Introduction** - Social withdrawal and social memory deficits are negative/cognitive symptoms of schizophrenia. Antipsychotics only showed minor or no improvement of such symptoms. Cannabinoid antagonists proved promising therapeutic potentials in schizophrenia, especially on the positive symptoms. However, little is know on their effects on cognitive and negative symptoms. Schizophrenics present with glutamate and cannabinoid system abnormalities and this may suggest an essential (and maybe cross-linked) role of these two systems in schizophrenia. We here induced a schizophrenic-like cognitive deficit by administering MK-801 to rats tested in a social interaction test to investigate whether THCV and risperidone can prevent cognitive deficits.

**Methods** - Male Lister hooded rats (Harlan UK) received either vehicle, risperidone (Otsuka, Japan; 0.1 mg/kg), or THCV (GW Pharmaceutical; 0.5, 3, 30 mg/kg) 1 hr prior to test, and saline or MK-801 (0.08 mg/kg) 30 minutes prior to test. Rats received 10 minutes habituation to the arena, 10 minutes sociability test with a juvenile rat enclosed into a transparent and perforated holder, and 10 minutes social recognition test where a second juvenile was placed opposite side to the first. Inter trial interval was 2 minutes. Social recognition would occur if the tested rat spends longer time with the novel co-specific during the recognition stage of test.

**Results** - Nor THCV or risperidone prevented the MK-801-induced social recognition deficit. THCV (30 mg) alone induced preference for familiarity. Sociability was normal in all groups, a part of THCV 30 mg/kg-treated rats. This dose of THCV may have induced unspecific side effects, since locomotor activity was reduced, this impacted on normal sociability.

**Conclusion** - This is the first investigation on effects of THCV in a social recognition deficit induced by NMDAR blockage as a model of schizophrenic-like symptoms in rats. At the tested dose-range, THCV had no effects on cognitive deficits, nor did risperidone. THCV is a CB1/CB2 receptor antagonist with a biphasic action in *in vivo* tests, with high doses acting as CB1R agonist; in the light of this, a wider dose-range including low doses is auspicial. Furthermore, THCV increases GABA release *in vitro* and this action correlates with a reduction in spontaneous excitatory transmission, this may indicate a possible glutamate release inhibition *in vivo*, which may contribute to explain the present results

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# THE GENOTOXICOLOGY OF PHYTOCANNABINOIDS

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**Introduction:** The toxicity of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) is well established and reported - however, little is known about the pharmacology and toxicity of the so-called "minor cannabinoids". Using standard plant breeding techniques, GW Pharma Ltd have bred specific strains (chemotypes) of *Cannabis sativa* L. which contain high levels of a number of principal cannabinoids including delta-9-tetrahydrocannabivarin (THCV), Cannabidivarin (CBDV), Cannabigerol (CBG), Cannabigerovarin (CBGV) and Cannabichromene (CBC). These compounds have differing pharmacology. Here, we report for the first time the genetic toxicology of novel Botanical Drug Substances (BDSs) prepared from the GW chemotypes.

**Methods:** CBD BDS, CBDV BDS, THCV BDS, CBG BDS, CBGV BDS and CBC BDS were evaluated for genotoxicity using standard regulatory genotoxicity assays: an in vitro bacterial reverse mutation assay (Ames Test, AT) and an in vivo model of genoxtoxicity (Rat Micronucleus Test, RMT).

The objective of the AT is to evaluate the mutagenic potential of a test article by its effects on one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system. Doses of principal cannabinoid up to 5000µg / plate were tested in the AT. In the RMT, the aim is to test whether the test compounds produce aneuploidy (affect spindle formation or function) in erythroblasts in the bone marrow undergoing their last chromosome replication. Chromosome fragments or whole chromosomes, which are unable to attach to the spindle, are left behind as micronuclei when the main nucleus is extruded in the production of the polychromatic erythrocyte (PCE) - micronuclei can be clearly seen in these cells and can be easily counted. Doses of BDSs ranged from 250-500mg/kg of principal cannabinoid in the RMT.

**Results:** None of CBD BDS, CBDV BDS, THCV BDS, CBG BDS, CBGV BDS and CBC BDS produced any evidence of genotoxicity in either the Ames Test or in the Rat Micronucleus Test. There was considerable plasma exposure of each compound in the RMT assay.

**Conclusion:** There was no evidence of genotoxicity of CBD BDS, CBDV BDS, THCV BDS, CBG BDS, CBGV BDS and CBC BDS, using standard genotoxicity assays which are compliant with European and FDA regulatory requirements. These compounds have differing pharmacology and are thus suitable for clinical development.

# FROM PLANT POLYPHENOL STRESS PATHWAYS TO THE ENDOCANNABINOID SYSTEM: CO-EVOLVED MODULATION OF BEHAVIOUR AND BIOENERGETICS

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It is now becoming clear that plants and animals have many similarities, including using redox and fatty acid signalling, both of which modulate mitochondrial function. Many 'stress molecules' in both also have multiple functions in signalling, development, growth modulation, stress resistance and inhibition of pathogens. This commonality may have very ancient origins, as indicated by the fact that both kingdoms utilise programmed cell death (PCD) for development, stress and pathogen resistance; critical in this is the mitochondrion. In plants, two well described groups of "wound response" compounds, the jasmonates and salicylates, modulate mitochondrial function in animal cells. Other polyphenols also share the same property. Chemically, many display both pro- and anti-oxidant activity, depending on context and concentration. Mild inhibition of mitochondrial function by polyphenols can, at the right dose, have a hormetic effect – co-ordinately upregulating mitochondrial biogenesis and resistance to oxidative stress. For instance, resveratrol can increase activity of the Sirt1, FOXO, AMPK and PGC-1α, mimicking calorie restriction, improving exercise capacity and increasing lifespan, but it can also kill cancer cells. Recent data now show it also has activity at CB receptors. Equally, phytocannabinoids can modulate mitochondrial function. We have suggested previously that the endocannabinoid system (ECS) could be viewed as endohormetic, transmitting stress signals in both an autocrine and paracrine fashion by modulating redox and mitochondrial function. We have also suggested that the metabolic syndrome may arise from a lack of hormesis, which results in decreased metabolic flexibility, increased oxidative stress and rising insulin resistance. Without hormesis in a calorie-rich environment, animals may have a 'sedentary' tipping point, which leads to a feed forward cycle of mitochondrial dysfunction, metabolic inflexibility, ectopic fat deposition and inflammation. This may also result in 'inflammatory-induced sickness behaviour' that reduces incentive to exercise. In contrast, calorie restricted animals are known to increase their exercise activity; thus, there may also be a 'movement' tipping point. Calorie restriction also induces mitochondrial biogenesis. As many polyphenols are increased in plants during periods of stress, it is possible that some animals, including humans, have adopted/become dependent on these signals to provide information about the environment. We propose that some polyphenols may not only induce mitochondrial biogenesis (readying the animal for migration), but also increase the urge to move to find new food sources, while suppressing excessive appetite. Hence, because of the many biochemical similarities between plants and animals, the polyphenol stress system in plants may modulate the ECS in animals and could have 'xeno-psychoergomimetic' properties. In summary, we propose that there may exist an as yet unidentified plant version of the ECS, which can play an important role in modulating the metabolism and behaviour of modern animals. This concept could shed further light on the mode of action of the phytocannabinoids and the function of the ECS, as well as future treatment paradigms for the metabolic syndrome.

# EVALUATION OF THE PURIFIED THCV AND PLANT EXTRACTS RICH IN THCV IN THE MOUSE IRWIN TEST

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**Introduction**: The hemp plant *Cannabis sativa* has acquired much interest over the last few years. However, a systematic observational analysis for most of the constituents (phytocannabinoids) has not been performed to date. Thus, we set out to determine the effects of some phytocannabinoids, purified Delta(9)-Tetrahydrocannabivarin (pure THCV) and its extract from the plant, on the central and the peripheral nervous system.

**Methods**: Pure THCV and THCV extract were evaluated in the neurobehavioral observation battery at 3, 10, 30, 100 mg/kg doses after *intraperitoneal (i.p.)* administration, using ICR mice aged 6-11 weeks (Harlan UK). Animals were examined in the Irwin test battery (Irwin S., Psychopharmacologia 1968;13:222-257) with modifications. They were analysed at several time points of up to 25hrs after administration. At each time point, the observations started in the home cage, then mice were transferred into a novel cage, where they were evaluated for signs of distress in behaviours (e.g., reactivity, locomotor activity). Then additional abnormalities were assessed by handling the mice (e.g., startle response, grip strength, reflexes).

**Results**: Neither cannabinoid caused serious toxicity such as epilepsy or death. Although Pure THCV led to changes in several parameters, most of these alterations were minor and not reliable. However THCV extract caused significant changes in several parameters (e.g. reactivity, body posture) over 10mg/kg.

**Conclusion**: Pure THCV can readily be assessed in doses up to 100mg/kg, although careful attention is required to dissociate side effects from direct effects on animal models. Minor cannabinoid constituents in THCV extract could cause several side effects, and behavioural tests utilizing THCV extracts may require special care to dissociate side effects.

# ANTI-CONVULSANT PROPERTIES OF $\Delta^9$-THCV IN ADULT RATS IN CHEMICALLY-INDUCED SEIZURES

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**Introduction***:* Cannabis has been ascribed both pro- and anti-convulsant properties in epilepsy patients, however little is known about the potential pro- or anti-convulsant properties of the more than 60 cannabinoids present in cannabis. Following our previous report (Weston et al., 2006) demonstrating anti-epileptiform effects of $\Delta^9$-tetrahydocannabivarin ($\Delta^9$-THCV) *in vitro*, we have investigated the anti-convulsant potential of $\Delta^9$-THCV in an *in vivo* rat model of generalised seizure induced by pentylenetetrazole (PTZ).

**Methods***:* 64 adult male rats were injected intra-peritoneally (I.P.) with either 0.025, 0.25 or 2.5mg/kg $\Delta^9$-THCV (GW Pharmaceuticals), or with the $\Delta^9$-THCV vehicle (1:1:18 ethanol:Cremophor:saline) alone. 30 minutes later, animals were injected with 80mg/kg PTZ I.P. to induce seizures. Seizure activity was observed and recorded to video (Farrimond et al., 2009). Seizure analysis was adapted from Pohl and Mares (1987) and coded using behavioural analysis software (The Observer Pro, Noldus). This yielded information describing median seizure severity, seizure incidence and latency from PTZ injection to specific markers of seizure activity. Animals that received $\Delta^9$-THCV were compared to vehicle-only controls.

**Results***:* Doses of 0.25 and 2.5mg/kg $\Delta^9$-THCV both exerted significant anti-convulsant effects following PTZ administration when compared to vehicle. 2.5mg/kg $\Delta^9$-THCV significantly delayed seizure activity onset (vehicle: 33.2±3.5s, 2.5mg/kg $\Delta^9$-THCV: 59.1±8.8s; p=0.02; Mann-Whitney U test) and also significantly delayed the development of more severe seizures (vehicle: 146.9±52.8s, 2.5mg/kg $\Delta^9$-THCV: 417.1±116.3s; p=0.013; Mann-Whitney U test). Additionally, animals that received 0.25mg/kg doses of $\Delta^9$-THCV exhibited a lower median maximum seizure severity although this difference was not significant. More interestingly, 0.25mg/kg $\Delta^9$-THCV significantly increased the percentage of animals exhibiting no seizure at all following PTZ administration. 12.5% of vehicle treated animals experienced no seizure activity; however this percentage rose to 33.3% in animals that received 0.25mg/kg $\Delta^9$-THCV (p=0.031).

**Conclusion***:* $\Delta^9$-THCV exerts significant anti-convulsant activity in this chemically-induced rat model of seizure. $\Delta^9$-THCV may thus be partially responsible for the anti-convulsant effects of cannabis reported by some epileptics. These data, combined with a reported absence of psychoactive effects suggest that $\Delta^9$-THCV represents a promising novel therapeutic strategy for the treatment of epilepsy.

# PHYTOCANNABINOID AFFINITIES AT $CB_1$ RECEPTORS IN THE MOUSE CEREBELLUM

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**Introduction:** $\Delta^9$-tetrahydrocannabivarin ($\Delta^9$-THCV) acts as a cannabinoid $CB_1$ receptor ($CB_1R$) antagonist in the CNS (Thomas *et al*., 2005; Ma *et al*., 2008). We have reported $\Delta^9$-THCV effects on [$^{35}$S]GTPγS binding in mouse cerebellar and piriform cortical membranes (Dennis *et al*., 2008), suggesting that $\Delta^9$-THCV exerts a non-competitive antagonist effect at $CB_1R$ at sub-micromolar concentrations. Here, radioligand binding assays were undertaken to further investigate $\Delta^9$-THCV's mode of action and compare the affinity of synthetic and other major phytocannabinoids at cerebellar $CB_1R$.

**Methods:** Cerebellar membranes were prepared from adult NIHS mice (9-11 weeks). Saturation binding assays were performed using [$^3$H]SR141716A (50 pM-10 nM). Competition binding assays *vs* 1 nM [$^3$H]SR141716A were carried out using the synthetic $CB_1R$ agonist WIN55212-2, the antagonists SR141716A and AM251, and the phytocannabinoids $\Delta^9$-THCV, cannabidiol (CBD) and cannabigerol (CBG) (1 pM-1 mM).

**Results:** Saturation binding experiments for [$^3$H]SR141716A yielded a $K_D$ of 1.61 ± 0.35 nM in cerebellar membranes, and a $B_{max}$ of 1.19 ± 0.62 pmol/mg (n=3, each in triplicate). $K_i$ values for cannabinoid ligands are detailed in the table below. Data were best fitted to a one-site binding model with the exception of WIN55,212-2 which best fitted to two-site binding. All ligands caused a concentration-dependent decrease in [$^3$H]SR141716A binding. Interestingly, at 100 μM $\Delta^9$-THCV, [$^3$H]SR141716A binding no longer fully plateaued. Moreover, competition curves for CBD and CBG did not fully plateau due to maximal solvent concentrations being reached.

| **Ligand** | **p$K_i$ ± s.e.m** | ***n*** |
|---|---|---|
| AM251 | 9.43 ± 0.08 | 4 |
| SR141716A | 8.95 ±0.04 | 3 |
| WIN55,212-2 - p$K_h$ (% $R_h$ = 33.31 ± 6.25) | 7.95 ± 0.18 | 4 |
| WIN55,212-2 - p$K_l$ | 6.03 ± 0.07 | 4 |
| $\Delta^9$-THCV | 6.44 ± 0.13 | 3 |
| CBG | 4.53 ± 0.19 | 3 |
| CBD | 4.25 ± 0.43 | 3 |

**Conclusions:** $\Delta^9$-THCV behaved similarly to synthetic antagonists in [$^3$H]SR141716A competition assays, but with a lower affinity for $CB_1Rs$. The present evidence does not determine the nature of antagonism by $\Delta^9$-THCV. To further investigate a potential allosteric mechanism, competition with an agonist radioligand may be warranted (Horswill *et al*., 2007, Thomas *et al*., 1998). Although CBD and CBG displaced [$^3$H]SR141716A, these phytocannabinoids showed only low affinity for $CB_1Rs$ in the cerebellum.

# PLANT WISDOM: CANNABIDIOL SYNERGIZES WITH $\Delta^9$-TETRAHYDROCANNABINOL TO INHBIT HUMAN GLIOBLASTOMA PROLIFERATION AND SURIVAL

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Naturally occurring plant cannabinoids have notable anticancer activity. $\Delta^9$-THC is currently being used in a clinical trial for the treatment of recurrent GBM. Of interest are potential compounds that can enhance the anti-cancer effects of cannabinoids. It has been shown that plant derived cannabinoids that do not interact efficiently with $CB_1$ and $CB_2$ receptors, can modulate the actions of $\Delta^9$-THC. The non-psychoactive cannabinoid, CBD has also demonstrated anti-cancer effects and can also modulate the effects of $\Delta^9$-THC *in vitro* and *in vivo.* We screened synthetic and natural $CB_1$ and $CB_2$ agonists for anti-proliferative effects against human GBM cell lines and found that CBD was the most potent inhibitor of GBMs. Therefore, we tested combinations of THC and CBD against GBMs.

In U251 and SF126 human cell lines, $\Delta^9$-THC co-administered with CBD, acted synergistically to inhibit GBM cell growth. The combination treatment also produced a greater induction of apoptosis. The inhibitory properties of the combination were mediated through the $CB_2$ receptor and relied on the inhibition of ERK and the G1/S transition of the cell cycle. Furthermore, an increase in production of oxygen radicals and activation of caspases was also observed. Signal transduction mechanisms associated with the synergistic effect of the combination treatment were significantly different from those observed with the individual compounds. This study suggests that the addition of CBD to $\Delta^9$-THC may improve the overall potency and efficacy of $\Delta^9$-THC in the treatment of patients with GBM.

# CB2 CANNABINOID RECEPTOR-MEDIATED S-NITROSYLATION OF MATRIX METALLOPROTEINASE IN ENDOTHELIAL CELLS: A NOVEL MECHANISM FOR THE REGULATION OF ANGIOGENESIS

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CB2 Cannabinoid receptors are known to express in a variety of endothelial cells. However, the role of CB2R in endothelial cells and vasculature is not well characterized. In the current study we showed that activation of CB2R in EAhy926 human endothelial cells stimulate endothelial nitric oxide synthase to produce nitric oxide. Using RT-PCR and zymography we showed that CB2R activation increased MMP-2 (matrix metalloprotease-2) mRNA expression and MMP-2 activity respectively in EAhy926 cells. We also showed that activation of CB2R produce in vitro angiogenic responses that can be blocked by CB2R antagonist SR144528 and NOS-inhibitor L-NAME. Further, we found that activation of CB2R produced S-nitrosylation of MMP-2 and thereby increased MMP-2 activity in EAhy926 cells. Blockade of NO production inhibited CB2R-mediated increase in MMP2 activity and blockade of S-nitrosylation resulted in a decrease in CB2R-mediated angiogenesis respectively. Collectively these results suggest that a) in endothelial cells CB2R is functionally coupled to nitric oxide production and MMP activation and b) S-nitrosylation of MMP2 can act as an angiogenic switch in the regulation of angiogenesis

# ANANDAMIDE METABOLISM IN GINGIVAL CREVICULAR FLUID AND PERIODONTAL TISSUE

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**Introduction**: The existence of cannabinoid receptors has been reported in human gingival fibroblast and endogenous levels of AEA have also been identified in the gingival crevicular fluid (GCF). The analysis of GCF - either in the absence or in the presence of periodontitis - may prove to be a useful approach, because of the simplicity of the constituents of the fluid and also in consideration of the fact that GCF samples may be collected through non-invasive methods. Here, we report on the role played by the elements of the ES that metabolize AEA in the periodontal inflammatory processes, based on the assumption that bacteria may be able to trigger the release of eCBs in the periodontal tissue, and in particular in the GCF, in order to exert an anti-inflammatory action.

**Methods**: The subjects were recruited from the patient population and clinically evaluated at the Department of Dentistry of the Tor Vergata University of Rome (Italy), after obtaining their informed consent.
Twenty-seven subjects were selected, 13 with periodontal inflammation and 14 as healthy controls. Samples of GCF were taken from the gingival sulcus. The samples of crevicular fluid were collected through micro-aspiration from periodontal locations at four sites per each selected tooth: mesio-vestibular, mid-vestibular, disto-vestibular, and mid-lingual. The gingival crevicular fluid collected was immediately transferred to airtight vials and stored at - 80°C, until the activity of enzymes responsible for the degradation (FAAH) and synthesis (NAPE-PLD) of anandamide was assayed.

**Results***:* Notwithstanding the small quantity of specimens that could be obtained from each patient, we were able to demonstrate the presence of active NAPE-PLD and FAAH, the two main enzymes responsible for the synthesis and degradation of anandamide, respectively. Based on our radiochromatography technique, we could demonstrate FAAH activity in tiny samples like gingival crevicular fluid, and could show that this activity is regulated in the context of an inflammatory process like periodontitis. Indeed, in patients suffering from this disease FAAH activity increased by ~3.5-fold compared with controls, reaching high statistical significance ($p<0.0001$). On the other hand we observed no differences in NAPE-PLD activity.

**Conclusion**: This work has demonstrated for the first time the presence of active FAAH and NAPE-PLD in gingival crevicular fluid. Taken together, these experimental findings suggest the existence of a functional endocannabinoid system in the gingival district, actively involved in inflammatory process.

# WIN55, 212-2 PREVENTS LEUKOTRIENE-INDUCED VOMITING IN THE LEAST SHREW (Cryptotis parva)

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Chemotherapy-induced vomiting (CIV) is a severe side effect of cisplatin-like drugs. These drugs activate multiple signaling systems, including inflammatory, and potentially emetogenic arachidonic acid (AA) metabolites such as prostaglandins and leukotrienes. In particular, the emetic role of the leukotriene family, including the cysteinyl leukotrienes ($LTC_4$, $LTD_4$ and $LTE_4$), has been neglected. The aims of this study were to test the emetic potential of key leukotrienes (LTA4, LTB4, LTF4, and the cysteinyl leukotrienes), and to investigate whether the $CysLT_1$ leukotriene receptor antagonist pranlukast or the cannabinoid $CB_{1/2}$ activation can prevent the induced emesis. For the determination of emetic dose-response curves, Least shrews were injected with varying doses (0.025 - 4 mg/kg, i.p., n= 7-12) of one of the six tested leukotrienes (n= 7-12 shrews per dose), and emetic parameters were recorded for the next 30 minutes. $LTC_4$ and $LTD_4$ were most efficacious, and significant increases in the frequency and percentage of animals vomiting occurred at their respective 0.1 and 0.05 mg/kg doses, while maximum emetic effects were seen at 1 mg/kg. The other tested leukotrienes were either weakly emetic or ineffective at doses up to 1 mg/kg. The profile of cysteinyl leukotriene-induced vomiting ($LTC_4$=$LTD_4$>$LTE_4$) suggests $CysLT_2$ receptors have a key role in emesis induction. However, the CysLT1 antagonist, pranlukast, dose-dependently, and at 10 mg/kg completely, blocked $LTC_4$-induced vomiting, implicating $CysLT_1$ receptor activation in leukotriene-induced vomiting. The cannabinoid $CB_{1/2}$ agonist WIN55, 212-2 (0, 0.5, 1 and 2.5 mg/kg, i.p., n = 8-12) also attenuated the induced emesis but in a more potent fashion, where complete blockade of vomiting was observed at its 2.5 mg/kg dose. Fos immunoreactivity was also measured subsequent to $LTC_4$-induced vomiting to define the anatomical substrates involved. Significantly more Fos-immunoreactive nuclei were noted in the enteric nervous system and medullary dorsal vagal complex following $LTC_4$ ($p < 0.05$) versus vehicle injections. In conclusion, this study is the first to show that some leukotrienes induce emesis, possibly involving both central and peripheral $CysLT_1$ and $CysLT_2$ receptors. In addition, activation of cannabinoid $CB_{1/2}$ receptors also prevents the cysteinyl leuktriene-induced vomiting.

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# CANNABINOID CB1 RECEPTOR BLOCKADE AT BIRTH CAUSES GROWTH FAILURE DUE TO ORAL MOTOR WEAKNESS AND IS MEDIATED BY PERIPHERAL MECHANISMS

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We have shown previously that a single exposure to cannabinoid CB1 receptor inverse agonists/antagonists within 24 h from birth, resulted in impaired milk suckling and growth failure. We proposed this syndrome in mouse pups as a first animal model for "non-organic failure-to-thrive" (NOFTT) which is a debilitating condition occurring in 2-5% of human infants. In the present study we wanted to further investigate the underlying physiological/psychomotor mechanisms underlying CB1 receptor blockade-induced growth failure.

**Methods**: Two series of experiments were performed using ICR mice. 1. In order to examine whether oral-motor competence is responsible for the inability of the experimental pups to suck milk, they were presented with a 'surrogate' nipple filled with milk (16% fat). 2. Since food intake-relevant CB1 receptors are expressed in hypothalamic areas and since food intake is also regulated by peripheral, including intestinal CB1 receptors, we also wanted to determine whether the growth failure induced by CB1 receptor antagonism is mediated by central or peripheral CB1 receptors. We injected SR141716 (SR1) (CB1 antagonist/inverse agonist) directly into the neonatal brain (within 24 h from birth), at 2 different depths (2 or 3 mm) and at various doses and compared growth, survival and milk ingestion with that in subcutaneously injected pups.

**Results**: 1. SR1-(s.c.) injected pups, displaying severe growth failure, ingested milk and gained weight after presentation with the surrogate nipple and thus gained significantly more weight than SR1-treated pups which were only allowed to ingest milk by sucking from the maternal nipple. 2. Control experiments, using Methylene-blue (ethidium bromide) or the anesthetic ketamine (1 mm lateral right from bregma and 0.5-1.0 mm posterior to bregma, volume 4 μl, using atlas by Paxinos et al 2007), indicated that the intracerebral injection technique delivered the compounds throughout a wide area from the injection point. Intracerebral SR1 injections (4.5-22.5 μg), did not result in growth failure, impaired milk ingestion or deficient suckling, as opposed to the severe FTT observed after peripherally delivered injections of SR1 (20-40 mg/kg).

**Conclusions**: These observations suggest that 1. Similarly to NOFTT infants, and supporting earlier data, oral-motor weakness seems to be responsible, at least partially, for the impaired milk suckling in CB1 receptor-blocked pups. 2. The deleterious effects of CB1 receptor blockade on neonatal milk ingestion is mediated by peripheral mechanism(s), perhaps through CB1 receptors found on peripheral nerves innervating the oral musculature. However, CB1 receptors located in organs such as the GI tract may also play a role, perhaps by interfering with 'Brain-Gut' bilateral communication. Thus, neonatal CB1 receptor blockade is a useful model to increase our understanding of NOFTT and to examine therapeutic approaches. Further, the putative role of CB1 receptors situated at various peripheral locations should be studied in more depth.

# CIRCULATING ENDOCANNABINOIDS ARE RELATED TO BLOOD PRESSURE IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA

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**Introduction:** Obstructive sleep apnea (OSA) episodes chronically increase blood pressure through sympathetic nervous system activation. In animals, arterial hypertension and sympathetic activity are restrained by the endocannabinoid system. Therefore, we hypothesized that the blood pressure increase found in OSA patients is associated with increased circulating endocannabinoid concentrations.

**Methods:** In 55 OSA patients, we recorded arterial oxygen saturation and hypnea/apnea epidsodes in the sleep laboratory. We measured seated blood pressure as well as fasting insulin, glucose, IL-6, and high-sensitive CRP. Insulin sensitivity was determined by euglycemic-hyperinsulinemic clamp testing. In addition, we conducted an oral glucose tolerance test. Anandamide (AEA), the sum of 1-AG and 2-AG, and OEA were measured by LC-MS/MS after organic extraction.

**Results:** The OSA cohort consisted of 29 patients with normal glucose tolerance and 26 patients with type 2 diabetes mellitus. The groups were well matched in terms of age, body composition, blood lipids, blood pressure and OSA parameters. AEA was correlated with blood pressure ($r = 0.59$ for systolic and $r = 0.58$ for diastolic blood pressure, $p < 0.001$). The correlation remained significant after adjusting for OSA severity, anthropometry, or inflammatory markers as possible confounders. The relationship was strongly attenuated after correction insulin sensitivity. 1/2-AG and OEA were not correlated with blood pressure.

**Conclusion:** OSA patients feature a positive correlation between blood pressure and venous AEA concentrations. Whether excessive endocannabinoid release predisposes to insulin resistance in this setting or *vice versa* remains to be shown.

# ALTERATIONS OF BASAL AND FASTING/REFEEDING-REGULATED TISSUE LEVELS OF ENDOGENOUS *N*-ACYL-ETHANOLAMINE LIGANDS OF PPAR-α IN OBESE ZUCKER RATS

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The peroxisome proliferator-activated receptor-α (PPARα) is a nuclear receptor deeply involved in the control of energy balance. The anandamide congeners, *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA), exhibit high and intermediate potency at PPARα and exert anorectic and anti-inflammatory effects in rodents, respectively. OEA also stimulates lipolysis in the adipose tissue and liver, and inhibits insulin release from the pancreas, and its tissue levels change during *ad lib* feeding, fasting and refeeding in these metabolism-controlling tissues. Here we provide new data on OEA and PEA tissue level regulation by the nutritional status in genetically obese rats.

Seven week-old wild type (WT) and Zucker rats fed *ad libitum* or following overnight food deprivation with and without refeeding were examined for their brainstem, duodenum, liver, pancreas and visceral (VAT) or subcutaneous (SAT) adipose tissue OEA and PEA levels, which were measured by liquid chromatography-mass spectrometry.

In WT rats, OEA, but not PEA, levels in the duodenum were reduced by food deprivation and re-augmented by refeeding, whereas the opposite was observed for OEA in the pancreas, and for both OEA and PEA in the liver and SAT. No changes were observed in the brainstem and VAT.

In *ad libitum* fed Zucker rats, PEA and OEA levels were up to 10-fold higher in the duodenum, slightly higher in the brainstem, and strongly reduced in the other analysed tissues. Fasting/refeeding-induced changes of OEA levels were maintained in the duodenum, liver and SAT of these obese rats, and lost in the pancreas, whereas food deprivation up-regulated this compound also in the VAT.

The observed changes in OEA levels in WT rats are potentially relevant to the described actions of this mediator on satiety, hepatic and adipocyte metabolism, and insulin release. The dysregulation of OEA in Zucker rats might represent an adaptive reaction to counteract hyperphagia, in the duodenum and brainstem, but might contribute to hyperinsulinemia, in the pancreas, and to increased basal lipogenesis, in the adipose tissues and liver. The observed decreases in PEA levels, particularly in the pancreas, liver and adipose tissue, instead, might be relevant to the typical inflammatory state of Zucker rats.

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# EFFECT OF HIGH-FAT DIET ON THE SENSITIVITY TO DELTA-9-TETRAHYDROCANNABINOL IN FEMALE LONG-EVANS RATS

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To date, the two most well-characterized endocannabinoids are anandamide (AEA) and 2-arachidoylglycerol (2-AG), with 2-AG being more abundant in the nervous system. Both 2-AG and AEA are lipids that are derived from the n-6 family of essential polyunsaturated fatty acids, which are readily available through diet. Consumption of different high-fat diets can significantly change levels of AEA and 2-AG in the rat brain and intestines High polyunsaturated and monounsaturated fatty diets have been shown to increase 2-AG in the rat brain, whereas AEA levels increased only after consuming a high monounsaturated fatty diet (Hansen and Artmann, 2008, Biochem. Biophys. Acta, 1781(4):200-12.). Furthermore, the observation of dietary influenced changes in endocannabinoid release has directed research towards examining the effects of a high polyunsaturated fat diet on CB1 receptor density. For example, mice fed a high fat diet for 20 weeks showed downregulation of CB1 receptors in the substantia nigra and the ventral tegmentum, but not in the hypothalamus (South and Huang, 2008, J. Neuroendocrinoly. 11:1288-94). The purpose of the present study was to determine whether consumption of a high polyunsaturated diet during adolescence would alter sensitivity to the pharmacological effects of delta-9-tetrahydocannabinol. These experiments fed a group (n=9) of female Long-Evans rats a high polyunsaturated diet (33% of total calories from fat) for a total of 14 weeks beginning on postnatal day (PD) 30 through PD 114. The control group received normal rat chow (13.5% of calories from fat) over the same developmental period. A cumulative dosing procedure (0, 3, 10, 30, 100 mg/kg) was used to determine an entire dose-effect curve during a single session that included the experimental triad tests measuring spontaneous locomotion, body temperature, and time spent with forepaws on an elevated bar. Testing was performed on PD30, PD44, PD68, and PD114. Results showed that the effects of THC in the three tests did not differ before initiation of different diets (i.e., on PD30). On PD68 and PD114, however, female rats fed a high fat diet were less sensitive to the effects of THC on locomotion and/or in the bar test. There were no significant differences between groups in THC-induced hypothermia on any test day. These results suggest that consumption of a high fat diet beginning in adolescence reduced sensitivity to some, but not all, of the pharmacological effects of THC in female rats. These results are consistent with the observation of decreased CB1 receptor binding in parts of the brain following consumption of a high fat diet. Further experimentation will evaluate pharmacological selectivity of these effects by examining sensitivity to the effects of other cannabinoids and non-cannabinoid drugs in female rats that have been fed a high fat diet.

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# FERTILIZING ABILITY OF HUMAN SPERM IS CONTROLLED BY TRPV1 VANILLOID RECEPTOR

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**Introduction:** Human spermatozoa express type-1 cannabinoid receptor (CB1R), whose activation by anandamide (AEA) affects motility and acrosome reaction (AR). In this study, we further characterized the AEA-related endocannabinoid system (ES) in human spermatozoa and we investigated the effect of capsazepine (CPZ) on sperm/oocyte fusion, highlighting the involvement of the AEA-binding vanilloid receptor (TRPV1) in the fertilizing ability of human spermatozoa.

**Methods:** Semen samples were collected from healthy normozoospermic donors, according to the World Health Organization-recommended procedure. Endocannabinoid levels were determined by high performance liquid chromatography-tandem mass spectrometry. ES were characterized by radiometric and immunochemical assays. The effect of CPZ on the sperm/oocyte fusion was tested by means of the progesterone (P)-enhanced hamster egg penetration test (HEPT), sperm motility was estimated with computer-assisted semen analysis (CASA) and AR assessment was evaluated by fluorescent technique.

**Results:** Human spermatozoa, besides expressing CB1R and TRPV1 receptors, exhibit all the other components of the ES needed to synthesize and degrade AEA, that are all functioning. Immunoreactivity for CB1R, NAPE-PLD and FAAH was localized in the postacrosomal region and in the mid-piece, while for TRPV1 it was restricted to the postacrosomal region. CPZ inhibited P-enhanced sperm/oocyte fusion, because of a reduction of the P-induced AR rate above the spontaneous AR rate, which was instead increased. The sperm exposure to OMDM-1, a specific inhibitor of AMT, prevented the promoting effect of CPZ on spontaneous AR rate and restored the sperm responsiveness to P. No significant effects could be observed on sperm motility.

**Conclusion:** This study provides unprecedented evidence that human spermatozoa exhibit a completely functional endocannabinoid system related to AEA and suggests a role of TRPV1 in modulating human sperm functions involved in the acquisition of its fertilizing ability.

# $CB_2$ RECEPTORS IN MOUSE MALE GERM CELL DIFFERENTIATION

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The exact role of the endocannabinoid system (ECS) during spermatogenesis has not been clarified. The ECS is modulated during cell proliferation, differentiation and apoptosis through alterations of the expression levels of cannabinoid receptors $CB_1$ and $CB_2$, and of the enzymes involved in the biosynthesis and degradation of the two agonists: anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The presence of an active ECS has been described both in testis and isolated spermatozoa of mammals, sea-urchins and *Rana esculenta*. The aim of this study was to investigate the presence and functional role of the ECS in germ cells at different stages of differentiation, utilizing purified germ cell fractions representative of each spermatogenesis phase.

Enriched spermatogonia (SPG) fractions were obtained from testes of immature 7-day-old Swiss CD-1 mice. Testes of adult CD1 mice were used to prepare meiotic (spermatocytes, SPC) and haploid germ cells (spermatids, SPT). Germ cells were separated on the basis of their size by centrifugal elutriation. AEA and 2-AG are quantified by LC-MS in the selected ion monitoring mode. The expression at transcriptional and transductional levels of endocannabinoid metabolic enzymes and receptors was assessed by qRT-PCR and Western immunoblot, immunofluorescence and enzymatic assays respectively.

Quantitative RT-PCR showed that $CB_1$ expression increases gradually in SPC and SPT. $CB_2$ showed elevated transcriptional levels in all stages of spermatogenesis, with a relative peak of expression in SPC. The expression levels of TRPV1 showed a strong increase in SPC. The expression of the receptors at the protein level, analyzed by Western blot, confirmed that $CB_1$ was very faint in SPG and increased in meiotic and post-meiotic germ cell extracts, whilst $CB_2$ was detected in all differentiative stages and TRPV1 was found in SPC and, in higher levels, in SPT. Immunofluorescence microscopy confirms the presence of $CB_2$ in differentiating SPG and its absence in spermatozoa (SPZ). The presence of a functional $CB_2$ in SPG was been evaluated treating primary cultures with the selective agonist JWH133, which induced Erk 1/2 MAPK phosphorylation and progression towards meiotic prophase in SPG primary cell cultures, in a way reversed by pre-treatment with the specific $CB_2$ antagonist AM630. The increase in meiotic prophase induced by JWH133was confirmed by analyzing the levels of selected pre-meiotic and meiotic markers by qRT-PCR and enzymatic assays. Finally, SPG exhibited high levels of 2-AG, which dramatically decreased in post-meiotic SPT. The mRNA levels of the two 2-AG biosynthetic isoenzymes DAGL$\alpha$ and $\beta$, decreased in meiotic and post-meiotic germ cells, paralleled by a down-regulation of the 2-AG-degrading enzymes, MAGL and FAAH.

Our data indicate that male mouse germ cells possess an active and complete ECS, which is modulated during meiosis, and suggest the presence of an autocrine endocannabinoid signal during spermatogenesis. Mitotic cells possess higher levels of 2-AG, which decrease in SPC and SPT. Accordingly, SPG express higher and lower levels of 2-AG biosynthetic and degrading enzymes, respectively, as compared to meiotic and post-meiotic cells. This endocannabinoid likely plays a pivotal role in promoting spermatogenesis via $CB_2$ receptor activation in SPG, stimulation of the Erk 1/2 MAPK phosphorylation cascade and subsequent progression of these cells towards meiosis.

# ESCALATING ADMINISTRATION OF CANNABINOIDS DURING ADOLESCENCE AUGMENTS MALE COPULATORY BEHAVIOR IN ADULTHOOD

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Studies in humans and other species have generally determined that cannabinoids are adverse to both male reproductive behaviour and biology. Additionally, several recent reports have indicated that cannabinoid administration during puberty can exert long-lasting changes on the brain and behaviour into adulthood. Given the profound effects cannabinoids can exert on neuroendocrine regulation, we examined whether administration of escalating doses of the cannabinoid $CB_1$ receptor agonist HU-210 could modulate the acquisition of sexual activity and testosterone levels in adulthood. Male rats were administered HU-210 at 0.025, 0.05 and 0.1 mg/kg daily on post-natal days 35-38, 39-42 and 43-46, respectively and then left undisturbed. On post-natal day 70, blood was collected for testosterone measurements and males subsequently began sexual training with estrogen/progesterone primed female rats. For sexual training, males were exposed to a sexually receptive female rat twice a week for three consecutive weeks, during which sexual behaviours were scored. Throughout the repeated exposures to female rats all males progressively increased their proficiency at sexual activity as denoted by the significant increase in ejaculations and significant reductions in latencies to engage in sexual activity from the initial sessions to the final session. Surprisingly, males which had received HU-210 during adolescence appeared to acquire sexual activity at a higher rate in that they exhibited more ejaculations in fewer sessions with a primed female, and ultimately exhibited more sexual activity across all six sessions than what was seen with the vehicle treated animals. In line with these findings, males administered HU-210 during adolescence also exhibited a strong trend to having elevated levels of basal testosterone at day 70, while not demonstrating any difference in testicular weight. Thus, against expectations, cannabinoid exposure during adolescence enhanced male sexual activity in adulthood.

**ABERRANT ENDOCANNABINOID SIGNALING COMPROMISES EPIDIDYMAL FUNCTION**

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It is known for decades that exposure to marijuana and its major psychoactive component $\Delta^9$-tetrahydrocannabinol (THC) alter both male and female reproductive functions in humans and laboratory animals. Long-term exposure to crude marijuana extracts reduces male fertility in humans. However, the mechanism by which cannabinoids confer suboptimal male fertility is not clearly understood. The discovery of endogenous cannabinoid-like molecules (endocannabinoids), anandamide (AEA) and 2-arachidonylglycerol (2-AG), as well as G-protein coupled cannabinoid/endocannabinoid receptors CB1 and CB2 have created an opportunity to study the role of these lipid molecules in male fertility. Fatty acid amide hydrolase (FAAH) is considered to be the main degrading enzyme for anandamide, and genetic loss of *Faah* results in elevated levels of anandamide. Using multiple approaches, we have shown that high levels of anandamide in the male reproductive system lead to compromised fertilizing capacity of sperm. Our recent work suggests that CB1 and CB2, which are expressed in the mouse epididymis, play a role in maintaining a functional epididymis. The loss of CB1 receptor in FAAH deficient mice disturbs the epididymal epithelial polarity, and thus interferes with absorptive and secretory functions of the epididymis. This defect causes blockage of sperm transportation and diminishes sperm maturation.

This work was supported in part by NIH grant DA06668.

# PALMITOYLTHENOLAMIDE CONTROLS BETA AMYLOID-INDUCED GLIAL HYPERACTIVATION

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**Introduction**. Beside cytotoxic mechanisms impacting on neurons, beta-amyloid (Ab) induces astroglial activation in Alzheimer's disease (AD) brain. In fact, glial cells undergo to a strong activation following Ab insult and release several pro-inflammatory and neuro-toxic mediators. Moreover, it has been demonstrated that glial cells are endowed with the complete machinery to trigger the angiogenetic process, since, following particular stimuli, they may release both vasoactive factors, such as VEGF, TNF and TGFβ, and several proteases degrading the extra-cellular matrix, thus allowing the progression of the newly formed vessels, as it happens in AD.
Therefore, the discovery of molecules, among which Palmitoylethanolamide (PEA), able to control glial activation, thus preventing in turn neurodegenerative disorders, is of huge interest. In fact, previous evidence showed that PEA is able to control neuro-inflammation in CNS.
PEA belongs to the family of "endocannabinoid-like" derivatives that exhibit cannabimimetic properties but are devoid of direct affinity for the CB receptors *in vitro*. As it is produced during inflammation, it has been proposed that PEA acts as an ALIAmide (Autacoid Local Inflammation Antagonism Amide).
The present study was aimed at investigating *in vitro* the role of PEA in the modulation of inflammatory response in Ab-challenged glial cells.

**Material and Methods.** C6 cells were cultured in 10% FCS supplemented DMEM and treated with 1 μg/mL of Ab (1-42) in the presence of PEA, at concentration of $10^{-8}$ to $10^{-6}$ M. Forty-eight hours after Ab challenge, NO was measured as nitrite ($NO_2^-$) released in cell supernatant by a colorimetric assay based on the Griess reaction, and cells vitality was tested with the MTT assay. Lysed cells subsequently underwent to Western Blot analysis with anti-iNOS, anti-S100B, anti-TNF-α, anti-MMP-9 antibodies respectively. In the same experimental conditions RT-PCR was used to evaluate VEGF transcription.

**Results.** The obtained results indicate that PEA reduces Ab-induced gliosis, evaluated both as cell viability and as inflammatory mediator release. In fact, PEA treatment decreased protein expression of iNOS and S100B, and nitrite production. Moreover PEA reduced both the expression of TNF-α and MMP-9, and the transcription of VEGF, a group of proteins strongly involved in the angiogenic process.

**Conclusions.** These data overall indicate that PEA is able to exert *in vitro* a significant effect on the control of Ab-induced gliosis and on the subsequent production of pro-inflammatory and pro-angiogenic mediators from activated glia. Our results prospect this compound as a useful tool in the attenuation of Ab-induced neuroinflammation.

**This work was partially supported by Epitech Group.**

# WIN 55,212-2 MEDIATED NEUROPROTECTION AND PREVENTION OF EPILEPTOGENESIS FOLLOWING GLUTAMATE-INJURY IN AN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURE MODEL

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Stroke is the most commonly identified cause of epilepsy in adults 35 and older and accounts for more than 50% of all new cases of acquired epilepsy (AE) in the elderly population, underscoring the need for further research of the mechanisms and prevention of seizures following stroke. It has been known for centuries that cannabinoids have anticonvulsant properties. More recent studies have demonstrated that these effects are mediated by CB1 receptor activation in the hippocampus. Our lab has also shown that cannabinoids act at the CB1 receptor to suppress epileptiform discharges in both in vitro and in vivo models of epilepsy. However, the role of the endocannabinoid system following stroke-induced epilepsy has not been investigated. To evaluate this, we have developed an in vitro model of AE following stroke-like injury utilizing an organotypic hippocampal slice culture (OHSC) system. OHSCs represent an advantageous model to study stroke-induced epilepsy because they maintain neuronal morphology, cellular and anatomical relations, and network connections. Exposure of OHSCs to 3.5mM glutamate for 35 minutes resulted in both immediate and delayed cell death in a small population (approx. 15-25%) of neurons as confirmed by propidium iodide staining, while a surviving, but injured neuronal population remained - thus mimicking a stroke-like injury in OHSCs. This sub-set of injured neurons is a substrate for neuronal plasticity and manifested epileptiform discharges as measured by extracellular field potential recordings. To investigate the role of the endocannabinoid system following stroke-like injury, OHSCs subjected to glutamate injury were then treated with the cannabimimetic compound WIN55,212-2 (1μM) for 72 hours. WIN 55,212-2 treatment significantly reduced delayed cell death in injured OHSCs compared to untreated glutamate injured controls. Further, treatment with WIN 55,212-2 also prevented the development of epileptiform discharges in glutamate injured OHSCs. Our result suggests that WIN 55,212-2 is neuroprotective and also prevents development of epileptogenesis following stroke-like injury in vitro. The results of this study further substantiate findings from previous work and implicate a role for CB1 receptor-dependent regulation of epileptiform discharges and neuroprotection in a novel model of stroke-induced AE. Further understanding of the role of the endocannabinoid system in models of AE may lead to novel treatment mechanisms for the prevention of AE following stroke.

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# THE GENETIC ABLATION OF FAAH MODIFIES THE ASTROCYTIC RESPONSE AGAINST BETA AMYLOID PEPTIDE

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**Introduction:** Alzheimer´s disease (AD) is the most common neurodegenerative disorder leading to dementia in the elderly. The amyloid peptide (Aβ) is thought to underlie the pathogenesis of AD since the aggregation of the peptide and the subsequent extracelular deposition as neuritic plaques in specific areas of the brain leads to neuronal death and triggers a glial inflammatory response. We have previously reported that both FAAH and $CB_2$ receptors are selectively overexpressed in glial cells located close to Aβ-plaques. Specifically, the expression and activity of the enzyme FAAH is selectively increased in reactive astrocytes.

**Methods:** To further explore the possible role of FAAH in the astrocytic response against Aβ, we have used primary culture of astrocytes from wild-type (WT) and FAAH knock-out (FAAH-ko) and exposed them to the pathogenic form of Aβ (5 micromolar). Next we performed a time course study by analyzing the profile of cytokine production at 8, 24 and 48 hours. In addition, we measured mRNA levels of some key pro-inflammatory genes.

**Results:** We found significant differences in the response against Aβ by WT and FAAH-ko astrocytes. Thus, genetic ablation of the enzyme made astrocytes more sensitive than WT astrocytes, as cytokine production was significantly higher in FAAH-ko astrocytes than in WT (IL-6, IL-1β, RANTES and MCP-1). In addition, an up-regulation of TNF-α, COX-2 and iNOS mRNAs was also evident. Finally, Aβ-induced cell death was more pronounced in FAAH-ko astrocytes than in WT astrocytes.

**Conclusion:** These data suggest that the increase in endocannabinoids levels by blocking FAAH-mediated degradation dramatically exacerbates the astrocytic response against Aβ.

**Acknowledgements:** CIBERNED (CB06/05/1109), Ministerio de Ciencia e Innovación Tecnológica (SAF2007-61565) and Comunidad Autónoma de Madrid (S-SAL/0261/2006).

# THE SELECTIVE DELETION OF FAAH ALTERS THE APPEARANCE OF BETA AMYLOID PLAQUES IN AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

Lourdes Ruiz-Valdepeñas[1], Cristina Benito[1], Alicia López[1], Cristina Otalora[1], Rosa M. Tolón[1], Benjamin F. Cravatt[2] and Julián Romero[1]

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**Introduction:** Alzheimer's disease (AD) is a neurodegenerative disorder very common in the elderly, characterized by the accumulation of beta amyloid (Aβ) in some regions of the brain, such as the cortex and the hippocampus. Several animal models currently exist that mimic the amyloid pathology characteristic of this disease. Among them, the 5xFAD mouse constitutes a very recent approach.

**Methods:** These mice contain five familial Alzheimer's disease mutations and exhibit amyloid deposits similar to those observed in AD patients. One important advantage of the 5xFAD model is that Aβ deposition is dramatically accelerated in respect to other transgenic models. Thus, even at 3 months of age, these animals exhibit abundant Aβ plaques, increasing with time. As previous reports suggest that FAAH may play a role in AD, we generated a new transgenic mouse by crossing the 5xFAD mice with FAAH-ko mice. In this way, an enhanced endocannabinoid tone will be implemented in the brains of these animals and its possible influence on the development of the pathologic estate will be observed with time.

**Results:** Our current data indicate that 5xFAD/FAAH$^{-/-}$ mice exhibit lower numbers of Aβ plaques in cortex, hippocampus and thalamus, brain regions known to posses high levels of neuritic plaques in 5xFAD/FAAH$^{+/+}$ mice. In addition, the increase in the expression level of several inflammatory cytokines as a consequence of the progression of the disease was lower in ko-mice.

**Conclusion:** We postulate that FAAH ablation and subsequent enhancement of the endocannabinoid tone may delay the development of amyloid pathology in this animal model of AD.

**Acknowledgements:** CIBERNED (CB06/05/1109), Ministerio de Ciencia e Innovación Tecnológica (SAF2007-61565) and Comunidad Autónoma de Madrid (S-SAL/0261/2006).

# MOLECULAR IMPACTS OF CHRONIC CANNABINOID TREATMENT IN HUNTINGTON'S DISEASE TRANSGENIC MICE

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R6/1 transgenic mice have many of the behavioural and molecular dysfunctions attributed to human Huntington's disease (HD), an inherited neurodegenerative disease caused by a mutation in the IT15 HD gene. While cell death is extensive in the basal ganglia and cortex in human HD, R6/1 mice exhibit minimal neuronal death making this a valuable model of the early pathogenic changes in the disease that may themselves be potential targets for development of pharmacological treatments. The cannabinoid system has been highlighted as a possible target due to the early loss of CB1 receptors from key brain regions, and the correlated upregulation of CB1 receptor levels and improvement in symptoms observed following housing in an enriched environment.

We have shown that R6/1 mice exhibit reduced CB1 receptor ligand binding and gene expression in the basal ganglia at 12 weeks of age, prior to the onset of motor symptoms, and with a greater loss seen at 20 weeks. Ligand binding of 5HT2A receptors showed a similar progressive loss, while $GABA_A$ receptor binding increased as the disease progressed. D1 and D2 dopamine receptor binding is decreased at 12 weeks but there is no further decrease by 20 weeks. At 12 weeks anandamide levels are decreased in the hippocampus and 2-AG levels are increased in the cortex.

Acute treatment with THC produced behavioral changes at 12 weeks of age in R6/1 mice, confirming the presence of active CB1 receptors. However, eight weeks of daily treatment with HU210 (0.01 mg/kg), THC (10.00 mg/kg) or URB597 (0.30 mg/kg) commenced at 12 weeks of age did not alter behavioral symptoms. Molecular characterization at the end of the study showed that chronic drug treatment did not significantly affect ligand binding of CB1, D1, D2, 5HT2A or $GABA_A$ receptors, nor CB1 or FAAH mRNA expression, inferring that the drug treatment protocol did not induce significant downregulation of receptors. Chronic desensitization cannot however be ruled out. Intriguingly, a significant increase (64%) in the number of ubiquitinated aggregates was observed in the striatum with agonist treatment. The role of aggregates in HD is controversial, however, the ability of the cannabinoid system to alter aggregate formation or clearance is an exciting finding and suggests that alternative modulation, for example, with an inverse-agonist, may prove to be beneficial in HD.

# RECEPTORIAL AND PHARMACOLOGICAL CHARACTERIZATION OF THE NEW CANNABINOID AGONIST NESS040C5

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A number of studies indicate that cannabinoids can reduce intraocular pressure (IOP) both in animals and humans, suggesting a putative efficacy of these compounds in the treatment of glaucoma.

Among the cannabinoid derivatives encompassed by Patent US 7,485,730 assigned to PharmaNess Scarl, different compounds characterized by low or absent permeability toward blood brain barrier evidenced agonism activity on both $CB_1$ and $CB_2$ sub-type cannabinoid receptors.

In this study we report the synthesis and pharmacological profile of a new compounds claimed by the above mentioned Patent: N-menthyl-6-methyl-1-(4"-methylbenzyl)-1,4dihydrothieno[2',3':4,5]cyclopenta[1,2-*c*]pyrazol-3-carboxamide (NESS040C5).

The affinity towards both $CB_1$ and $CB_2$ receptors was determined through radioreceptor binding assays according to the procedure reported by Ruiu et al. in *J.P.E.T.,* 2003, 306, 363−370, while the activity at those receptors was evaluated using an *in vitro* cell culture model.

To evaluate the pharmacological application of the compound in glaucoma treatment, a validated animal model based on the IOP determination in aged glaucomatous DBA/2J mice was adopted.

NESS040C5 showed high affinity at both $CB_1$ and $CB_2$ receptors, which were positively modulated suggesting an agonistic activity of the derivative. In addiction, the compound reduced IOP in DBA/2J glaucomatous mice suggesting a potential efficacy of NESS040C5 in the treatment of glaucoma.

# CHARACTERIZATION OF THE NEUROPROTECTIVE EFFECT OF CANNABIDIOL AFTER OXYGEN AND GLUCOSE DEPRIVATION OF NEWBORN MICE FOREBRAIN SLICES

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**Introduction**: we reported that cannabidiol (CBD) affords neuroprotection in an in vitro model of newborn hypoxic-ischemic brain damage –oxygen and glucose deprivation (OGD) of mice forebrain slices-. In the present work we aimed to determine the mechanisms and receptors involved in such an effect.

**Methods:** 500 μm brain slices obtained from 7-day-old C57BL6 mice were exposed to OGD for 30 min, being incubated with vehicle (VEH), or CBD alone or together with SR141716 as a CB1 antagonist, AM630 as a CB2 antagonist, DPCPX as a adenosine type A1 receptor (A1AR) antagonist, or SCH58261 as an A2AR antagonist (all 0.1 mM). Slices without OGD served as controls (CTL). Samples of the incubation medium were obtained every 30 min to determine LDH efflux by spectrophotometry, as well as glutamate or IL-1 release by ELISA; LDH efflux was quantified by calculating the Area under the Curve (AUC). At the end of the experiment (120 min) brain slices were collected and homogenated to determine iNOS, COX-2 and TNFa expression by RT-PCR, as well as caspase-9 concentration by ELISA in brain tissue.

**Results:** OGD led to severe tissue damage, reflected by the increase in LDH efflux (AUC 23.9±1.3 vs 53.7±3 a.u. for CTL and VEH, respect., $p<0.01$) and caspase-9 (2.9±0.3 vs 4.1±0.4 ng/mL, $p<0.01$), and associated with an increase of glutamate (0.5±0.1 vs 3.5±0.3 mcg/mL, $p<0.01$), IL-6 (29.2±3 vs. 57.6±4 pg/mL, $p<0.01$), and TNFa (0.08±0.002 vs. 0.135±0.013 a.u., $p<0.01$) production and COX-2 (0.06±0.005 vs. 0.212±0.02 a.u., $p<0.01$) and iNOs (0.14±0.005 vs. 0.18±0.01 a.u., $p<0.01$) expression. CBD reduced tissue damage (LDH 37.2±2 a.u.; caspase-9 2.8±0.3 ng/mL), by reducing glutamate (1.1±0.1 mcg/mL), IL-6 (15.3±2 pg/mL) and TNFa (0.06±0.008 a.u.) production and COX-2 (0.07±0.009 a.u.) and iNOS (0.12±0.01 a.u.) expression (all $p<0.01$ vs. VEH). CBD-induced reduction of tissue damage was unaffected by SR141716, but was reversed by co-incubation with AM630 (LDH 46.8±1.4 a.u.), CPCPX (LDH 65.3±1.7 a.u.) or SCH58261 (LDH 65.6±2.1 a.u.)

**Conclusions**: CBD reduces early and programmed cell death after OGD in newborn mice brain slices, in association with robust anti-inflammatory and anti-excitotoxic effects. This neuroprotective effect is mediated by $CB_2$ and adenosine receptors.

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**CYANOAMIDINE ANALOGS OF *N*-THIAZOLYLIDENE CARBOXIMIDE: POTENT, SELECTIVE CANNABINOID $CB_2$ AGONIST AS NOVEL PAIN THERAPEUTICS**

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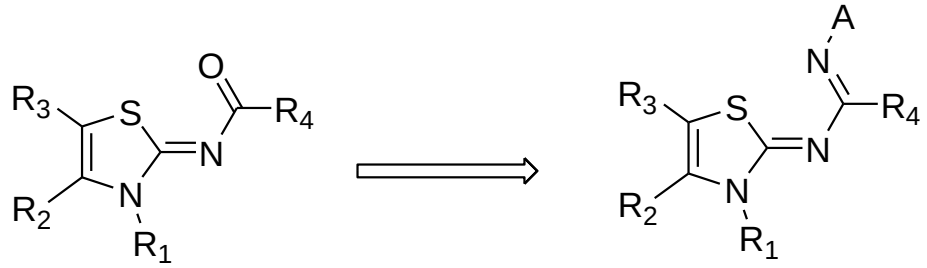
The $CB_1$ and $CB_2$ cannabinoid receptors belong to the class A rhodopsin-like GPCR family. The $CB_1$ receptor is predominantly found in the CNS and is thought to be responsible for most of the overt undesired pharmacological effects induced by nonselective cannabinoid receptor agonists. The $CB_2$ receptor is primarily expressed in peripheral immune tissues. Recent studies have demonstrated that $CB_2$-selective agonists are analgesic in preclinical models of nociceptive and neuropathic pain without causing the adverse side effects associated with $CB_1$ receptor activation. In our research, efforts to identify potent and highly selective $CB_2$ agonists, *N*-thiazol-2(3H)-ylidene carboxamide analogs, generically represented by structure **1**, have exhibited robust efficacy in various pain models at doses that do not elicit undesirable CNS effects. However, partial occupancy of $CB_1$ receptors in the CNS by agonists are manifested behaviorally, and it has been demonstrated that at higher doses, the residual $CB_1$ activation of $CB_2$–selective agonists typically induce a $CB_1$ receptor-mediated decrease in locomotor activity. The design and synthesis of agonists that specifically activate the $CB_2$ receptor with little residual $CB_1$ agonist function remains a challenge and is desirable in order to maximize the therapeutic window in regards to CNS safety. In this poster, we describe the identification of amidine-compounds with structure **2**, which have similar $CB_2$ agonist potency as **1**, but drastically improved subtype selectivity. The structure activity relationships and relevant pharmacological characterization will be disclosed.




**1** **2**

# STRUCTURE-ACTIVITY RELATIONSHIP STUDIES ON A NOVEL SERIES OF IMIDAZOPYRIDINE DERIVATIVES AS CANNABINOID RECEPTOR 2 ($CB_2$) AGONISTS

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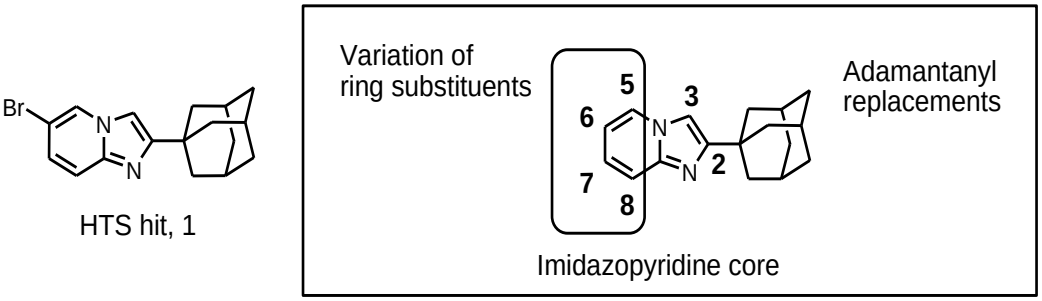
**Introduction**: Two class A GPCR cannabinoid receptors, $CB_1$ and $CB_2$, have been reported to be mainly responsible for the physiological actions of cannabis. The $CB_1$ receptor is predominantly found in the CNS, while the $CB_2$ receptor is primarily distributed in peripheral immune tissues under non-pathologic conditions. Multiple structurally distinct $CB_2$-selective agonists have demonstrated efficacy in preclinical models of inflammatory and neuropathic pain. These compounds produce analgesic activity at doses that do not elicit CNS-related side effects, which are thought to be mediated through $CB_1$ receptor activation. Herein we describe a unique class of imidazopyridine derivatives from which potent and selective $CB_2$ agonists were identified.

**Methods**: A high-throughput screen identified compound **1** as a hit having modest $CB_2$ binding affinity and weak agonist activity. The imidazopyridine scaffold present in screening hit **1** was then exploited in attempts to design and synthesize potent, highly selective $CB_2$ agonists. Compounds were characterized in radioligand binding assays and in *in vitro* functional assays to assess ligand affinity, agonist activity, and selectivity for $CB_2$ vs. $CB_1$.

**Results**: Medicinal chemistry investigations, including replacement of the adamantanyl moiety and variation of the imidazopyridine core substituents, provided several $CB_2$ agonists with increased levels potency relative to screening hit **1**. These efforts resulted in the identification of A-994619, a potent, efficacious, and selective $CB_2$ agonist that exhibited *in vivo* efficacy in an experimental pain model.

**Conclusion**: We have explored SAR on an imidazopyridine pharmacophore based on a compound discovered in a high-throughput screen. Adamantanyl imidazopyridines containing a variety of different core substituents were prepared and several derivatives were identified that exhibit potent affinity and agonist activity at the $CB_2$ receptor.



# PHARMACOLOGICAL CHARACTERIZATION OF PUTATIVE CANNABINOID $CB_2$ AGONISTS FROM THE CANNABILACTONE CLASS: ANTINOCICEPTION WITHOUT CENTRAL NERVOUS SYSTEM SIDE-EFFECTS

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Cannabinoid $CB_2$ agonists produce antinociception without central nervous system (CNS) side-effects. This study was designed to pharmacologically characterize the antinociceptive profiles of AM1714 and AM1710, agonists from the cannabilactone class of cannabinoids. The effects of AM1714 and AM1710 were compared with the $CB_2$ specific agonist (*R,S*)-AM1241 in tests of antinociception. CNS side-effects were evaluated in a modified tetrad (tail-flick, rectal temperature, locomotor activity and rota-rod). AM1714 and AM1710 (0.1-10 mg/kg i.p.) produced antinociception to thermal but not mechanical stimulation of the hindpaw. Antinociception produced by the low (0.1 mg/kg i.p.) dose of AM1710 was blocked selectively by the $CB_2$ antagonist SR144528, demonstrating pharmacological specificity similar to that observed with the aminoalkylindole $CB_2$ agonist (*R,S*)-AM1241. Antinociception produced by AM1714 (5 mg/kg i.p.) was blocked by either AM630 (6 mg/kg i.p.) or SR141716 (6 mg/kg), but not multiple doses of SR144528 (6 and 10 mg/kg i.p.). Effects of AM1710 (5 mg/kg i.p.) were blocked by either SR144528 (6 mg/kg i.p.) or SR141716 (6 mg/kg i.p.). Neither AM1714 nor AM1710 produced hypothermia, tail-flick antinociception, or motor ataxia. AM1714 produced hypoactivity at a dose 50-fold higher than that needed to achieve maximal antinociception. Our studies suggest that the $CB_2$-preferring cannabinoids AM1714 and AM1710 produce antinocieption in the absence of CNS-side effects. The *in vivo* pharmacological profile would not be expected based upon the *in vitro* binding profile of these compounds. Biologically active metabolites may contribute to the *in vivo* pharmacological profile of systemically administered AM1714 and AM1710.

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# FAAH INHIBITION REVERSES LIPOPOLYSACCHARIDE-INDUCED MECHANICAL ALLODYNIA

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Inflammatory pain is generally a secondary response to an infection or wound healing. The onset of inflammatory pain is often characterized by edema and allodynia to mechanical stimuli. New treatments for hyperalgesia are needed. One potential target is the endocannabinoid system (ECS), which has been implicated in a variety of acute pain models. The ECS is comprised of two primary ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), two receptors ($CB_1$ and $CB_2$) that bind these endocannabinoids, and the enzymes responsible for the biosynthesis and degradation of the endocannabinoids. AEA signaling is short-lived, due to its rapid degradation by the enzyme fatty acid amide hydrolase (FAAH). Additionally, genetic deletion or pharmacological inhibition of FAAH increases AEA levels in the brain and reduces nociception in a wide range of preclinical pain models. In the present study, we investigated whether genetic deletion or pharmacological inhibition of FAAH reverses inflammatory pain induced by intraplantar (ip.l) lipopolysaccharide (LPS) into a hind paw. LPS elicited a concentration-dependent increase in mechanical allodynia (1 -25 μg) with significant differences at 2.5 μg and 25 μg of LPS per paw. Gabapentin dose-responsively reversed the mechanical allodynia, $ED_{50}$ (95% Confidence Interval)= 8.3 mg/kg (4.8-14.4). In order to determine the involvement of FAAH, we used genetically modified knockout mice which have no substantial levels of FAAH expression, termed FAAH (-/-) mice. FAAH (-/-) mice displayed an anti-allodynic phenotype to LPS compared to control FAAH (+/-) mice, which have normal functioning FAAH levels. Next, to differentiate between neuronal and non-neuronal FAAH, we used genetically modified mice which express FAAH only in neuronal tissue (FAAH-NS). FAAH-NS mice did not show an anti-allodynic phenotype to ip.l LPS. Using a pharmacological approach to block FAAH, we treated mice with PF-3845, an irreversible FAAH inhibitor. The novel FAAH inhibitor, PF-3845, significantly reduced the mechanical allodynia elicited by LPS up to 3-fold. Rimonabant completely blocked the anti-allodynic effects of PF-3845, implicating the involvement of a $CB_1$ receptor mechanism of action. Importantly, the contralateral paw responses of FAAH (-/-) mice as well as PF-3845-treated mice were not affected by drug treatments, indicating that the anti-allodynic effects were specific to inflammatory pain. Taken together, these data suggest that neuronal FAAH is a key regulator involved in inflammatory pain, and pharmacological inhibition of FAAH will reduce mechanical allodynia caused by inflammation through a $CB_1$ receptor mechanism of action.

# BIOCHANIN A: A NATURALLY OCCURRING, PERIPHERALLY ACTING INHIBITOR OF FATTY ACID AMIDE HYDROLASE

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Use of genetically modified mice lacking $CB_1$ receptors in nocicecptive neurons have suggested that these receptors are the major mediators of the analgesic effects of cannabinoids (Agarwal et al., Nature Neurosci 10 [2007] 870-879). Although FAAH inhibitors like URB597 produce analgesic effects in animal models without producing behavioural effects reminiscent of a central CB receptor activation, central effects such as alteredethanol preference have been reported, making a case for the design of peripherally restricted FAAH inhibitors. The naturally occurring isoflavones genistein and daidzein have limited CNS penetration and have previously been shown to inhibit FAAH with reasonable (low micromolar) potencies (Thors et al., Br J Pharmacol 152 [2007] 744-50). In the present study, we have investigated the properties of the related isoflavone biochanin A, which is found in red clover.

Biochanin A inhibited 0.5 µM anandamide hydrolysis by mouse liver, rat brain and recombinant human FAAH with $IC_{50}$ values of 1.8, 1.4 and 2.4 µM, respectively. In the rat homogenates, biochanin A acted as a mixed-type inhibitor, with Ki(slope) and Ki(intercept) values of 1.1 and 8.2 µM, respectively. In RBL2H3 basophilic leukaemia cells, Biochanin A reduced the URB597-sensitive accumulation of membrane tritium following incubation of the cells with 0.1 µM anandamide with an $IC_{50}$ value of 0.22 µM. The compound had modest effects upon the binding of [$^3$H]CP55,940 to $CB_1$ and $CB_2$ receptors. Administration of biochanin A (10 mg/kg i.v.) did not affect brain levels of anandamide. However, it potentiated the behavioural effects (inhibition of locomotion, hypothermia, catalepsy and antinociception) of i.v. administered anandamide, consistent with a reduction in the peripheral metabolism of this endocannabinoid. In order to assess its efficacy in a peripheral pain pathway, the effect of i.pl. biochanin A upon the spinal pERK response to formalin was assessed in anaesthetised mice. In this model URB597 (100 µg/paw) reduced the increased pERK formation seen following formalin in a manner antagonised by AM251. Biochanin A (300 µg/paw) behaved in the same manner.

It is concluded that biochanin A acts as a FAAH inhibitor with limited CNS penetration. The compound is by no means selective for FAAH and interacts with other targets, not the least being oestrogen receptors. Nonetheless, biochanin A may be a useful template for the design of reversible, peripherally acting FAAH inhibitors.

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# SUBSTRATE SPECIFICITY IN THE ACTIVATION OF COX-2

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**Introduction**: The endocannabinoid 2-arachidonoyl glycerol (2-AG) is a selective substrate for the inducible isoform of cyclooxygenase (COX), COX-2, *in vitro*. Its turnover leads to formation of glyceryl analogs of traditional prostaglandins (PG-Gs), a subset of which elicit agonism at unique receptors, at picomolar to nanomolar concentrations. While 2-AG is approximately one-tenth as abundant as arachidonic acid (AA) in zymosan-stimulated macrophages, the ratio of PGs to PG-Gs recovered from these macrophages is about 1000:1 (Rouzer, C.A. and Marnett, L.J. (2008) *J. Biol. Chem.* **47**:3917.). The activation of oxygenase activity in COX is dependent on the turnover of the peroxide product of AA or 2-AG oxygenation ($PGG_2$ and $PGG_2$-G respectively). We hypothesized that $PGG_2$-G is a less efficient POX substrate than $PGG_2$ and therefore a poor activator of 2-AG turnover. Here we demonstrate that while both peroxide intermediates are reduced at comparable rates, the turnover of 2-AG is exquisitely sensitive to peroxide tone in activating and sustaining oxygenase activity.

**Methods**: Peroxidase activity was measured by monitoring the consumption of the peroxidase reductant 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at 414 nm. Cyclooxygenase activity was measured by monitoring oxygen consumption of pure enzyme with an Instech fiber optic oxygen probe, in a reaction chamber held at 37 °C.

**Results**: 15-HpETE and 15-HpETE-G were used as stable surrogates for $PGG_2$ and $PGG_2$-G and were reduced at comparable rates by the peroxidase sites of ovine COX-1 ($k_{cat}/K_m$ = 6.9 $S^{-1}.\mu M^{-1}$ and 10.9 $S^{-1}.\mu M^{-1}$ respectively) and human COX-2 ($k_{cat}/K_m$ = 3.2 $S^{-1}.\mu M^{-1}$ and 3.4 $S^{-1}.\mu M^{-1}$ respectively). However, 15-HpETE-G is a significantly less efficient substrate than 15-HpETE for mouse COX-2 ($k_{cat}/K_m$ = 1.9 $S^{-1}.\mu M^{-1}$ and 6.4 $S^{-1}.\mu M^{-1}$) (**$p<0.001$). 15-HpETE and 15-HpETE-G demonstrated equivalent ability to activate the oxygenase activity of ovine COX-1 and mouse COX-2. To test the ability of $PGG_2$ and $PGG_2$-G to sustain the turnover of AA and 2-AG respectively, we measured oxygen consumption in the presence of 250 mM NaCN. We found that the turnover of 2-AG exhibited significant sensitivity to competition for the peroxidase active site (**mouse COX-2, $p<0.001$ and ***human COX-2, $p<0.0001$). The turnover of 2-AG relative to AA demonstrated significant sensitivity to suppression by the endogenous peroxide detoxification system, gluthathione and glutathione peroxidase, in a glutathione peroxidase concentration-dependent manner (**mouse COX-2 and **human COX-2, $p<0.001$). A mouse COX-2 mutant (H388Y) with ~ 300 fold diminished peroxidase activity, demonstrated severely diminished 2-AG turnover compared to AA; this phenomenon was reversed by increasing concentrations of exogenous peroxide.

**Conclusions**: We have shown that 2-AG demonstrates a substrate-specific deficiency in the activation of its own turnover, relative to AA. These results could partly account for the paucity of 2-AG derived prostaglandins from cellular settings.

**PROTEOMIC ANALYSIS OF ANANDAMIDE-INDUCED APOPTOSIS IN HUMAN NEURONAL CELLS**

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Introduction: Anandamide (AEA) is an endogenous agonist of type 1 cannabinoid receptors (CB1R) that, along with the enzymes responsible for the synthesis, transport and hydrolysis of AEA and congeners, compose the "endocannabinoid system (ECS)". In this study, we report the biochemical, morphological and functional characterization of the ECS in human neuroblastoma SH-SY5Y cells, that are an experimental model for neuronal cell damage and death, as well as for major human neurodegenerative disorders.

Methods: SH-SY5Y cells were cultured with AEA in the presence or absence of CB1 antagonist. Activity of FAAH and NAPE-PLD, MAGL, DAGL and cannabinoid receptors were assessed by standard methods. mRNA expression, siRNA evaluation, apoptosis analysis and proteomic assays were measured by traditional procedures.

Results: We show that AEA induced apoptosis of SH-SY5Y cells via CB1R. Through proteomic analysis, we demonstrate that AEA-induced apoptosis was paralleled by a ~3 to ~5-fold up-regulation or down-regulation of five genes (i.e., BiP/GRP78). Interestingly, only the effect of AEA on BiP was reversed by SR141716, and silencing or over-expression of this gene increased or reduced, respectively, AEA-induced apoptosis of SH-SY5Y cells. In addition, the expression of the BiP-related apoptotic markers was increased by AEA.

Conclusion: In conclusion, we show a complete and fully functional ECS in human SH-SY5Y cells, and report an unprecedented proteomic analysis that identifies BiP as a key-protein in the death programme induced by AEA in human neuronal cells.

# EFFECT OF PHYTOCANNABINOIDS ON HUMAN PROSTATE CARCINOMA CELLS

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Work carried out in our and other laboratories showed that, together with the previously reported agonist effect of cannabidiol (CBD) on Transient Receptor Potential cation channels of the Vanilloid-type 1 (TRPV1), other phytocannabinoids affect the activity of TRP channels. In particular, CBD and cannabichromene (CBC) are potent agonists at TRP channels of the Ankyrin type-1 (TRPA1), whereas CBD and cannabigerol (CBG) are potent antagonists of TRP channels of the Melastatin type-8 (TRPM8) (De Petrocellis et al., *J. Pharmacol. Exp. Ther.* 2008). TRPV1 and TRPM8 are non-selective cation channels that are emerging as a novel targets for the treatment of cancer. In particular, activation of TRPV1 can cause apoptosis in several types of carcinoma as well as skin cancer, and TRPV1 null mice are more prone to develop skin cancer, whereas high expression of TRPV1 is associated with better prognosis of patients with hepatocellular carcinoma. TRPV1 activation has been suggested to cause pro-proliferative and pro-apoptotic effects on androgen-dependent and androgen–independent human prostate cancer cells (HPCCs), respectively. TRPM8 was instead suggested to be an androgen-dependent factor necessary for the survival of HPCCs. Based on this background, and on our previous finding that CBD, CBG and/or CBC can inhibit the proliferation in vitro of several human cancer cell lines (Ligresti et al., *J. Pharmacol. Exp. Ther.* 2006), we studied the effects of these and other phytocannabinoids on HPCC proliferation and apoptosis.

Cell viability was assessed with the MTT assay after 3-day incubation of LNCaP (androgen-responsive) and DU-145 (androgen unresponsive) HPCCs with increasing concentration of compounds, in the presence or absence of TRP channel agonists or antagonists. Apoptosis was assessed with a chemoluminescence-based assay of caspase-3/7 activity, after 24 and 48 hour incubation of LNCaP or DU-145 cells with the compounds. Experiments investigating the role of TRP channels in the effects of the phytocannabinoids were carried out using canonical agonists and/or selective synthetic agonists and antagonists of TRPV1, TRPA1 and TRPM8. The expression of TRP channels in DU-145 and LNCaP cells was evaluated by quantitative RT-PCR.

We found that CBD and CBG, but not CBC, increase and reduce DU-145 and LNCaP cell viability at low ($\leq$1 μM) and high ($\geq$10 μM) concentrations, respectively. CBD (1-10 μM), but not CBG or CBC, induced caspase-3/7 release from LNCaP, but not DU-145, cells. TRPM8 was expressed in LNCaP, but not DU-145, cells, whereas TRPV1 and TRPA1 were expressed in both cell lines. We suggest that TRPA1 plays a minor role in the effects of phytocannabinoids, whereas antagonism at TRPM8 might be the major underlying mechanism for CBD pro-apoptotic-like action.

If confirmed in vivo, these studies might suggest the testing of non-psychotropic phytocannabinoids for the treatment of human prostate carcinoma.

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# DIFFERENTIAL EFFECTS OF THE ANTIOXIDANTS ALPHA TOCOPHEROL AND BUTYLATED HYDROXYANISOLE (BHA) ON AM251-INDUCED ENDOTHELIAL CELL GROWTH INHIBITION

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**Introduction**: Cannabinoid ligands have been known to inhibit the growth of both normal and tumor cells via apoptotic processes. We have recently observed that both agonists and antagonists of cannabinoid receptors, including AM251 inhibit the growth of EA.hy926 cells derived from human umbilical vein endothelium. Here we present evidence that AM251-induced endothelial cell growth inhibition is blocked by alpha tocopherol while BHA accelerates the AM251's effect. **Method**: Subconfluent EA.hy.926 endothelial cells were incubated with 1 ml of fresh medium containing 5% charcoal-stripped FBS with or without 15 µM of AM251. To determine if AM251's effect can be reversed, the cells were first preincubated for 30-60 minutes with reagents known to affect cannabinoid signaling. After 48-hour culture the medium was removed and the attached cells were processed for staining with 0.5% crystal violet. Subsequent to solubilization in 1% SDS, absorbance was measured at 595 nm. Cell lysates were also prepared and subjected to SDS-PAGE and Western transferred, and probed with a monoclonal antibody against thrombospondin-1 (TSP-1). **Results**: Within 24 hours of incubation, in contrast to flatly attached control cells, cells exposed to 15 µM of AM251 appeared phase bright and showed blebs, characteristic of apoptotic cells. A few cells were also swollen and were completely detached from the dish. These changes were further pronounced after 48 hours. A caspase 3/7 inhibitor was unable to block the apoptotic changes induced by AM251. Other reagents such as L-NAME, capsazepine, trehalose or trans-resveratrol, which have been previously implicated in some aspects of cannabinoid signaling, were also not able to prevent the inhibitory effect of AM251. Only alpha tocopherol at 200 µM and above was able to prevent AM251's growth inhibitory effect. Surprisingly, when co-incubated with 200 µM of BHA, another intracellular antioxidant, the growth inhibitory effect of AM251 was accelerated. BHA alone did not affect the cells. In confluent monolayer AM251 also caused a dose-dependent increase in TSP-1, a protein known to inhibit angiogenesis through inhibition of both cell growth and migration. **Conclusion**: The ability of alpha tocopherol to abrogate the growth inhibitory effect of AM251 suggests that it increases reactive oxygen species (ROS) in these cells. However, the enhancement of AM251 toxicity by BHA suggests that BHA may increase the intracellular bioavailability of AM251 leading to increase in cellular ROS. Further, the inability of executioner caspase 3/7 inhibitor to block AM251's effect suggests that once committed, the cells may use alternate pathways to undergo cell death. Since TSP-1 is also an inducer of apoptosis and inhibitor of cell adhesion, induction of TSP-1 by AM251 suggests that AM251's effects on these cells may be partly mediated by TSP-1.

# EXPRESSION OF ANANDAMIDE-RELATED ENZYMES IN HUMAN PROSTATE CANCER CELLS

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Anandamide is present in human prostate cancer cells, and the cells express the cannabinoid receptors CB1 and CB2 and the anandamide-hydrolyzing enzyme fatty acid amide hydrolase (FAAH). Endocannabinoids are noted because of their anti-proliferative effect on prostate cancer cells. However, the presence of anandamide-related enzymes other than FAAH has not been examined. In the present studies, we investigated the expression of *N*-acylphosphatidylethanolamine- hydrolyzing phospholipase D (NAPE-PLD) and lysosomal *N*-acylethanolamine- hydrolyzing acid amidase (NAAA) in human prostate cancer cells (PC-3, DU-145, and LNCaP) and prostate epithelial cells (PrEC). As analyzed by reverse transcriptase-PCR, mRNAs of NAPE-PLD and NAAA were detected in all of these cells. More interestingly, among various human tissues the prostate showed the highest expression level of NAAA. In LNCaP cells, both of NAAA and FAAH were expressed, and *N*-[$^{14}$C]palmitoylethanolamine-hydrolyzing activity of NAAA was distinguishable from that of FAAH, based on their different pH dependency profiles, solubilization of NAAA by freezing and thawing without detergent, and specific inhibition of FAAH by URB597. These results showed that both the enzymes in LNCaP cells are functionally active. However, hydrolysis of anandamide was mostly attributed to FAAH. NAAA was partly secreted from LNCaP cells to the medium, suggesting its extracellular role. These results indicated that human prostate cancer cells express not only FAAH but also NAPE-PLD and NAAA, which are involved in the formation and degradation of anandamide and other *N*-acylethanolamines.

# DECREASE OF TUMOR CELL VIABILITY VIA INHIBITION OF ENDOCANNABINOID DEGRADATION BY URB597

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**Introduction:** The antitumoral effects of the endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamine (anandamide, AEA) received a particular attention these last few years. The cannabinoids are known to exert cytostatic, apoptotic and antiangiogenic effects in different tumor cell lines and tumor xenografts. Other bioactive lipids, like palmitoylethanolamide (PEA), also exhibit indirect anti-proliferative effects by acting as "entourage" compounds for endocannabinoids. These "endocannabinoid-like" belong to the class of *N*-acyl-ethalonamines and share similar properties with endocannabinoids without binding the cannabinoid receptors. The endocannabinoids are metabolised by several hydrolases including the fatty acid amide hydrolase (FAAH), the monoacylglycerol lipase (MGL) and the *N*-acylethanolamine-hydrolysing acid amidase (NAAA).

**Results**: The neuroblastoma cell line N1E115 expresses the $CB_1$, but not $CB_2$, cannabinoid receptor. By RT-PCR, other receptors thought to play a role in the endocannabinoid system were also detected: the G protein-coupled receptors GPR55 and GPR119, the vanilloid cation channel TRPV1 and the nuclear receptors PPARα and PPARγ. The expression of the enzymes responsible for the degradation of the endocannabinoids was also measured. We detected the presence of the FAAH, the MGL and the NAAA. The N1E115 cells are able to metabolise AEA and 2-AG. The experiment relies on the hydrolysis of radiolabelled anandamide and 2-oleoylglycerol, an analogue of the 2-arachidonoylglycerol. This enzymatic metabolisation is inhibited by the addition of URB597, a selective FAAH inhibitor, and MAFP, a mix inhibitor of FAAH and MGL. In a second time, was evaluated the cytotoxic potential of the endocannabinoids AEA, 2-AG, PEA and a fourth one, the oleoylethanolamide (OEA). All these molecules are able to decrease the N1E115 cell viability in a MTT test. When we incubated AEA, PEA and OEA with the URB597, a diminution of the cell viability compared to the endocannabinoids alone was observed, while this was not the case with MAFP. Noteworthy, URB597 and MAFP exhibit a cytotoxic effect by themselves.

**Conclusion:** We showed that the endocannabinoids AEA, 2-AG and the "endocannabinoid-like" PEA and OEA can alterate tumor cell viability. The cytotoxic effect can be increased by the inhibition of endocannabinoid metabolisation by the addition of URB597 but not MAFP. This could reflect a dissimilar inhibitory profile of these coumpounds in the cell.

# WIN55,212-2 PROTECTS HUMAN DOPAMINERGIC NEURONS AGAINST GP120-INDUCED DAMAGE

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**Introduction:** Despite the therapeutic impact of anti-retroviral therapy (ART), HIV-1-associated dementia (HAD) remains a serious threat to AIDS patients, and there currently remains no specific therapy for the neurological manifestations of HIV-1. The DA-rich midbrain is known to be a key target of HIV-1. Based upon findings in our laboratory that synthetic cannabinoids inhibit HIV-1 expression in human microglia, as well as a substantial literature demonstrating neuroprotective properties of cannabinoids in other systems, experiments were performed using a human midbrain cell culture model, which contains dopaminergic (DAergic) neurons, microglia, and astrocytes to test the hypothesis that synthetic cannabinoids will protect DAergic neurons against the toxic effects of gp120 through a mechanism involving inhibition of activated microglia.

**Methods:** Human brain tissues from 10-22 week-old fetuses were obtained at the time of elective termination of intrauterine pregnancy under a protocol approved by the Human Subjects Research Committee at our institution. Assays included measurement of $^{3}$H-dopamine, apoptosis, oxidative damage (8-isoprostane levels), production of intracellular ROS and $O_2^-$ production. We used the prototypic synthetic $CB_1/CB_2$ receptor agonist WIN55,212-2 (WIN2) to test whether pretreatment with a synthetic cannabinoid would protect against gp120 toxicity

**Results:** In our human midbrain culture model, DAergic neurons appear to be more sensitive to the detrimental effects of gp120 than other neurons of the midbrain, both in terms of cell count and function. Gp120 induced apoptosis (specifically DAergic apoptosis), increased 8-isoprostane levels (a measure of lipid peroxidation), and elicited both ROS and O2- in our midbrain cultures. Pretreatment with WIN2 blunted each of these effects. Pretreatment with WIN2 also protected against the structural damage to DAergic neurons by gp120 in both loss of numbers and loss of dendrites, as well as protecting against loss of DA transporter function. These protective effects of WIN2 were at least in part $CB_2$ mediated. We also showed that the addition of microglial cells enhances gp120-induced damage, and that inhibition of activated microglial cells lessens this damage. Finally, we demonstrated that gp120 induces $O_2$- production in purified microglial cells and that WIN2 blunts this production.

**Conclusions:** In this study, we showed that DAergic neurons are particularly sensitive to gp120-induced damage, and that pretreatment with WIN2 was able to protect against gp120-induced structural, functional, and apoptotic damage to DAergic neurons. This gp120-induced damage was elicited in part by oxidative damage, the source of which may be microglial cells. Further studies will be directed at identifying additional mechanisms by which synthetic cannabinoids may blunt gp120 induced damage.

# DELTA-9-TETRAHYDROCANNABINOL ($\Delta^9$-THC) SUPPRESSES CD40 LIGAND (CD40L) UPREGULATION IN ACTIVATED $CD4^+$ T CELLS

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Plant-derived cannabinoids, such as $\Delta^9$-THC and cannabidiol, possess immunomodulatory properties by affecting a variety of immune cells, including T cells. As demonstrated by this laboratory, treatment with $\Delta^9$-THC selectively inhibits T cell function including T cell-dependent humoral immune responses, T cell proliferation, mixed lymphocyte responses, and production of T cell-derived cytokines. The mechanism of immune suppression involves, at least in part, the disruption of the nuclear factor of activated T cells (NFAT) as well as the extracellular signal-regulated kinase (ERK) MAPK activation. Even though $\Delta^9$-THC binds both known cannabinoid receptors, CB1 and CB2, the role of these receptors in immune regulation remains poorly understood. CD40L, one of many co-stimulatory molecules expressed on the cell surface of activated T cells, has been characterized extensively as the critical component in the generation of appropriate T cell-dependent humoral immune responses. Furthermore, optimum expression of CD40L on activated T cells requires the activation of the transcription factors NFAT and early growth response-1 (Egr-1). In light of the above, the objective of this study was to investigate whether $\Delta^9$-THC impairs the up-regulation of CD40L expression in T cells activated with different stimuli by using mouse splenocytes as a model. Time course studies demonstrated that peak cell surface CD40L expression after either anti-CD3/CD28 or phorbol ester plus calcium ionophore (PMA/Io) was 8 hr post stimulation on activated $CD4^+$ T cells, as determined by flow cytometry. Despite the similarity in kinetic profiles of cell surface CD40L expression induced by either anti-CD3/CD28 or PMA/Io, the kinetics of CD40L mRNA induced by these two stimuli were quite different with peak CD40L mRNA level at 2 hr post PMA/Io stimulation, and 4 hr post anti-CD3/CD28 stimulation, as determined by real time PCR. Pretreatment with $\Delta^9$-THC significantly suppressed the up-regulation of both cell surface CD40L expression and CD40L mRNA level induced by anti-CD3/CD28, but not the PMA/Io-induced up-regulation of the cell surface CD40L expression on $CD4^+$ T cells. Furthermore, pretreatment with $\Delta^9$-THC also attenuated anti-CD3/CD28-induced CD40L expression on $CD4^+$ T cells derived from $CB1^{-/-}/CB2^{-/-}$ mice, which suggests that the $\Delta^9$-THC-mediated impairment of anti-CD3/CD28-induced CD40L expression is a cannabinoid receptor-independent mechanism. Studies are currently underway to further characterize the signaling pathways and mechanisms that are responsible for the suppressive effect of $\Delta^9$-THC on CD40L expression by initially focus on the involvement of NFAT and Egr-1, as well as the differential effect of $\Delta^9$-THC in the presences of different T cell stimuli.

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# EFFECTS OF CHRONIC THC TREATMENTS ON ADAPTIVE IMMUNE RESPONSES TO *CANDIDA ALBICANS* INFECTED C57BL/6 MICE

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Delta-9-tetrahydrocannabinol (THC) has been shown to suppress host resistance to bacterial and viral infections presumably through the 2 receptors associated with the endocannabinoid system: central cannabinoid receptor ($CB_1R$) and peripheral cannabinoid receptor ($CB_2R$). In order to better understand the relationship between host resistance to pathogenic infections and THC treatments, we designed a systemic *Candida albicans (C. albicans)* infection model. We first established a chronic THC regimen in which c57BL/6 female mice received either vehicle (1:1:18, ethanol, cremophor, PBS) or THC (16mg/kg) via intraperitoneal injection (i.p.) on day 1. On day 2, the mice received PBS or $5X10^6$ *C. albicans* cells/ml intravenously (i.v.) followed by vehicle or THC (i.p.) injections for 12 more days. The mice were sacrificed on day 13 and their kidneys, brains and spleens were collected. The kidneys were used to determine colony forming units per gram of tissue (CFU/g). The brains were used to determine $CB_1R$ and $CB_2R$ expressions. In addition, splenic T cells were isolated and $CB_1R$ and $CB_2R$ expression was also determined. We found that mice receiving THC exhibited lower kidney CFU/g. The $CB_1R$ expression was equally high in the brain of vehicle and THC treated mice. The $CB_2R$ expression was equally low in the brain of vehicle and THC treated mice. Similarly, there was no $CB_1R$ expression in the splenic T cells. However, $CB_2R$ expression was slightly higher in splenic T cells from mice treated with THC than those treated with vehicle. We next looked at the effects of a chronic THC treatment on survival to a systemic *C. albicans* infection. For this study, 8 week old C57BL/6 female mice were first injected with vehicle or THC (16mg/kg, i.p.) on days 0-3, 7-11 and 15-19. The mice were injected with *C. albicans* (0.75 X $10^6$ cells/ml, i.v) on day 1 and again on day 18 (5 $X10^6$ *C. albicans* cells/ml, i.v). Mice were monitored daily for survival for 15 days after the last injection. We found that mice receiving the THC experienced a higher survival rate than those receiving vehicle. To determine if THC has antifugal activities, a minimum inhibitory concentration assay was performed. We found that at all the concentration tested, there was no antifungal activity by THC. To further investigate the effects of THC on the memory response, we used different doses of THC. In this study, 8 week old C57BL/6 female mice were injected with vehicle or THC (4, 8, 16, 32 or 64mg/kg in vehicle, i.p.) on days 1-4, 8-11, 15-18. On day 2, they were injected with *C. albicans* (0.75 X $10^6$ cells/ml, i.v) and on day 19 with *C. albicans* (5 X $10^6$ cells/ml, i.v). The mice were then monitored. Our preliminary results show that there is no significant correlation between THC treatment and % weight loss. In conclusion, chronic THC treatments seem to suppress the *C. albicans* infection.

# THE EFFECTS OF THC ON THE INNATE IMMUNE RESPONSE TO SYSTEMIC *CANDIDA ALBICANS* INFECTION IN MICE

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Delta-9-tetrahydrocannabinol (THC) suppresses the immune resistance to viral, parasitic and bacterial infections. However, the effect of THC on fungal infections is unknown. Systemic Candidiasis is most common among individuals whose immune systems are compromised due to transplants, cancer, AIDS, and other diseases. The objective of this study was to establish a model to investigate the effects of THC on the innate immune response to $1.0x10^7$ yeast cells/ml of *Candida albicans* (*C. albicans* strain CP 680) infection. C57BL/6 (wild type) and $CB_2R$ knockout ($CB_2R^{-/-}$) female mice 8 weeks of age weighing approximately 18-20 grams (n=7 mice/group) were used to investigate the involvement of $CB_2R$ on *C. albicans* infection. *C. albicans* or PBS controls were injected i.v. The fungal load was measured as *C. ablicans* colony forming units per gram of kidney or per gram of liver (CFU/g). The mice were sacrificed and the kidneys, liver, spleen and serum were collected from each mouse for analysis. Serum levels of interleukin 6 (IL-6) were measured by enzyme linked immunosorbent assay (ELISA). Our preliminary results show that the IL-6 production between wild type and $CB_2R^{-/-}$ mice were not significantly different. Similarly, the liver and kidney CFU/g were not significantly different between wild type and $CB_2R^{-/-}$ mice. Next, we explored the effects of chronic THC treatment on the innate immune response to a systemic *C. albicans* infection. Vehicle (1:1:18, ethanol, cremophor, PBS) or THC (4,8,16,20 mg/kg, i.p.) was given at days 1 through 4, day 8 through 11 and day 15 through day 19. On day 20, $1x10^7$ *C. albicans* cells/mouse was administered and the mice monitored for an additional 3 days. On day 23, the mice were sacrificed and the kidneys, liver, spleen and serum were collected for analysis. Serum levels of IL-6 were measured by ELISA. In addition, splenic macrophages (CD11b $Mac^+$ cells) were isolated and IL-6 mRNA levels determined by reverse transcriptase polymerase chain reaction (RT-PCR). Thus far, we found that THC treatment did not alter the effect of *C. albicans* on mouse body weight. Our findings will advance our knowledge on the effects of THC on systemic fungal infections.

# PUTATIVE ABBERATION OF THE ENDOGENOUS CANNABINOID SYSTEM: A CASE STUDY

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Cannabinoids, terpenes and flavinoids have numerous functions, but play a central role in modulating the immune system. They have been shown to be effective as anti-oxidants, analgesics, and as an anti-inflammatory; they are anxiolytic, effective in treating OCD, are active in cellular glucose metabolism, and appear to inhibit aggressive tumor cell growth. The terpenes are known to act synergistically as allosteric modulators at the CB2 receptor. Abnormal function of the ECS can arise from a Clinical Endocannabinoid Deficiency (CECD), from an as yet unidentified Endogenous Terpene Deficiency, as well as from structural / functional deficits in the synthetic, import and degradative enzymes. Aberrant ECS modulation ranges from over activity to under activity.

I am a female patient that was diagnosed with Rheumatoid Arthritis and Systemic Lupus Erythematosus at age 16. I was diagnosed with Interstitial Cystitis and unsuccessfully treated with chemotherapeutic installations, urethral dilatations, and bladder dilations under general anesthesia to mechanically avulse the nerves enervating the bladder, six times. Bladder failure required daily self-catherization for several years. Additionally, I have marked hypoglycemia, asthma, endometriosis, chronic sinusitis, chronic bacterial infections, severe allergies to almost all medications, allergic dermatitis, pre-cancerous cell growths were removed twice from my cervix, and I was also diagnosed as sterile. I underwent 14 surgical procedures and treatment in 5 states, including Mayo Clinic. Despite these efforts, I was bedridden for 3 ½ years, taking over 40 medications a day, such as Plaquinyl, Methotrexate, Methadone, Vioxx, Morphine and Prednisone, to alleviate symptoms, boost the immune system and control side effects of other medications. ECS abnormalities could account for this cluster of immunologic disorders.

I moved from St. Charles, Illinois to California, in order to saturate my receptors with phytocannabinoids. Clinical cannabis was taken over a 30-month period, in which the fresh, green cannabis leaf was the primary source of cannabinoids. The study period included a healthy, normal pregnancy, during which I continued green leaf therapy after consulting with my OBGYN and rheumatologist at UCSF. I was closely evaluated and physiological assessments of my medical progress were conducted frequently.

Phytocannabinoids established and sustained my remission from lupus, IC and RA. My ANA and SED rate both decreased significantly last year. The pre-cancerous skin flap has disappeared. My RA titer was negative for the first time in 13 years. I avoided back surgery and a third sinus surgery. I was able to get off regular antibiotic usage for the first time in 6 years. My recent pregnancy was full-term and produced a perfectly health baby girl. No allergic reaction has occurred with clinical cannabis allowing long-term treatment. Analysis of ECS synthetic, primary, and allosteric binding, import, and degradative proteins for substitutions, deletions or additions may explain my immunological abnormalities. The DHHS US Patent 6,630,507 recommends 20mg/kg of CBD for my conditions, which I would like to try next for continued improvement.

# REDUCED CYTOTOXIC T LYMPHOCYTE (CTL) EFFECTOR RESPONSES IN CANNABINOID RECEPTORS 1 AND 2 DEFICIENT MICE *IN VITRO*

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Cannabinoid compounds exhibit effects on the immune system through cannabinoid receptor-dependent and -independent mechanisms. Cannabinoid receptor 1 (CB1) is highly expressed in the central nervous system, while cannabinoid receptor 2 (CB2) is highly expressed in the periphery, including the immune system. Previous studies showed that marijuana derived compound, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), regulated immune responses only partially through CB1 and CB2 in the presence of receptor antagonists or with $CB1^{-/-}/CB2^{-/-}$ (KO) mice generated from C57Bl/6 wild type (WT) background. In addition, it has been demonstrated that CB1 and CB2 play a critical role in the generation of the IgM anti-sheep red blood cell *in vitro* antibody response. Moreover, an *in vivo* influenza virus challenge model previously employed in our laboratory showed a more robust immune response to influenza virus challenge in KO mice compared to WT mice. Also exacerbated allergic contact dermatitis associated inflammation has been reported in the cannabinoid receptors deficient mice by Karsak and coworkers. Thus, the objective of the present study was to investigate the role of CB1 and CB2 in the generation of functional CTL effectors *in vitro*. In an allogeneic model, splenocytes (SPLC) from WT and KO mice were co-cultured with major histocompatability complex (MHC)-mismatched P815 (DBA/2) cells. The kinetics of CTL activity against P815 target cells was examined on day 3, 5, 7 and 9 post elicitation with different CTL effectors to targets ratios using a standard $^{51}$Cr-release assay. A peak response was observed on day 5. In addition, the ability to lyse P815 target cells was decreased in CTLs derived from KO mice compared to WT mice. In a syngeneic model, CTLs were elicited in response to an immunodominant peptide from influenza virus ($PB1_{703-711}$) presented in the context of MHC I. The PB1 peptide was loaded onto antigen presenting RMA-S cells which were then co-cultured with SPLC from WT and KO mice. Five days after elicitation, the resulting population was assayed for CTL activity using IFN-γ ELISPOT to enumerate peptide specific CTLs. As a result, lower numbers of IFN-γ producing cells were observed in peptide-specific CTL populations elicited from SPLC of KO mice compared to WT mice. Overall, these results imply that CB1 and CB2 play an important role in the generation of functional CTLs.

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# $\Delta^9$-TETRAHYDROCANNABINOL DIRECTLY IMPAIRS CYTOTOXIC T LYMPHOCYTE EFFECTOR FUNCTION DURING DIFFERENTIATION

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Our laboratory previously investigated the effect of $\Delta^9$-tetrathydrocannabinol ($\Delta^9$-THC) on host resistance to influenza virus (A/PR8/34) challenge. Influenza virus elicits a strong antiviral immune response in which the presence of influenza-specific $CD8^+$ T cells parallels the clearance of the virus from the lungs. Our studies show that viral lung burden was increased by $\Delta^9$-THC administration when compared to the vehicle control group. Furthermore, $\Delta^9$-THC significantly decreased $CD8^+$ T cell numbers in bronchoalveolar lavage. According to this endpoint, $CD8^+$ T cells were more sensitive to $\Delta^9$-THC compared to $CD4^+$ T cells. We hypothesized that there is a direct effect of $\Delta^9$-THC on $CD8^+$ T cell function and is responsible, at least in part, for the $\Delta^9$-THC-mediated increase in lung viral burden. We employed two *in vitro* models of $CD8^+$ T cell elicitation to test the effect of $\Delta^9$-THC on the differentiation of $CD8^+$ T cells into cytotoxic T lymphocytes (CTL). We used splenocytes of C57Bl/6 mice as a source for the T cells. In an allogeneic model, CTL were elicited in response to an MHC mismatch of the P815 cell line originating from DBA/2 mice. Also a syngeneic model was employed in which CTL were elicited to an immunodominant peptide epitope ($PB1_{703\text{-}711}$) derived from A/PR8/34. This epitope was exogenously loaded onto RMAS cells, which act as antigen presenting cells. In the allogeneic model, $\Delta^9$-THC reduced the cytolytic activity of CTL when present during the elicitation but not when introduced at the effector phase. In the syngeneic model, $PB1_{703\text{-}711}$ stabilized MHC I on the surface of RMAS cells, indicating presentation of the peptide in the context of MHC I. Furthermore, $\Delta^9$-THC reduced the cytolytic activity as well as impaired the production of IFNγ, an important cytokine in the antiviral response, when added during the elicitation phase. In conclusion, $\Delta^9$-THC directly impairs CTL effector function *in vitro* during the course of differentiation, but not after differentiation.

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# FORMATION AND IMMUNE MODULATING ACTIVITIES OF THE ETHANOLAMIDE FROM DOCOSAHEXAENOIC ACID IN 3T3L1 ADIPOCYTES AND RAW 264.7 MACROPHAGES

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Omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosa eicosapentaenoic acid (EPA) have been linked to several positive health effects. In relation to inflammation and other immunological processes this could be explained by their role in the synthesis of endocannabinoids. To investigate the biosynthesis of potentially immune modulating endocannabinoids, 3T3 L1 adipocytes were exposed to DHA. The formation of the ethanolamide of DHA (DHEA) was measured by LC-MS/MS and its effect on IL-6 release and NO production was investigated in 3T3-L1 adipocytes and RAW264.7 macrophages, respectively.

3T3L1 pre-adipocytes were differentiated using the MDI-protocol. Cells were used at day 8-10 after induction of differentiation. Adipocytes were exposed to DHA for 24 and 48 hr. Medium was analyzed for DHEA as described previously (Balvers et al, *J. Chrom. B* 2009). To evaluate the effect of DHEA on cytokine production, 3T3-L1 adipocytes were pre-incubated for 1 hr with DHEA and subsequently exposed to medium containing DHEA in the presence of LPS for 4 and 8 hrs. IL-6 levels in medium were determined using ELISA. NO production by RAW264.7 macrophages was assessed using the Griess assay.

Exposing 3T3-L1 cells for 24 hr to 10 µM DHA resulted in increased DHEA levels to $4.2 * 10^{-5}$ µg/mL compared to $2.1 * 10^{-5}$ µg/mL (control; $P = 0.08$), whereas 30 and 50 µM DHA resulted in $9.0 * 10^{-5}$ µg/mL and $14 * 10^{-5}$ µg/mL DHEA, respectively ($P < 0.0001$ for both concentrations). DHEA levels increased significantly after 48 hr exposure to 10 µM DHA to $6.5 * 10^{-5}$ µg/mL compared to $2.4 * 10^{-5}$ µg/mL (control, $P < 0.05$), whereas 30 and 50 µM DHA resulted in $9.6 * 10^{-5}$ µg/mL and $15.3 * 10^{-5}$ µg/mL DHEA, respectively ($P < 0.0001$ and $P < 0.001$, respectively). DHEA reduced IL-6 production by 3T3-L1 cells after LPS stimulation from 3.3 ng/mL (control) to 2.8 and 2.6 ng/mL after 4 hr co-incubation with 10 and 50 µM DHEA, respectively ($P < 0.05$ and $P < 0.001$, respectively). Co-incubation for 8 hr also resulted in a decrease from 6.7 to 3.9 and 3.6 ng/mL for 10 and 50 µM, respectively ($P < 0.05$ and $P < 0.001$, respectively). In addition, 48 hr incubation with 1 and 10 µM DHEA significantly decreased NO production by RAW264.7 macrophages after LPS challenge.

It can be concluded that 3T3-L1 cells can form DHEA after exposure to DHA which has potentially immune modulating properties.

# PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR γ IS ACTIVATED BY 15d-PGJ2-G, A PUTATIVE METABOLITE OF 2-ARACHIDONYL GLYCEROL

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Previous published results from our laboratory demonstrated that 2-arachidonyl glycerol (2-AG), an endocannabinoid, suppressed IL-2 secretion in activated T cells by the activation of peroxisome proliferator activated receptor γ (PPARγ) independent of the cannabinoid receptors, CB1 and CB2. 2-AG is structurally similar to arachidonic acid and a comparison of the eicosanoid pathway of arachidonic acid and 2-AG predicts that the potential metabolites of 2-AG are glycerol esters of various prostaglandins and thromboxanes produced from arachidonic acid. One of the putative metabolites of 2-AG is a glycerol ester of a prostaglandin, 15d-PGJ2-G. Treatment of Jurkat T cells with increasing concentrations of 15d-PGJ2-G followed by activation with phorbol ester and ionomycin demonstrated that 15d-PGJ2-G suppressed IL-2 secretion in activated T cells in a concentration-dependent manner. Since one of the known ligands for PPARγ is 15d-PGJ2, it was investigated if 15d-PGJ2-G served as a ligand for PPARγ. Cotransfection of HEK293T cells with PPARγ-LBD plasmid and pFR-luc reporter plasmid followed by treatment with 15d-PGJ2-G demonstrated that 15d-PGJ2-G transcriptionally activated PPARγ-LBD in a concentration-dependent manner. In addition, the increase in luciferase activity caused by increasing concentrations of 15d-PGJ2-G was inhibited in the presence of a PPARγ antagonist, T0070907. We hypothesize that 15d-PGJ2-G is a metabolite of 2-AG and is responsible for IL-2 suppression by acting in a CB1/CB2 independent manner through the activation of PPARγ. (Supported in part by NIH RO1 DA12740 and Student Research Fellowship from Pfizer Global Research).

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