CB_2 CANNABINOID RECEPTORS: NEW VISTAS

The first International Conference devoted to studies of the CB_2 cannabinoid receptor

Organised by:

Keith A. Sharkey, Canada (Chair)

Marnie Duncan, Canada
Ken Mackie, USA
Ruth Ross, UK
Betty Yao, USA

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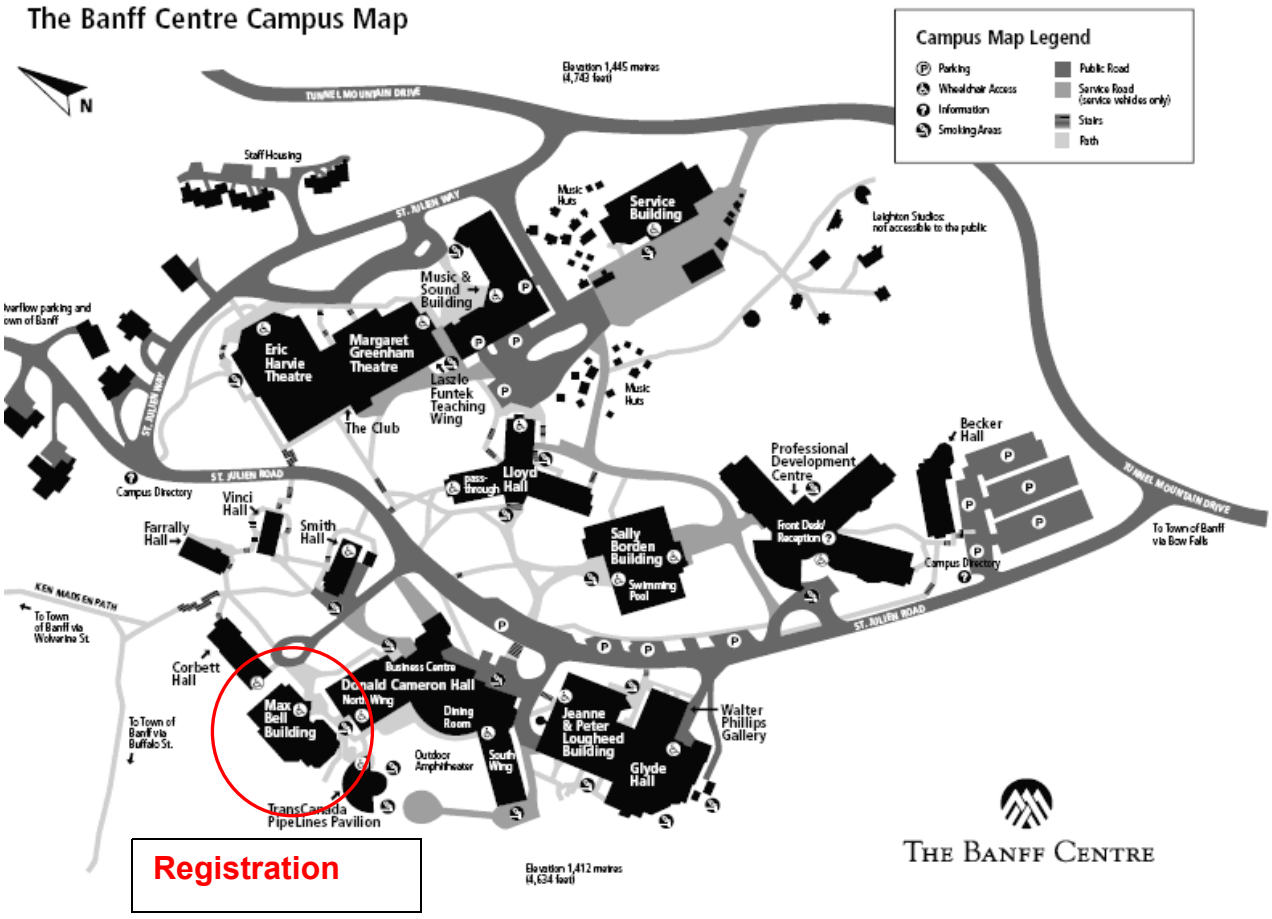

Daily Program

All presentations are in the Max Bell Building.

Thursday, May 31

Arrival		
2.30 - 5.30 p.m.	Registration (Max Bell Foyer)	
5.30 – 7:00 p.m.	Buffet dinner (Banff Centre Dining Room)	
7.15 - 7.30 p.m.	Welcome to Banff – **Dr. Keith Sharkey**, University of Calgary	
7.30 - 8.30 p.m.	*Keynote address:* The enzymatic regulation of endocannabinoid signaling – our state of understanding and the future challenges that remain	***Dr. Benjamin Cravatt*** *Scripps Research Institute*
8.30 - 10:00 p.m.	Opening reception / mixer	

Friday, June 1

7:00 - 8:00 a.m.	Breakfast (Banff Centre Dining Room)	

Session I ***Structural and molecular determinants of CB_2 receptors and CB_2 receptor signaling***

Session Moderator: **Dr. Alex Makriyannis**, Northeastern University

8:15 – 8:30 a.m.	Opening remarks – **Dr. Ken Mackie**, Indiana University	
8:30 – 9:00 a.m.	Pharmacological interactions between phytocannabinoids and the cannabinoid CB_2 receptor	***Dr. Roger Pertwee*** *University of Aberdeen*
9:00 – 9:30 a.m.	Structural determinants of CB_2 receptor selective ligands	***Dr. John Huffman*** *Clemson University*
9:30 – 10:00 a.m.	Inverse agonists of CB_2 receptors	***Dr. Charles Lunn*** *Schering-Plough Research Institute*
10:00 –	Modeling and development of CB_2	***Dr. Antti Poso***

10:30 a.m.	selective ligands	*University of Kuopio*
10:30 - 11:00 a.m.	Coffee Break	
11:00 – 11:30 a.m.	Agonist-directed trafficking of CB_2 receptors	***Dr. Paul Prather*** *University of Arkansas*
11:30 – 12:00 p.m.	Mapping the effects of CB_2 agonists on regional brain activity using pharmacological magnetic resonance imaging	***Dr. Gerard Fox*** *Abbott Laboratories*

Session II ***Lunch and poster session***

12:00 - 3:00 p.m.	Posters and working lunch
3:00 – 3:30 p.m.	Coffee Break

Session III ***CB_2 receptor in osteoporosis***

Session moderator: **Dr. Nephi Stella**, University of Washington

3:30 – 4:00 p.m.	CB_2 receptor and the regulation of bone mass	***Dr. Itai Bab*** *Hebrew University of Jerusalem*
4:00 – 4:30 p.m.	CB_2 receptors modulate macrophage, microglia and osteoclast activities	***Dr. Andreas Zimmer*** *University of Bonn*
4:30 – 5:00 p.m.	Endocannabinoids are produced by bone cells and stimulate activity of human osteoclasts in vitro	***Dr. Ruth Ross*** *University of Aberdeen*
6:00 p.m.	Buffet Dinner (Banff Centre Dining Room)	

Saturday, June 2

8:00 - 9:00 a.m.	Breakfast (Banff Centre Dining Room)	

Session IV *CB_2 receptors and immune modulation*

Session moderator: **Dr. Ruth Ross,** University of Aberdeen

9:00 – 9:30 a.m.	CB_2 receptors in brain: role in central immune function	***Dr. Guy Cabral*** *Virginia Commonwealth University*
9:30 – 10:00 a.m.	Cannabinoid CB_2 receptors and Alzheimer's disease: interaction with Aβ peptide	***Dr. Julian Romero*** *Fundación Hospital Alcorcón*
10:00 – 10:30 a.m.	CB_2 receptors regulate microglia and astrocytoma migration	***Dr. Nephi Stella*** *University of Washington*
10:30 – 11.00 a.m.	Direct suppression of autoreactive lymphocytes in the brain via the CB_2 receptor	***Dr. Bonnie Dittel*** *Blood Centre of Wisconsin*
11:00 – 1:00 p.m.	Coffee, Informal Discussions and Lunch (Lunch 12:00 – 1:00, Banff Centre Dining Room)	

Session V *CB_2 receptor and the modulation of pain*

Session moderator: **Dr. Ken Mackie**, Indiana University

1:00 – 1:30 p.m.	Cannabinoid CB_2 modulation of persistent pain states: self-administration of a CB_2 agonist in an animal model of neuropathic pain	***Dr. Andrea Hohmann*** *University of Georgia*
1:30 – 2:00 p.m.	Role of peripheral, spinal and supraspinal CB_2 receptors in a model of neuropathic pain	***Dr. Victoria Chapman*** *University of Nottingham*
2:00 – 2:30 p.m.	Mechanism of CB_2 agonists in the treatment of neuropathic pain: functional and behavioral consequences of receptor upregulation along pain pathways	***Dr. Michael Meyer*** *Abbott Laboratories*
2:30 – 3:00 p.m.	Development of agonists to the cannabinoid receptor type 2 as drugs for neuropathic pain	***Dr. Arnon Aharon*** *Pharmos Ltd*
3:00 –	Comparative roles of CB_2 and CB_1	***Dr. Jennifer Laird***

3:30 p.m.	receptors in animal models of pain	*AstraZeneca R&D*
3:30 – 4:00 p.m.	Coffee Break	

Session VI ***Talks from submitted abstracts***

Session moderator: **Dr. Keith Sharkey**, University of Calgary

4:00 – 6:00 p.m.

1. *Dr. Nele Evans*
   Comparison of two PET-radioligands with affinity for theCB_2 receptor

2. *Dr. David Thomson*
   Discovery of a novel class of CB_2 receptor agonists

3. *Dr. Ming Huang*
   CB_2 agonists regulate LPS-stimulated cytokine production in mice via a non-CB_2 receptor mechanism

4. *Mr. Aaron Miller*
   CB_2-mediated chemotaxis of astrocytoma cells

5. *Dr. Pal Pacher*
   Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury

6. *Dr. Neta Rimmerman*
   Compartmentalization of endogenous 2-AG into lipid rafts in a dorsal root ganglion cell line

7. *Dr. Brendan Bingham*
   The effects of AM1241 and its separated enantiomers at human, mouse and rat CB_2

8. *Dr. Niklas Schuelert*
   Selective activation of cannabinoid CB_2 receptors modulates mechanosensitivity of afferent nerve fibres in control but not osteoarthritic rat knee joints

6:30 p.m. Conference Dinner (Banff Centre Dining Room)

Sunday, June 3

8:00 – 9:00 a.m.	Breakfast (Banff Centre Dining Room)	

Session VII ***Role of CB_2 receptors in reproductive, cardiovascular, liver and gastrointestinal systems***

Session moderator: **Dr. Betty Yao**, Abbott Laboratories

9:00 – 9:30 a.m.	Role of the CB_2 receptor in reproduction	***Dr. Mauro Maccarrone*** *University of Teramo*
9:30 – 10:00 a.m.	Antifibrogenic role of the CB_2 cannabinoid receptor in the liver	***Dr. Sophie Lotersztajn*** *INSERM U841*
10:00 – 10:30 a.m.	Implications of CB_2 receptor in cardio-vascular disease	**Dr. *François Mach*** *Geneva University*
10:30 - 11:00 a.m.	Coffee Break	
11:00 – 11:30 a.m.	CB_2 in gastrointestinal inflammation	***Dr. Karen Wright*** *University of Bath*
11:30 – 12:00 a.m.	CB_2 receptor in the enteric nervous system and its role regulating intestinal motility	***Dr. Marnie Duncan*** *University of Calgary*
12:00 – 1:00 p.m.	Lunch (Banff Centre Dining Room)	

Depart Sunday PM

Abstracts of Invited Talks

Keynote address

THE ENZYMATIC REGULATION OF ENDOCANNABINOID SIGNALING – OUR STATE OF UNDERSTANDING AND THE FUTURE CHALLENGES THAT REMAIN

Benjamin F. Cravatt

The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA

Endogenous cannabinoids (endocannabinoids) constitute an emerging class of signaling lipids that act on both central and peripheral cannabinoid receptors, which also mediate the effects of delta9-tetrahydrocannabinol, the active component of marijuana. The magnitude and duration of endocannabinoid signaling are tightly controlled in vivo by the action of multiple biosynthetic and degradative enzymes. Much progress has been made in understanding the identity and function of these endocannabinoid-related enzymes. Nonetheless, many challenges remain, including confirming the contribution made by specific enzymes to endocannabinoid metabolism in vivo, developing selective pharmacological tools to perturb the function of these enzymes in living systems, and ascertaining the behavioral consequences of short- and long-term alterations in endocannabinoid tone. The vigorous pursuit of these objectives promises to not only enrich our understanding of the role that the endocannabinoid system plays in mammalian physiology and pathology, but also offers exciting possibilities for the development of next-generation therapeutics for a range of human disorders.

Session I

PHARMACOLOGICAL INTERACTIONS BETWEEN PHYTOCANNABINOIDS AND THE CANNABINOID CB_2 RECEPTOR

Roger Pertwee, Lisa A. Gauson, Lesley A. Stevenson, Adèle Thomas, Gemma L. Baillie & Ruth A. Ross

School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, Scotland, UK

Aims: There is evidence that the phytocannabinoids, cannabidiol (CBD) and cannabigerol (CBG), are anti-inflammatory and also that inflammation can be alleviated by compounds that target the CB_2 receptor as agonists or inverse agonists. The aim of this investigation was to establish whether CBD and CBG activate CB_2 receptors or behave as CB_2 receptor inverse agonists and to compare these compounds both with a third phytocannabinoid, Δ^9-tetrahydro-cannabivarin (THCV), and with the CB_1/CB_2 receptor agonist, CP55940.

Methods: CB_2 receptor binding and agonism/inverse agonism were measured *in vitro* using Chinese Hamster Ovary (CHO) cells transfected with the human CB_2 receptor (hCB_2) or membranes prepared from these cells.

Results: [^{3}H]CP55940 was displaced from hCB_2 CHO cell membranes more potently by CBG (K_i = 337 nM) and THCV (K_i = 63 nM) than by CBD (K_i = 4200 nM). At submicromolar concentrations, all three compounds inhibited forskolin-induced stimulation of cyclic AMP production by hCB_2 CHO cells, their rank orders of potency and intrinsic activity being CBD > THCV > CBG and THCV > CBD = CBG respectively. The intrinsic activity of each compound was significantly less than that of CP55940. Whereas THCV also behaved as a partial agonist in the [^{35}S]GTPS binding assay performed with hCB_2 CHO cell membranes, CBD (1 & 10 μM) behaved as an inverse agonist in this bioassay, as indicated by its ability to inhibit [^{35}S]GTPS binding to these membranes.

Conclusions: These findings suggest that the anti-inflammatory effects of CBG and CBD may be due at least in part to CB_2 receptor activation (CBG and CBD) or to inverse agonism (CBD). Additional experiments are required to establish first, whether CBG, THCV and/or CBD also behave as agonists or inverse agonists at CB_2 receptors in a cell line or tissue in which these receptors are less over-expressed than in the hCB_2-CHO cells used in this investigation, and second, whether THCV has anti-inflammatory properties.

Funding: GW Pharmaceuticals, BBSRC and NIDA.

STRUCTURAL DETERMINANTS OF CB_2 RECEPTOR SELECTIVE LIGANDS

John W. Huffman

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Although many compounds representing a variety of structural types are selective ligands for the CB_2 receptor, the structural features that give rise to this selectivity have not been established. Structure-activity relationships (SAR) at the CB_1 receptor have been determined for traditional cannabinoids related to ^{9}-THC, non-traditional cannabinoids such as CP-55,940 and cannabimimetic indoles, however, there is little published information concerning SAR at the CB_2 receptor for any of these major groups of cannabinoid receptor ligands. There is evidence, primarily from modeling studies of both the CB_1 and CB_2 receptors, combined with site-directed mutagenisis applied to both receptors, that ligands interact somewhat differently with each receptor. Cannabimimetic indoles and related compounds interact with both receptors primarily by aromatic stacking while traditional cannabinoids and the CP-compounds interact with the CB_1 receptor by a combination of hydrogen bonding and lipophilic interactions. This latter group of compounds interacts with the CB_2 receptor by a similar combination of hydrogen bonding and lipophilic interactions, however the hydrogen bonding involves a serine on helix-3 of the receptor, rather than a lysine as in the CB_1 receptor.

Our group has prepared several series of 1-methoxy and 1-deoxy-^{8}-THCs, 1-bromo-^{8}-THC analogs and 1-methoxy and 1-deoxy-11-nor-9-hydroxyhexahydrocannabinols. Many members of these series of compounds are highly selective ligands for the CB_2 receptor and we have been able to develop preliminary SAR not only for CB_2 receptor affinity, but also for receptor selectivity. A series of 1-deoxy analogs of CP-47,497 and CP-55,940 also exhibit selectivity for the CB_2 receptor, but have little affinity for either receptor. The effects upon receptor selectivity of side chain structure, C-1 substituent and hydroxylation at C-11 will be presented.

The CB_1 and CB_2 receptor affinities of several hundred cannabimimetic indoles have been reported and a number of these compounds are highly selective ligands for the CB_2 receptor. Principal structural variations that influence receptor selectivity include the substituent at the indole nitrogen and the nature of the substituent at C-3 of the indole nucleus. The manner in which various substituents at these positions affect CB_1 and CB_2 receptor affinity as well as selectivity for the CB_2 receptor will be discussed.

There are a number of other structural classes of ligands that exhibit selectivity for the CB_2 receptor, including pyrazoles, sulfones and 2- and 4-quinolones. Although these compounds are structurally interesting, at the present time there are too few examples of each structural type to develop SAR or criteria for receptor selectivity.

Funding: The work at Clemson University is supported by the NIDA grant DA03590.

INVERSE AGONISTS OF CB_2 RECEPTORS

Charles A. Lunn

Department of Immunology and New Lead Discovery, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, New Jersey 07033

Aims: We have identified a novel set of orally active triaryl bis sulfones that behave as cannabinoid CB_2-specific inverse agonists in standard binding and activity assays. These compounds modulate immune cell mobility in vitro and in vivo, and we postulate that such compounds may have broad therapeutic effects on many systemic immune diseases. In this report, we review our data characterizing the biology of these cannabinoid CB_2-specific inverse agonists.

Results: The triaryl bis sulfones moderate several disease models that require immune cell migration to a site of insult. For example, the compounds block lung eosinophilia in an asthma model induced with an aerosolized antigen. The compounds also ameliorate bone damage in an arthritis model induced by injecting antigen directly into the joint. New data will show that oral administration of a cannabinoid CB_2-specific inverse agonist can modulate symptoms of antigen-induced experimental allergic encephalomyelitis (EAE), a model of human multiple sclerosis. This data complements data showing that mice lacking the cannabinoid CB_2 receptor exhibit diminished symptoms associated with induction of EAE. We will also describe initial experiments to determine the cellular mechanism by which triaryl bis sulfones modulate cell mobility. We show that these compounds modulate a constellation of phosphoproteins unique from those altered by standard cannabinoid agonists.

Conclusions: Characterization of the proteins will lead to a detailed understanding of the mechanism of action of the triaryl bis sulfones in vivo, and will add to our understanding of the biology of the cannabinoid CB_2 receptor.

MODELING AND DEVELOPMENT OF CB_2 SELECTIVE LIGANDS

Antti Poso[1], Heikki Käsnänen[1], Outi Salo-Ahen[2], Tapio Nevalainen[1]

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Aims: The aim of this study was to understand possible ways to create selective CB_2 ligands.

Methods: There are only limited information available concerning the structures of both CB_1 and CB_2 receptors. In addition to our own rhodopsin (inactive state) based models (O. Salo et al, 2004 and 2006) there are also some other $CB_{1/2}$ models published. Unfortunately not a single one of those models can be stated to be in an "active" state. Recently published active state X-ray structure of rhodopsin is a new way to understand the selectivity issues. Unfortunately the resolution of active rhodopsin X-ray structure is far from perfect. In spite of this we decided to use this X-ray structure as a template for activated CB_1 and CB_2 models. Sequence alignment was done as described earlier. The models were created and refined using standard methods.

After construction of the models we used GRID method to study the differences between CB_1/CB_2 active and inactive state binding cavities and automated molecular docking methods (GOLD, Surflex-Dock) to gain unbiased receptor-ligand interaction profiles. We also used molecular dynamics to produce a set of possible receptor conformations (simulation done within a lipid-bilayer environment).

Results:
1. There is not so big difference between active and inactive state of CB_2 receptors (and the same holds true also for CB_1). The main difference is close the intracellular region, while most of the ligand binding domain seems to be only slightly influenced by the activation of the receptor.
2. The detailed analysis of selectivity issue (using active state of CB_1 and CB_2) is not finished at the moment of submitting this abstract. The results will be presented during the meeting.

Conclusions: To be presented during the meeting.

Funding: University of Kuopio, National Technology Agency Tekes, Finnish Academy, ISB graduate school.

AGONIST-DIRECTED TRAFFICKING OF CB_2 RECEPTORS

Jennifer L. Shoemaker, Michael B. Ruckle, Philip R. Mayeux and Paul L. Prather

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA 72205

Aims: Different cannabinoid agonists, acting through CB_2 receptors, have distinct effects in regulating specific functions of immune and inflammatory cells. The differential actions of these agonists at CB_2 receptors could be due to their ability to selectively activate distinct signaling pathways. The purpose of this study was to determine whether agonists acting at CB_2 receptors selectively direct the trafficking of intracellular responses.

Methods: This study examined the ability of the endocannabinoids, 2-arachidonoyl glycerol (2-AG) and noladin ether, as well as the synthetic cannabinoid CP-55,940 to regulate three intracellular effectors via CB_2 receptors in transfected CHO cells.

Results: Although the three agonists regulate all effectors with equivalent efficacy, the rank order of potencies differs depending on which effector is evaluated. Noladin ether and CP-55,940 most potently inhibit adenylyl cyclase, requiring higher concentrations to stimulate the extracellular signal-regulated kinase subgroup of the mitogen-activated protein kinases (ERK-MAPK) and Ca^{2+}-transients. In contrast, 2-AG most potently activates ERK-MAPK, necessitating greater concentrations to inhibit adenylyl cyclase, and even higher amounts to stimulate Ca^{2+}-transients. The CB_2 antagonist AM630 reverses the actions of all agonists except Ca^{2+}-transient stimulation by 2-AG. However, the effect of 2-AG on Ca^{2+}-transients is attenuated by a second CB_2 antagonist SR144528. This suggests that 2-AG stimulates Ca^{2+}-transients by binding to sites on CB_2 receptors distinct from those occupied by AM630 and the other cannabinoids examined. Agonists produce no effects in pertussis toxin-treated cells.

Conclusions: Cannabinoid agonists distinctly bind to CB_2 receptors and display different rank order of potencies for regulation of intracellular effectors. These data provide direct evidence for agonist-directed trafficking of response by endocannabinoids acting at CB_2 receptors.

Funding: National Institute on Drug Abuse (DA13660).

MAPPING THE EFFECTS OF CB_2 AGONISTS ON REGIONAL BRAIN ACTIVITY USING PHARMACOLOGICAL MAGNETIC RESONANCE IMAGING

Chih-Liang Chin, Terese R. Seifert, Ann E. Tovcimak, Vincent P. Hradil, Michael J. Dart, Gin C. Hsieh, Bryan F. Cox, Michael D. Meyer, Betty B. Yao and Gerard B. Fox.

Abbott Laboratories, Global Pharmaceutical Research & Development, Abbott Park, IL 60064

Cannabinoid (CB) receptors are currently under investigation as potential drug targets for pain. Subtypes identified to date include CB_1 and CB_2 receptors, both of which mediate analgesic effects in animal models. Recent publications indicate that selective activation of CB_2 receptors produces analgesia without the undesirable psychotropic side effects associated with activation of CB_1 receptors. Thus, it is important to demonstrate functional selectivity *in vivo* prior to developing selective CB_2 agonists for pain.

Pharmacological MRI (phMRI) is a relatively new and powerful tool in Drug Discovery that can provide *in vivo* functional information on effects of drugs in the CNS with high temporal and spatial resolution. Using a high field (7T) MRI scanner, we examined the effects of non-selective CB_1/CB_2 and selective CB_2 agonists in awake rats. In addition, pharmacological specificity was determined using selective CB_1 or CB_2 antagonists.

The non-selective CB_1/CB_2 agonist produced a dose-related activation of brain regions that agrees well with published autoradiographic CB_1 receptor density binding maps, PET studies in baboons, as well as human phMRI data with the non-selective agonist tetrahydrocannabinol. Pretreatment with a CB_1 antagonist but not with a CB_2 antagonist, abolished these activation patterns, suggesting an effect mediated by CB_1 receptors alone. In contrast, no significant changes in brain activity were found with relevant doses of CB_2 selective ligands. However, at a high dose, CB_1-like activation patterns were evident with one CB_2 selective ligand and these effects were blocked by pretreatment with a CB_1 antagonist, indicating that this compound lacks sufficient selectivity for CB_2 receptors at this dose. This study therefore provided useful information for determining *in vivo* functional selectivity. Finally, as the presence of CB_2 receptors in the brain remains controversial, our data suggest that, if CB_2 receptors are expressed, they are not functional under normal physiological conditions.

Session III

CB_2 RECEPTOR AND THE REGULATION OF BONE MASS

Itai Bab

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In vertebrates, bone mass is maintained constant between the end of skeletal growth and gonadal failure by a continuous destruction/formation process termed bone remodeling. This process consists of a resorption phase of pre-existing bone by a specific cell type, the osteoclast, followed by a phase of bone formation by another bone-specific cell type, the osteoblast. The physiologic importance of bone remodeling is best illustrated in osteoporosis, the most common degenerative disease in developed countries, which results from an impaired remodeling balance that leads to bone loss and increased fracture risk. The osteoblasts and osteoclasts express CB_2. Both osteoblast number and activity are enhanced by synthetic CB2-specific agonists and by anandamide, but not by 2-AG. The CB_2 stimulation of osteoblasts involves a MAP kinase-CRE pathway. A CB_2 specific agonist also restrains osteoclastogenesis by inhibiting proliferation of osteoclast precursors and receptor activator of NF-B ligand (RANKL) expression. RANKL is expressed in bone marrow-derived stromal cells and osteoblasts and activates RANK, which is present on osteoclasts and on their precursors. CB_2-deficient mice exhibit a markedly accelerated age-related bone loss, reminiscent of human osteoporosis. The CB_2 specific agonist, HU-308, partially prevents and rescues ovariectomy-induced bone loss by suppressing bone resorption and stimulating bone formation. Hence, CB_2 is essential for maintaining normal bone mass thus offering a molecular target for the diagnosis and treatment of osteoporosis.

CB_2 RECEPTORS MODULATE MACROPHAGE, MICROGLIA AND OSTEOCLAST ACTIVITIES

Andreas Zimmer[1], Judith Alferink[1], Itai Bab[3], Evelyn Gaffal[2], Andras Bilkei-Gorzo[1], Meliha Karsak[1], [4]Raphael Maldonado, [4]Xavier Nadal, Ildiko Racz[1] and Thomas Tüting[2]

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[4]Laboratory of Neuropharmacology, Department of Experimental Sciences and Health, University Pompeu Fabra, 08013 Barcelona, Spain

Interactions between the nervous and immune systems occur at different levels from peripheral tissues to the brain. They are important for normal physiology, but also contribute to pathological processes, including neurodegenerative and chronic pain disorders. A number of years ago we hypothesized that endocannabinoids released from nerve cells may be involved in the regulation of immune cell responses through activation of CB_2 receptors. In order to test this hypothesis, we generated CB_2 receptor knockout mice. Mice deficient of CB_2 receptors have an essentially normal number of immune cells as evaluated by flow cytometry analysis of primary and secondary lymphoid organs. However, we found that THC did not suppress the co-stimulatory capacity of macrophages from CB_2 knockout mice in an assay for T helper cell activation. We have now studied CB_2 receptor knockout animals in animal models for pain, contact skin hypersensitivity, and osteoporosis. Collectively, these studies suggest that cannabinoids modulate the activity of cells from the monocyte/macrophage cell lineage via CB_2 receptors. Furthermore, as we observed an osteoporotic phenotype in CB_2 receptor deficient mice, we performed a genetic study in human patients with osteoporosis and identified disease-associated CB_2 variants. CB_2 activation restrains the formation of osteoclasts, the bone resorbing cells which are derived from monocytes/macrophages. Because the inhibition of osteoclastogenesis involves downregulation of osteoclast precursors, these studies indicate that CB_2 polymorphisms may also affect the responsiveness of monocytes/macrophages to cannabinoid signaling in humans.

ENDOCANNABINOIDS ARE PRODUCED BY BONE CELLS AND STIMULATE ACTIVITY OF HUMAN OSTEOCLASTS *IN VITRO*

Ruth A. Ross, Susan A. Ridge, Lorna Ford, Lauren Whyte, Gary Cameron & Mike J. Rogers

Institute of Medical Sciences, University of Aberdeen, Scotland. UK. AB25 2ZD

Aims: The recent demonstration of abnormal bone phenotypes in cannabinoid receptor (CB_1 and CB_2) knockout mice implies a role for these receptors in bone physiology. However, the effects of cannabinoids on human osteoclasts are unknown and it is unclear whether endocannabinoids are produced in the bone microenvironment.

Methods: The production of anandamide and 2-AG in primary bone cells and cell lines using LC-MS/MS. Endocannabinoids were extracted from cultured cells with methanol/acetonitrile and levels were normalised to total protein content. The extraction efficiency was >95% and the limit of quantification were determined as 0.01pmol for anandamide and 25pmol for 2-AG. To study the possible role of these endocannabinoids in bone physiology, we investigated the effects of 2-AG, anandamide and CP55950 on the function of human osteoclasts derived from M-CSF-dependent peripheral blood monocytes.

Results: 2-AG was detected in mouse calvarial osteoblasts, osteoblast (MC3T3-E1 and MG-63), osteocyte (MLOY4) and macrophage cell lines (J774) and in human and mouse primary macrophages/osteoclast precursors and osteoclast-like cells. Anandamide was detected in calvarial osteoblasts, osteoblast and osteocyte cell lines but was not detectable in human and mouse primary macrophages or in osteoclasts. Addition of the calcitropic factor parathyroid hormone (PTH) caused a significant increase in the levels of 2-AG in the osteoblast-like cell line MC3T3-E1 (1.96 0.59nmol/mg protein to 3.66 1.03 nmol/mg protein). PTH also caused a significant increase in anandamide levels in calvarial osteoblasts (2.251.65 pmol/mg protein to 3.34 2.56 pmol/mg protein), while treatment of mouse osteoclasts with the bacterial endotoxin LPS caused a significant increase in 2-AG levels (1.37 0.44 nmol/mg protein to 4.81 3.35 nmol/mg protein). Treatment of human osteoclasts with 2-AG resulted in a 6.5-fold increase in bone resorption and a 2-fold increase in the number of F-actin rings in cultures of osteoclasts compared to treatment with vehicle control. Similarly, treatment with anandamide resulted in a 3.5-fold increase in resorption and a 1.7-fold increase in the number of F-actin rings in osteoclast cultures. Furthermore, the non-selective CB_1/CB_2 receptor agonist, CP55940 also significantly increased the proportion of actively-resorbing osteoclasts and increased resorption; at 1μM, cells with actin rings were 229% ± 31 of control ($P<0.05$); resorption area was 334% ± 50 of control ($P<0.01$).

Conclusions: We conclude that 2-AG and anandamide can be produced locally by bone cells and can be regulated by osteotropic factors. These endocannabinoids and CP55940 are activators of bone resorption by human osteoclasts.

Funding: Allergan Inc and The Nuffield Foundation.

Session IV

CB_2 RECEPTORS IN BRAIN: ROLE IN CENTRAL IMMUNE FUNCTION

G. A. Cabral, E. S. Raborn, and F. Marciano-Cabral

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Aims: There is increasing evidence that the receptor CB_2 plays a functionally relevant role in the central nervous system (CNS) during inflammation. This role appears to be exerted primarily through microglia, a resident population of macrophage-like cells. Microglia undergo a process of multi-step activation that is characterized by differential gene expression, including that of the CB_2, that correlates with signature functional activities. These observations suggest a functional window for microglia that may be linked to the CB_2. The aim of this study was to employ *in vitro* and *in vivo* models of neurotropic infection to examine the linkage of the CB_2 to microglial responsiveness.

Methods: 1A $(B_6C_3)F_1$ mouse model was developed for assessment of *in vivo* effects of cannabinoids on neuropathogenesis induced by *Acanthamoeba culbertsoni* (*A. culbertsoni*), an opportunistic free-living ameba that causes granulomatous amebic encephalitis (GAE) in immune deficient humans. Microglia were purified from neonatal Sprague-Dawley rat cerebral cortices for *in vitro* evaluation of the chemotactic response to amebae. Mutagenic RT-PCR, ribonuclease protection assay (RPA), and Western immunoblotting were used to assess for levels of CB_2 and cytokine mRNA and protein.

Results: Mice treated with delta-9-tetrahydrocannabinol (THC) or with CP55940 and infected with *A. culbertsoni* experienced exacerbated disease and higher mortalities than similarly infected vehicle controls. Immunofluorescence microscopy of brain tissue demonstrated colocalization of amebae and Mac1+ cells for vehicle-treated mice but not for THC- or CP55940-treated mice. Light microscopy revealed granulomas surrounding amebae for vehicle-treated mice but not for cannabinoid-treated mice. Purified microglia expressed CB_2 mRNA and cognate protein when in a "responsive" state of activation, a state for which chemotaxis is a signature activity. THC and CP55940 inhibited significantly the chemotaxis of these cells to ameba extract or to their released factors. Inhibition of chemotaxis was obtained also when the CB_2-selective ligand O-2137 was used while the CB_1-selective ligand ACEA had a minimal effect. The THC-mediated inhibition was reversed by the CB_2 antagonist SR144528 but not by the CB_1 antagonist SR141716A. THC and CP55940 also exerted a dose-related inhibition of microglial production of pro-inflammatory cytokines in response to *A. culbertsoni*. However, no pharmacological linkage to this action was obtained for either the CB_1 or CB_2.

Conclusions: The results implicate the CB_2 as playing a relevant role in early "immune responsiveness" to brain infection, namely the directed migration of microglia toward pathogens. They also articulate a mode by which THC may interfere in this process.

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CANNABINOID CB_2 RECEPTORS AND ALZHEIMER'S DISEASE: INTERACTION WITH ASS PEPTIDE

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Aims: Beta amyloid (A) deposition in the brain is one of the main hallmarks of Alzheimer's disease (AD) and is known to trigger a powerful local inflammatory response. Glial cells and, specifically, microglia play a prominent role in this process. Previously reported data suggest that CB_2 receptors are expressed by A-associated microglial cells and are capable of modulating microglial activity *in vitro*. The aim of the present work is to further characterize the link between A deposition and the microglial expression of CB_2 receptors and to explore their role in the microglial response against the pathological form of this peptide.

Methods: Two different approaches were used: i) a time-course immunohistochemical study of CB_2 expression in Down's syndrome (DS), a natural model of AD; and ii) an analysis of the consequences of CB_2 activation on A-removal from human AD tissue sections by THP1-derived macrophages.

Results: Immunohistochemical analysis of human DS samples obtained from individuals at different ages confirm a direct link between A-deposition and CB_2 induction in microglial cells. Control tissue samples as well as those from young DS showed no staining for CB_2, while adult DS exhibited significant A deposits (or "plaques") with associated CB_2-positive activated microglia. In addition, CB_2 activation by the selective agonist JWH-015 potently induced A removal from AD tissue sections by THP1-derived macrophages.

Conclusions: The present data confirm that the selective expression of CB_2 receptors by microglial cells is an A-linked event in AD and DS. In addition, these receptors are able to modulate cellular functions that may have potential therapeutic interest by enhancing A removal from the cerebral parenchyma.

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CB_2 RECEPTORS REGULATE MICROGLIA AND ASTROCYTOMA MIGRATION

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Several studies have shown that activation of CB_2 receptors can either stimulate or inhibit cell migration. We sought to test this possibility in microglial cell and astrocytomas, because of the therapeutic relevance of non-psychotropic CB_2 receptor pharmacology. Using RT-PCR, we found that both primary microglial cells in culture and the microglial cell line BV-2 express CB_2 receptor mRNA. Using an antibody that recognizes the carboxy-terminal portion of CB2 receptors, we found that this receptor is expressed at the leading edge of microglial cell lamellipodia, suggesting their ability to regulate cell migration. Accordingly, 2-AG-induced activation of CB_2 receptors expressed by BV-2 cells increases their migration.

Recently, we revisited these results by using Q-PCR and found that primary microglia actually express much less than one copy of CB_2 receptor mRNA *per* cell. Using diverse combinations of cytokines, we were not able to activate primary microglial cells in such a way that CB_2 receptor mRNA levels would reach more than one copy *per* cell. Using a newly developed cell migration assay based on near-infrared fluorescences and Licor Odyssea imaging, 2-AG or other synthetic cannabinoids did not significantly stimulate the migration of primary microglial cells, while other classic chemokines, including ATP and C5a, did.

Using Q-PCR, we found that the mouse astrocytoma cell line DBT does not express CB_1, CB_2 or GPR55. Thus, we generated stable DBT cell lines expressing either of these receptors. Activation of CB_2-DBT cell lines with CP55940 induces an SR144528-dependent increase in Erk phosphorylation, confirming the functionality of this receptor. Interestingly, CP55940 did not induce cell migration, while 2-AG did. This 2-AG induced migration of CB2-DBT cells was prevented by SR144258.

These results suggest that different agonists acting on CB_2 receptors might differentially regulate glial cell migration and that the mere activation of the Erk pathway is not sufficient to trigger migration.

DIRECT SUPPRESSION OF AUTOREACTIVE LYMPHOCYTES BY THE CNS MICROENVIRONMENT VIA THE CANNABINOID CB_2 RECEPTOR

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Aims: The cannabinoid system has been shown to have immune modulatory activity, and has been targeted as a treatment strategy for the central nervous system (CNS) autoimmune disease multiple sclerosis (MS). The aim of this study was to investigate the role of the CB_2 receptor in the regulation of autoimmune inflammation in the CNS.

Methods: Experimental autoimmune encephalomyelitis (EAE), the animal model of MS, was induced by the adoptive transfer of myelin basic protein-specific encephalitogenic T cells. EAE was induced in both wild-type (WT) and CB_2 receptor-deficient mice ($CB_2^{-/-}$) using either WT or $CB_2^{-/-}$ encephalitogenic T cells. The severity of EAE clinical disease was assessed daily. On day 13 after EAE induction, total mononuclear cells were isolated from the CNS and spleen, and the absolute number of encephalitogenic T cells was determined along with their level of apoptosis, proliferation and pro-inflammatory cytokine production.

Results: When EAE was induced with WT encephalitogenic T cells in WT and $CB_2^{-/-}$ mice, no difference in clinical disease severity was observed between the two groups. In contrast, when EAE was induced in WT mice with $CB_2^{-/-}$ encephalitogenic T cells, the mice exhibited very severe clinical disease as compared to WT. The more severe disease was associated with increased numbers of encephalitogenic T cells in the CNS undergoing reduced levels of apoptosis and a higher rate of both proliferation and inflammatory cytokine production. No difference in encephalitogenic T cell number or their effector function was observed in the periphery in WT and $CB_2^{-/-}$ mice.

Conclusions: Our results demonstrate that the CNS plays a critical role in the active suppression of immune responses via the cannabinoid system, in particular via the CB_2 receptor on T cells. These cumulative results suggest that cannabinoid-based therapeutics specifically targeting the CB_2 receptor would be beneficial in the treatment of MS by suppressing inflammation within the CNS.

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Session V

CANNABINOID CB_2 MODULATION OF PERSISTENT PAIN STATES: SELF-ADMINISTRATION OF A CB_2 AGONIST IN AN ANIMAL MODEL OF NEUROPATHIC PAIN

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Aims: Activation of cannabinoid CB_2 receptors suppresses acute, inflammatory and nerve-injury induced nociception. CB_2 receptors are localized predominantly outside the central nervous system (CNS) and are upregulated in the CNS by traumatic nerve injury. This distribution is consistent with the failure of CB_2 agonists to induce centrally-mediated side-effects associated with activation of CB_1. These observations support the hypothesis that analgesics targeting CB_2 would be unlikely to be psychoactive or addictive. This hypothesis however, remains to be empirically tested.

Methods: We evaluated the propensity of rats to self-administer the CB_2 agonist (R,S)-AM1241 in the presence and absence of neuropathic pain. A unilateral spared nerve injury was performed to induce neuropathic nociception. Control rats received a sham surgery and naive animals were left intact. All animals were surgically implanted with an indwelling jugular catheter to permit intravenous drug self-administration. Mechanical withdrawal thresholds were evaluated before and after surgical procedures and each drug self-administration session. Pressing the active, but not the inactive, lever elicited an intravenous infusion.

Results: Self-administration of (R,S)-AM1241, but not vehicle, increased mechanical paw withdrawal thresholds in neuropathic rats. Changes in withdrawal thresholds were absent in naive and sham-operated rats permitted to self-administer (R,S)-AM1241. (R,S)-AM1241 self-administration was observed in neuropathic and sham-operated groups. Naive animals showed no preference for responding on the active as opposed to the inactive lever. Naive, sham and neuropathic groups allowed to self-administer vehicle also failed to respond preferentially on the active as opposed to the inactive lever. The CB_2 antagonist SR144528 blocked the (R,S)-AM1241-induced suppression of nerve injury-induced mechanical hypersensitivity and attenuated responding on the active but not the inactive lever. The CB_1 antagonist SR141716A attenuated responding on both levers and consequently, partially attenuated (R,S)-AM1241 self-administration-induced changes in paw withdrawal thresholds.

Conclusions: Self-administration of a CB_2 agonist suppresses nerve injury-induced mechanical hypersensitivity. The contribution of central and peripheral CB_2 receptors to (R,S)-AM1241 self-administration remains to be determined .

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ROLE OF PERIPHERAL, SPINAL AND SUPRASPINAL CB_2 RECEPTORS IN A MODEL OF NEUROPATHIC PAIN

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Aims: Neuropathic pain states are associated with aberrant pain responses including touch-evoked pain (allodynia) and hyperalgesia, which are difficult to treat with conventional analgesics. Cannabinoid CB_1 receptor agonists attenuate nociceptive processing in animal models of neuropathic pain, but are associated with CNS mediated side-effects. Recent studies have reported the presence of CB_2 receptor mRNA and protein in the spinal cord of neuropathic rats and the brain of naïve rats. The aim of our recent studies was to investigate the effects of a selective CB_2 receptor agonist on nociceptive processing in the selective spinal nerve ligation model of neuropathic pain, compared to sham operated controls.

Methods: Rats received selective spinal nerve ligation (SNL) or sham surgery. 14 days following surgery, rats were anaesthetised and prepared for electrophysiological recordings at the level of the spinal cord, or the ventral posterolateral (VPL) nucleus of the thalamus. Spontaneous and mechanically-evoked responses of spinal and VPL neurones were recorded and the effects of local administration of the CB_2 receptor agonist, JWH133, on these responses were recorded in SNL and sham operated rats. JWH133 was administered either via intraplantar, spinal or intra-thalamic routes of administration.

Results: Intraplantar injection of JWH133 significantly attenuated innocuous and noxious mechanically-evoked responses of spinal neurones in both SNL and sham-operated rats. Effects of JWH133 were blocked by the CB_2 receptor antagonist SR144528. In contrast to these data, local spinal and intra-thalamic administration of JWH133 significantly attenuated innocuous and noxious-evoked responses of spinal and VPL neurones in SNL rats, but not sham operated rats. The effects of JWH133 were blocked by the CB_2, but not the CB_1 receptor antagonist.

Conclusions: These data demonstrate that there is a novel functional role of CB_2 receptors in the spinal cord and VPL nucleus of the thalamus in neuropathic rats, compared to sham operated rats. The lack of effect of JWH133 following thalamic administration in sham operated rats indicates that the recently described presence of CB_2 receptors in the thalamus of naïve rats are unlikely to modulate nociceptive processing at this level. The ability of the CB_2 receptor agonist to attenuate both innocuous and noxious-evoked responses of neurones in SNL rats suggests that these compounds may be able to modulate both allodynia and hyperalgesia associated with neuropathic pain states.

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MECHANISM OF CB_2 AGONISTS IN THE TREATMENT OF NEUROPATHIC PAIN: FUNCTIONAL AND BEHAVIORAL CONSEQUENCES OF RECEPTOR UPREGULATION ALONG PAIN PATHWAYS

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Although the analgesic properties of non-selective cannabinoid receptor agonists have been realized for many years, there is now an increasing body of evidence to support the potential utility of selective cannabinoid CB_2 receptor agonists for the treatment of pain. Strong supporting evidence for this hypothesis is provided from knockout studies and studies with a variety of CB_2-selective agonists, which have demonstrated efficacy in preclinical models of inflammatory, moderate to severe post-operative, and neuropathic pain.

Both the mechanism and site of action of CB_2 agonists in pain remains elusive. Studies using the selective CB_2 agonist AM1241 have implicated modulation of endogenous opioid systems as the underlying mechanism for CB_2mediated analgesia, whereas studies with other selective CB_2 agonists (GW405833, A-796260) have failed to demonstrate an opioid mechanism dependence. Several studies have demonstrated up-regulation of CB_2 receptor gene expression in pain pathways (spinal cord, dorsal root ganglia) in models of neuropathic pain, but the functional consequences of these changes in expression have not been reported.

Herein we report on a series of studies demonstrating differential changes in gene expression across different pain models. The functional consequences of those changes are demonstrated using wide dynamic range (WDR) neuron recording in anesthetized rats after both systemic and site injection of selective CB_2 agonists. These results are then extended to the observation of behavioral effects across different pain models after both systemic and site injection studies. From these studies, we conclude that the site of action of CB_2 agonists varies across different painful states, and correlates to changes in CB_2 receptor gene expression.

DEVELOPMENT OF AGONISTS TO THE CANNABINOID RECEPTOR TYPE 2 AS DRUGS FOR NEUROPATHIC PAIN

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The cannabinoid receptor CB_2, expressed by immune and peripheral nerve cells, has been recognized as a valid drug target for pain and inflammatory diseases. Pharmos CB_2 receptor agonists have demonstrated analgesic activity in neuropathic pain and ability to attenuate disease progression in multiple sclerosis and rheumatoid arthritis models.

Pharmos is focused on designing and developing novel CB_2 selective compounds. Data on Cannabinor, Pharmos lead compound, would be presented. Cannabinor has been evaluated in a phase I and phase IIa clinical trials for pain. Cannabinor was found to be safe and well tolerated. In the Phase IIa study using capsaicin induced pain model, cannabinor demonstrated a general analgesic effect measured in the non sensitized skin.

COMPARATIVE ROLES OF CB_2 AND CB_1 RECEPTORS IN ANIMAL MODELS OF PAIN

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There is overwhelming evidence that activation of cannabinoid receptors results in reduction in pain, both in animals and in man. CB_1 and CB_2 cannabinoid receptors have low homology and very different expression profiles, with CB_1 mostly expressed on CNS and peripheral neurones, and CB_2 predominately expressed on immune cells. Earlier studies in pain models suggested that the predominant receptor for analgesia was the CB_1 receptor, but there is accumulating evidence for a role for CB_2 receptors in certain pain conditions.

The evidence for either CB_1 or CB_2 receptor involvement requires, ultimately, the use of selective agonists and/or antagonists. Earlier studies were predominantly limited to using mixed CB_1 and CB_2 receptor agonists, for example WIN55,212,2, CP99,940, or nabilone. Distinguishing between a CB_1 or CB_2 receptor-mediated effect thus had to rely on the use of selective receptor antagonists. More recently, CB_2-selective ligands have been described in the literature and these compounds have been tested in a variety of pain models by several laboratories. The results obtained have been mixed but analgesic effects have been observed in many cases. We have tested several of these CB_2 selective agonists including GW405833 and HU308 and also observed analgesic effects in neuropathic and inflammatory models of pain in rats and mice.

However, before drawing firm conclusions from these results we realized that whilst the CB_2 ligands described are selective *in vitro*, the margin of separation from CB_1 is generally relatively small (<100 fold). This is not an issue for *in vitro* studies as the concentration can be controlled sufficiently precisely to exclude CB_1 receptor-mediated effects. However, when *in vivo* studies are conducted not only is it much more difficult to control exposure precisely, but, also, in the vast majority of studies, plasma or tissue concentrations have not been measured, the interpretation of the results relying on the assumption that the ligand(s) used retain the same selectivity *in vivo* as seen *in vitro*. The picture is further complicated as similar caveats apply to the widely used selective CB_1 and CB_2 receptor antagonists, rimonabant and SR144582. We have conducted a series of studies aimed at correlating *in vivo* efficacy with measures of plasma and tissue exposure to both agonists and antagonists in order to try and elucidate the true relative contribution of CB_1 and CB_2 receptors to the analgesia observed in different types of pain and after acute and chronic treatment. In addition, we have used CB_1 and CB_2 knockout mice to provide complementary evidence to support the agonist/antagonist efficacy studies.

I will present the results of these studies and the insight they have given us into the potential for CB_1 and/or CB_2 receptor-mediated analgesia.

Session VII

CB_2 RECEPTORS IN REPRODUCTION

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Mammalian reproduction is a complicated process designed to diversify and strengthen the genetic complement of the offspring. An emerging concept in mammalian reproduction is the role of endocannabinoids, a group of endogenously produced lipid mediators that bind and activate cannabinoid receptors. Although adverse effects of cannabinoids on fertility have been implicated for years, the mechanisms by which they exert these effects are not clearly understood. With the identification of cannabinoid receptors, endocannabinoid ligands, their key synthetic and hydrolytic pathways, and the generation of mouse models missing cannabinoid receptors or metabolic enzymes, a wealth of information on the significance of cannabinoid/endocannabinoid signaling in spermatogenesis, fertilization, preimplantation embryo development, implantation and postimplantation embryonic growth has been generated. This lecture will focus on the involvement of type-2 (CB_2) cannabinoid receptors in reproductive biology, covering both male and female fertility. It is hoped that a deeper insight would lead to potential clinical applications of the CB_2-dependent endocannabinoid signaling as a target for correcting infertility and improving reproductive health in humans.

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ROLE OF CB_2 RECEPTORS IN HEPATIC WOUND HEALING

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We have demonstrated that CB_2 receptors are markedly induced in cirrhotic human samples and that inactivation of CB_2 receptors enhances liver fibrogenesis, indicating that CB_2 receptors display antifbrogenic properties (Julien et al, 2005, Gastroenterology), in contrast to the profibrogenic role of CB_1 receptors in the liver (Teixeira-Clerc et al, 2006, Nature Medicine).

We recently investigated the role of CB_2 receptors in the wound healing response in two models of liver injury. Acute carbon tetrachloride (CCl_4) administration is a model of acute hepatitis characterized by parenchymal necrosis and liver inflammation, followed by hepatic regeneration from hepatocytes, and matrix remodelling following activation of liver fibrogenic cells. Choline-deficient ethionine supplemented (CDE) diet is a model of hepatic wound healing associated with regeneration from oval cells, a population of hepatic progenitors with potential differenciation into hepatocyte and biliary cells, that proliferate only in context where hepatocyte proliferation is impaired, and may also favor matrix remodelling and fibrogenesis. Results were compared in WT and CB_2 KO mice.

Hepatic CB_2 receptor expression was markedly induced in both models of liver injury. In CB_2 KO mice, acute CCl_4 administration was associated to increased liver injury, as well as to an enhanced wound healing response, as shown by higher induction of SMA and TGF-ß1. Moreover, hepatocyte proliferation was impaired in $CB_2^{-/-}$ mice as compared to WT mice, indicating that CB_2 stimulates liver regeneration following acute liver injury. CB_2 KO mice exposed to CDE diet also showed exacerbated liver injury and inflammation as compared to WT animals, and exhibited enhanced matrix remodelling, associated wih increased oval cell proliferation.

Taken together, these results indicate that CB_2 receptors decrease the matrix remodelling process associated to liver injury, in keeping with our previous data demonstrating the antifibrogenic properties of CB_2 receptors. Importantly, our data also unravel that CB_2 receptors regulate hepatic regeneration, with a positive effect on regeneration from hepatocytes, and an inhibitory effect on regeneration from progenitor cells. This differential effect of CB_2 receptors on liver regeneration needs further investigation, in particular in light of the potential role of hepatic progenitor cells in liver carcinogenesis.

IMPLICATIONS OF CB_2 RECEPTOR IN CARDIOVASCULAR DISEASE

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Atherosclerosis is a chronic inflammatory disease of the large arteries that represents the primary cause of heart disease and stroke. Derivatives of cannabinoids such as delta-9-Tetrahydrocannabinol (THC) modulate immune functions and therefore have a therapeutic potential for the treatment of inflammatory diseases. We tested THC on established atherosclerosis in a murine model. Oral administration of THC (1 mg kg^{-1} per day) resulted in the significant inhibition of disease progression, as demonstrated by reduced atherosclerotic plaque development within the aortic roots. This effective dose is lower than the dose required for psychotropic effects of THC. Furthermore, CB_2 receptor, the main cannabinoid receptor expressed on immune cells, was detected in both human and mouse atherosclerotic plaques. Lymphoid cells isolated from THC-treated mice exhibited diminished proliferation capacity and IFN- secretion, whereas IL-10 and TGF- production were not significantly altered. In vitro, THC also inhibited macrophage chemotaxis, a crucial step for the development of atherosclerosis. These effects were completely blocked by the specific CB2 antagonist SR144528, and absent when macrophages isolated from CB_2 knock-out mice were used. Our data demonstrate that oral treatment with a low dose of THC, through its pleiotropic immunomodulatory effects on lymphoid and myeloid cells, is a potent inhibitor of atherosclerosis progression in mice. Thus, oral THC therapy may prove beneficial for the treatment of patients with clinically manifested atherosclerosis.

CB_2 RECEPTORS IN GASTROINTESTINAL INFLAMMATION

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Aims: Crohn's Disease (CD) is a chronic inflammatory disease, with fibrosis and stricture formation being common complications that require surgical intervention. Recent studies report that CB_2 receptor agonists inhibit intestinal motility and modulate intestinal pain. The expression pattern and role of the CB_2 receptor has not been widely characterised in human small bowel, particularly in relation to Crohn's Disease. The aim of the present study is to determine the distribution of CB_2 receptors in human ileal sections comparing healthy and non-involved tissue with that from active CD and fibrotic CD.

Methods: Paraffin-embedded human ileal biopsies from patients with or without CD were used for immunohistochemical staining of CB2 receptors in mucosal and submucosal regions.

Results: Immunohistochemical studies demonstrate differential CB_2 receptor expression in areas of active disease. Healthy mucosal epithelium was CB_2 negative. Non-involved epithelium showed weak/negative immunostaining for CB_2, whereas positive membrane staining was seen at the ulcerative margins of active disease. This progressed from patchy to negative as distance from the ulcer increased. In addition, the ulcer-associated cell lineage (UACL), which appears in mucosal tissue during active disease and promotes healing of the damaged mucosa in the small intestine, was CB_2 positive. Submucosal fibrotic regions displayed smooth muscle metaplasia and fibroblast hyperplasia. Smooth muscle cells and submucosal fibroblasts were weakly CB_2 positive in healthy and non-involved ileal sections, but loss of CB_2 expression was evident in both cell types in nearly all cases of diseased tissue. CB_2 expression was also evident in submucosal and myenteric ganglia; as well as on plasma cells, neutrophils, activated macrophages and subsets of T cells.

Conclusions: This information indicates that in active human Crohn's Disease, CB_2 receptors are upregulated in the epithelium at the ulcerative margins and in the UACL implying potential involvement in mucosal regenerative processes. The functional consequence of CB_2 receptor loss from the submucosal fibroblasts and smooth muscle cells in fibrotic CD is currently under investigation. Increased CB_2 expression on immune cells is more likely to be related to the increased immune cell infiltrate, which is characteristic of intestinal inflammation, rather than to upregulation per se. The recent finding in an animal model of inflammation that reduced intestinal motility was mediated by CB_2 receptors on myenteric neuronal cells supports the idea that enteric ganglial CB_2 could be a potential target for motility disorders in humans.

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CB_2 RECEPTOR IN THE ENTERIC NERVOUS SYSTEM AND ITS ROLE REGULATING INTESTINAL MOTILITY

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Novel protective properties of CB_2 receptors in the gastrointestinal tract have recently been reported. The CB_2 receptor agonist JWH133 is able to normalize enhanced gastrointestinal motility in a model of lipopolysaccaride (LPS)-induced inflammation (Mathison *et al.,* BJP;142:1247-54). In addition, Hornby and colleagues have shown JWH133 to be protective in two distinct models of colitis (Hornby *et al.,* AJP;291:G364-G371). The localization or mechanism of actions of CB2 receptors in the gut is poorly understood and so the aim of the present study was to characterize CB2 receptors in the gastrointestinal tract and to understand how CB_2 receptor agonists correct motility dysfunction induced by LPS.

An *in vivo* LPS challenge (65 μgkg^{-1}, i.p.) results in enhanced gastrointestinal motility. Rats were either treated with vehicle or LPS, after 80 minutes animals were injected with drug or vehicle, at 120 minutes ileum tissues were removed for c-Fos immunohistochemistry. Tissues from either vehicle or LPS treated rats were mounted in organ baths and electrical field stimulation was applied in the presence or absence of CB_1 and CB_2 receptor agonists.

Immunohistochemical studies demonstrate that *in vivo* LPS treatment significantly enhances c-Fos expression in both enteric glia and neurons compared to control tissues. Treatment with JWH133 significantly attenuates the c-Fos activation in both enteric glia and neurons, and this action was reversed by the CB_2 receptor antagonist AM630. In the *in vitro* functional studies, JWH133 had no effect on the electrically-evoked twitch in the vehicle treated tissues compared to control responses, whereas the CB_1/CB_2 receptor agonist WIN55, 212 reduced the amplitude of the twitch responses in a concentration-dependant manner. In the LPS treated tissues, JWH133 was able to reduce the twitch response in a concentration dependant manner compared with vehicle control. WIN55, 212 reduced the amplitude of the twitch responses in a concentration-dependant manner and there was no significant difference in the actions of WIN55, 212 between the vehicle and LPS-treated tissues.

These data indicate that in a LPS model of inflammation, both enteric glia and neurons are activated, indicating a possible novel role of enteric glia in LPS-enhanced gastrointestinal motility. Furthermore, activation of CB_2 receptors under these conditions attenuate neuronal and glial activation as well as cholinergic neurotransmission in LPS-treated tissues. It would appear that LPS over-stimulates the enteric nervous system which results in enhanced motility, CB_2 receptors act to normalize this by reducing neuronal excitability.

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Abstracts of Submitted Presentations

Session VI

1. COMPARISON OF TWO PET-RADIOLIGANDS WITH AFFINITY FOR THE CB_2 RECEPTOR

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Aims: At this moment, no radioligand has been described for in vivo visualization of the cannabinoid-type 2 receptor (CB2R). We have synthesized and preliminarily evaluated (2,3-dichloro-phenyl)-[5-[^{11}C]methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]methanone **(1)** and N-[(1s)-1-[4-[[4-[^{11}C]methoxy-2-[(4-methoxyphenyl)sulfonyl)-phenyl] sulfonyl] phenyl]ethyl]methyl sulfonamide **(2)** as potential CB2 receptor radiotracer agents for positron emission tomography (PET).

Methods: Demethylation of **(1)** and **(2)** was performed using BBr_3 at -70°C to provide the phenol precursors for labelling. Radiolabelling with carbon-11 was achieved by methylation of the phenol compounds with [^{11}C]-methyl triflate or [^{11}C]-methyl iodide. The reaction product was purified by RP-HPLC and the log $P_{oct/buff}$, biodistribution and metabolism of **(1)** and **(2)** in mice were studied.

Results: Radiochemical yield in the optimised conditions (see table) was around 30% for both compounds. Using $^{11}CH_3I$ as a methylation agent to obtain **(1)** a yield of only 15% was obtained, while the alkylation of **(2)** went equally well with both alkylation agents.

compound	precursor amount	reagent	reaction time	base	temperature
(1)	200 µg	[^{11}C]-methyl triflate	2 min	Cs_2CO_3 (3 mg)	90°C
(2)	200 µg	[^{11}C]-methyl iodide	4 min	Cs_2CO_3 (3 mg)	90 °C

(1) has a log P value of 2.6 while **(2)** is a more polar compound with a log P value of 2.2. In spite of the favourable log P value, **(2)** did not show brain uptake in normal mice. In contrast, **(1)** showed high brain wash-in (1.44 % of ID at 2 min post injection (p.i.)) and rapid brain wash-out (0.13 % of ID in brain at 60 min p.i.). Plasma clearance proceeds mainly through the hepatobiliary pathway for both compounds. Plasma metabolite analysis showed 89, 51 and 15 % intact product for **(1)** and 93, 63 and 60 % intact product for **(2)** at 2, 10 and 30 min p.i. resp. The affinity of **(1)** and **(2)** for the human CB_2R was found to be 35 nM and 4.5 nM resp., for the human CB_1 receptor 6450 nM and 79 nM resp.

Conclusions: **(2)** shows higher affinity for CB2R and slower metabolisation than **(1)**, but is less selective (relative to CB_1) and does not pass the blood brain barrier. Both compounds will be further evaluated as PET tracers for in vivo visualisation of CB_2 expression where **(2)** will only serve for peripheral applications.

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2. DISCOVERY OF A NOVEL CLASS OF CB_2 RECEPTOR AGONISTS

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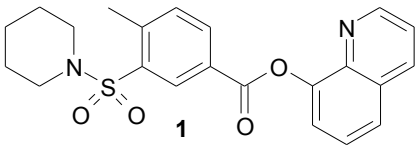
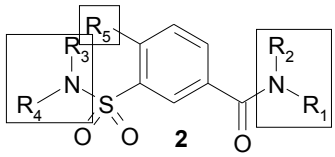
Aims: Literature evidence suggests that selective CB_2 agonists are potentially valuable therapeutics for the treatment of inflammatory diseases and chronic pain. Our goal is the identification of CB_2 selective agonists and their evaluation of pharmacological and pharmacokinetic properties. .

Methods: CB_2 and CB_1 agonistic activity is determined by their ability to inhibit cAMP production in forskolin stimulated CB_2 and CB_1 transfected CHO cells, respectively. Anti-inflammatory properties are tested in a mouse zymosan induced paw inflammation (ZIPI) model, measuring the efficacy of compounds to prevent paw swelling after zymosan injection. Oral exposure is tested in mouse by measuring plasma levels at various time points after oral administration.

Results: We identified commercially available arylsulfonamide **1** in a high-throughput screen (cAMP assay) as a CB_1/CB_2 dual agonist. Recently Lambeng et al. have also reported **1** and closely related analogs as novel highly potent CB_1 agonist.



1



2

Using **1** as a starting point our two primary goals were to find a replacement for the ester to eliminate a metabolic liability and to build in selectivity for CB_2 over CB_1. We have identified numerous amides **2** that possess the combination of potent CB_2 activity (cAMP EC_{50}<10nM) and high selectivity versus the CB_1 receptor (S > 1,000 fold). The structure-activity relationships of **2** will be described. Representative amides **2** demonstrated anti-inflammatory efficacy in the ZIPI model after i.p. administration. The compounds however show low oral bioavailability due to rapid metabolism. Optimization towards oral bioavailability will also be discussed.

Conclusions: The ZIPI data confirms the anti-inflammatory activity of CB_2 selective agonists. Further optimization of the arylsulfonamide class of compounds for oral bioavailability is required for the potential use of these compounds as orally available anti-inflammatory agents.

3. CB_2 AGONISTS REGULATE LPS-STIMULATED CYTOKINE PRODUCTION IN MICE VIA A NON-CB2 RECEPTOR MECHANISM

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Previously we observed that the CB_2 receptor-selective partial agonist AM1241 (CB2 EC50 of 12 nM; efficacy of ~20% relative to CP55,940) inhibited LPS-stimulated TNF-production in rats and the selective CB_2 antagonist SR144528 failed to fully abolish the in vivo effect of AM1241 (Huang et al. Soc. Neurosci. 686.1, 2006). Although the inability of SR144528 to block the *in vivo* activity of AM1241 could be due to its high plasma protein binding resulting in a low level of free plasma concentrations (<10 nM), it is also possible that the AM1241 effect was mediated by a non-CB_2 receptor mechanism. To test this hypothesis, we examined the ability of two structurally distinct CB_2 agonists AM1241 and HU308 to inhibit LPS-stimulated TNF-α production in wild type and CB_2 knockout mice (C57BL/6). AM1241 (50 mg/kg, ip) and HU308 (30 mg/kg, ip) were administered 60 minutes before LPS (0.15 mg/kg, iv), and blood was collected by cardiac puncture 90 minutes after LPS. Plasma samples were analyzed for cytokine levels (Rules Based Medicine, Inc.) and drug exposure by LC/MS/MS. AM1241 and HU308 significantly inhibited LPS-induced TNF-α production in both wild-type ($p<0.01$) and CB_2 knockout mice ($p< 0.001$). Furthermore, AM1241 and HU308 significantly elevated plasma IL-10 levels in both wild type and CB_2 knockout mice ($p < 0.001$ for both CB_2 agonists in both mouse genotypes). The plasma concentrations were 1.5 ± 1.0 and 0.8 ± 0.1 μM for AM1241, and 2.1 ± 0.9 and 3.0 ± 2.0 μM for HU308 in wild-type and CB_2 knockout mice, respectively. The results indicate that AM1241 and HU308 regulate cytokine release *in vivo* through a non-CB_2 receptor mechanism in the acute inflammation model.

4. CB_2-MEDIATED CHEMOTAXIS OF ASTROCYTOMA CELLS

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Aims: Cannabinoid CB_2 receptors have been shown to regulate cell migration and affect astrocytoma cell viability. In order to further characterize the ability of endogenous and synthetic cannabinoids to specifically elicit chemotaxis *via* CB_2 receptors in astrocytomas, we generated a cell line stably expressing this receptor and screened a battery of compounds for their ability to induce chemotaxis.

Methods: DBT mouse astrocytoma cells, which do not express detectable levels of CB_1, CB_2 or GPR55, were stably transfected to express CB_2. Cell migration was measured using a technique newly developed by our laboratory. Specifically, cells were labeled with the near infrared dye DRAQ5, loaded into a 96-well chemotaxis chamber containing a collagen-coated filter and cannabinoids in the lower well, and allowed to migrate for three hours. The lower portion of the filter was then scanned on a LI-COR Odyssey near-infrared scanner, and fluorescence (an index of the number of cells that migrated) was quantified.

Results: Several DBT clones were generated and CB_2 expression and functionality were quantified by Q-PCR, radioligand binding and coupling to Erk phosphorylation. Here, we focused on one such cell line (2G5), which demonstrated functional coupling and expression of CB_2 at 31 copies mRNA *per* cell. While the endocannabinoid 2-arachidonoylglycerol induced a dose-dependent increase of 2G5 cell migration, which reached 216% of basal at 1 μM, anandamide and the two endocannabinoid candidates HEA and DEA increased migration to only 120-140% of basal when tested at 1 μM. The 2-AG response was absent from untransfected DBT cells and completely blocked by SR144528 (1 μM). The synthetic agonist CP55,940 (1 μM) produced no effect in either untransfected or 2G5 cells, while WIN55,212-2 (1 μM) inhibited migration in both cell lines.

Conclusions: These results demonstrate differential signaling of the CB_2 receptor in regard to endocannabinoids, synthetic agonists and chemotaxis. Some endogenous cannabinoids, notably 2-AG, produced robust migration, while other eCBs and synthetic ligands produced no significant CB_2-dependent response. Interestingly, our cell line may express an uncharacterized cannabinoid receptor as revealed by the inhibition of migration by WIN55, 212-2 independent of CB_2 expression.

Funding: NIH DA014486 and T32 DA07278-12.

5. CANNABINOID-2 RECEPTOR MEDIATES PROTECTION AGAINST HEPATIC ISCHEMIA/REPERFUSION INJURY

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Aims: Hepatic ischemia-reperfusion (I/R) injury continues to be a fatal complication that can follow liver surgery or transplantation. We aimed to investigate the involvement of the endocannabinoid system in hepatic I/R injury.

Methods: An *in vivo* mouse model of hepatic I/R and *in vitro* cell systems were used to study the role of endocannabinoid system in hepatic ischemia/reperfusion.

Results: Here we report that I/R triggers several-fold increases in the hepatic levels of the endocannabinoids anandamide and 2-arachidonoylglycerol, which originate from hepatocytes, Kupffer and endothelial cells. The I/R-induced increased tissue endocannabinoid levels positively correlate with the degree of hepatic damage and serum TNF-α, MIP-1α and MIP-2 levels. Furthermore, a brief exposure of hepatocytes to various oxidants (H_2O_2 and peroxynitrite) or inflammatory stimuli (endotoxin and TNF-α) also increases endocannabinoid levels. Activation of CB_2 cannabinoid receptors by JWH133 or HU308 protects against I/R damage by decreasing inflammatory cell infiltration, tissue and serum TNFα, MIP-1α and MIP-2 levels, tissue lipid peroxidation and expression of adhesion molecule ICAM-1 *in vivo*. JWH133 and HU308 also attenuates the TNF--induced ICAM-1 and VCAM-1 expression in human liver sinusoidal endothelial cells (HLSECs) and the adhesion of human neutrophils to HLSECs*in vitro*. Consistent with the protective role of CB_2 receptor activation, $CB_2^{-/-}$ mice develop increased I/R-induced tissue damage and pro-inflammatory phenotype.

Conclusions: These findings suggest that oxidative/nitrosative stress and inflammatory stimuli may trigger endocannabinoid production, and indicate that targeting CB_2 cannabinoid receptors may represent a novel protective strategy against I/R injury.

Funding: Intramural Research Program of NIH/NIAAA (to. P.P.)

6. COMPARTMENTALIZATION OF ENDOGENOUS 2-AG INTO LIPID RAFTS IN A DORSAL ROOT GANGLION CELL LINE

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Aim: 2-Arachidonoyl glycerol (2-AG) is an endogenous cannabinoid that binds CB_1 and CB_2 receptors and may affect synaptic transmission in a retrograde fashion. Increases in 2-AG by inhibition of monoacylglycerol lipase in the periaqueductal gray or lumbar dorsal horn are linked to a CB_1-mediated stress-induced analgesia (Hohmann et al, 2005). In addition, peripheral 2-AG application induces CB_2-mediated antinociception in the formalin test (Guindon et al, 2006). 2-AG appears to be derived from three putative precursors (reviewed by Sugiura et al, 2006): 1) diacylglycerol (via diacylglycerol lipase); 2) lysophosphatidylinositol (via a phospholipase C); and, 3) 2-arachidonoyl lysophosphatidic acid (via a phosphatase). To investigate the cellular dynamics of 2-AG biosynthesis we studied the partitioning of endogenous 2-AG, diacylglycerols (1-stearoyl-2-arachidonoyl-*sn*-glycerol), diacylglycerol lipase α, phosphatidic acid and CB2 receptors into lipid raft fractions isolated from F-11 cells. F-11 cells are a fusion of rat embryonic (day 13-14) dorsal root ganglia and mouse N18TG2 neuroblastoma cells and express both CB_1 and CB_2 cannabinoid receptors (Platika et al., 1985; Ross et al, 2001).

Methods*:* F-11 cells were homogenized and fractionated using a detergent-free OptiPrep density gradient. All lipids were partially purified from methanolic extracts of the fractions on solid phase disc cartridges and quantified using liquid chromatography tandem mass spectrometry (LC/MS/MS). Protein localization was determined by western blotting.

Results*:* Under basal conditions, the endogenous cannabinoid 2-AG, but not *N*-palmitoyl ethanolamine or *N*-arachidonoyl glycine, was localized to lipid rafts. The CB_1 cannabinoid receptor, if present, occurred at levels below our detection limit, but CB_2 receptors were expressed in high levels in F-11 cells localized to non-lipid raft fractions. In contrast, the 2-AG precursor 1-stearoyl-2-arachidonoyl-*sn*-glycerol as well as the enzyme diacylglycerol lipase α (DAGLα), which cleaves diacylglycerol to form 2-AG, and 2-AG itself were co-localized with the lipid raft markers caveolin-1, flotillin-1, cholesterol, sphingomyelin and the glycolipid ganglioside GM3.

Conclusions: The biochemical machinery for the production of 2-AG via the putative diacylglycerol pathway is localized within lipid rafts, suggesting that 2-AG synthesis occurs within lipid rafts. Whether the 2-AG is then released into the cytoplasm from the rafts or travels laterally within the membrane to engage CB_2 receptors within the non-raft regions of the same cells remains a question for future investigation.

Funding: The authors are grateful for the support of the National Institute on Drug Abuse (DA-018224, DA-020402, DA-011322, F32-DA-016825), the Gill Center for Biomolecular Science, Indiana University, Bloomington, and the Lilly Foundation Inc, Indianapolis, IN.

7. THE EFFECTS OF AM1241 AND ITS SEPARATED ENANTIOMERS AT HUMAN, MOUSE, AND RAT CB_2

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Aims: Two published forms of rat CB_2 exist, differing in the size and composition of the intracellular C-terminus of the molecule. AM1241 is a widely used racemic compound that is reported to be a CB_2-selective agonist. The aim of this study was to provide an *in vitro* characterization of AM1241 an its enantiomers in recombinant human, rat, and mouse CB_2 cell expression systems and an *in vivo* characterization in rodent pain models.

Methods: Rat and mouse CB_2 were PCR amplified, cloned, and stably expressed in CHO-K1 cells. These cell lines, as well as commercially available human CB_2-expressing cell lines, were used in the characterization of AM1241 and its enantiomers in in ligand binding and cAMP inhibition assays. AM1241 and its enantiomers were also assessed in a mouse model of visceral pain and a rat model of inflammatory pain.

Results: CB_2 amplified from rat spleen cDNA revealed only short isoform-encoding message. Sequence corresponding to the C-terminus of the longer isoform was present, but only as part of the 3' UTR of messages encoding the shorter isoform. In cAMP inhibition assays performed in cell lines expressing human, mouse, and short isoform rat CB_2, racemic AM1241 was shown to be an agonist at the human receptor, but an inverse agonist at the mouse and rat receptors. R-AM1241 had a functional profile very similar to the racemate, whereas S-AM1241 was an agonist at all three species of CB_2, but with a more than 40-fold lower binding affinity than the R-enantiomer. In two rodent pain models, S-AM1241 was more efficacious than either the racemate or R-AM1241. In the rat inflammatory pain model, the antihyperalgesic effects of S-AM1241 were shown to be CB_2 mediated, as evidenced by substantial blockade by the CB_2 antagonist AM630.

Conclusions: The *in vitro* findings reveal species-specific and stereoisomer-specific aspects to the functional consequences of the AM1241-CB_2 ligand-receptor interaction. The *in vivo* results largely corroborate the *in vitro* characterization and are consistent with the expectation that CB_2 agonists should be effective in the relief of pain.

Funding: Wyeth Pharmaceuticals was the sole source of funding for this work.

8. SELECTIVE ACTIVATION OF CANNABINOID CB_2 RECEPTORS MODULATES MECHANOSENSITIVITY OF AFFERENT NERVE FIBRES IN CONTROL BUT NOT OSTEOARTHRITIC RAT KNEE JOINTS

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Aims: The present study examined whether the synthetic CB_2 cannabinoid receptor agonist GW 405833 could modulate joint nociception in osteoarthritic (OA) rat knee joints.

Methods: OA was induced in male Wistar rats by intraarticular injection of 3 mg sodium monoiodo-acetate with a recovery period of 14 days. A parallel control group of rats received an intra-articular injection of sterile saline. Animals were deeply anaesthetised with ethyl carbamate (urethane; 2 mg kg^{-1} *i.p.*) and prepared for electrophysiological recording from knee joint primary afferents. Afferent firing rate was determined in response to normal rotation and noxious hyper-rotation of the joint both before and following close intra-arterial injection of different doses of the CB_2 agonist GW405833 (10^{-12}, 10^{-10}, 10^{-8} mol).

Results: In control joints, application of GW405833 significantly reduced firing rate in 52% of the recorded afferent nerve fibres during joint rotation. In 28% of recorded fibres application of GW405833 had no effect and increased firing rate in 20% of recorded fibres. In the OA joint, a desensitizing effect of GW405833 was observed in just 20% of recorded fibres, whereas 45% of recorded fibres showed increased firing rate. The effect was found to be maximal 7 min after drug application.

Conclusions: These findings indicate that peripheral injection of GW405833 into control knee joints reduces afferent mechanosensitivity in 50% of the fibres. Conversely in OA knee joints, GW405833 induced peripheral sensitization of joint afferents indicative of a pro-nociceptive response. Thus, peripheral CB_2 receptors may not be useful targets for the management of OA pain.

Funding: Canadian Arthritis Network (CAN)
Alberta Heritage Foundation for Medical Research (AHFMR)
Arthritis Society of Canada
Canadian Institutes of Health Research (CIHR)

Session II

9. IDENTIFICATION AND CHARACTERIZATION OF ENDOGENOUS PROSTAGLANDIN E_2 GLYCEROL ESTER (PGE_2-G), A COX-2 METABOLITE OF 2-ARACHIDONOYLGLYCEROL (2-AG), IN PAIN AND INFLAMMATION

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Aims: Previous research demonstrated that the endocannabinoid 2-arachidonoylglycerol (2-AG) can be oxygenated by COX-2 to produce prostaglandin glycerol esters (PG-Gs) *in vitro*. One of these oxygenated 2-AG metabolites, PGE_2-G, but not PGE_2 or glycerol, triggered intracellular Ca^{2+} mobilization via the IP_3 and PKC pathways in RAW264.7 macrophage cells. To date, the oxygenated products of 2-AG by COX-2 have not been found *in vivo*. Therefore, the aim of the present study was to isolate and identify endogenous PGE_2-G from mammalian tissues and to examine its role in pain and inflammation.

Methods: Male Sprague-Dawley rats served as subjects in the experiments. RAW264.7 cells were used as a model of inflammatory responses in immune cells. To identify endogenous PGE_2-G in the mammalian periphery, rat hindpaws were homogenized in 100% methanol and centrifuged for 20 min. The paw extracts were partially purified by solid phase extraction methods and analyzed with triple quadrupole mass spectrometry (LC/MS/MS) and quadrupole time of flight mass spectrometry (qq-TOF LC/MS/MS). To test if PGE_2-G affects pain sensitivity, PGE_2-G, PGE_2-G combined with the prostanoid receptor antagonists, or vehicle were injected intraplantarly (i.pl.) and the animals were tested with the Hargreaves and von Frey tests. Finally, RAW264.7 cells were transiently transfected with an NFκB-luciferase plasmid and activated by LPS. PGE_2-G or vehicle was added for 30 min and followed by the substrate for luciferase. The emitted light was measured by a scintillation counter.

Results: Mass spectrometry revealed that a constituent of the rat hindpaw extract has the same exact mass, HPLC retention time, fragmentation pattern, and structural components as synthetic PGE_2-G. Therefore, we concluded that PGE_2-G is present in the rat hindpaw. PGE_2-G caused thermal hyperalgesia and mechanical allodynia, and its effects were only partially mediated by PGE_2, a metabolic product of PGE_2-G. PGE_2-G caused an apparent bell-shaped dose-response curve in the activation of NFκB activity in the immune cells.

Conclusions: Here, it was shown for the first time that the COX-2 metabolite of 2-AG PGE_2-G a naturally occurring molecule in the rat hindpaw. Our behavioral data suggested that PGE_2-G binds to a unique receptor on the skin cells to induce pain. It appears thatPGE_2-G is a member of a new class of lipid signaling molecules with a role in pain and inflammation.

10. PLASMA ENDOCANNABINOID CONCENTRATIONS IN ALZHEIMER'S DISEASE AND ELDERLY CONTROLS

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The endocannabinoid system is emerging as a worthy target of intervention in inflammatory disease. Both neuropathological and animal model studies point to the possible role of the endocannabinoid system in Alzheimer's disease pathology and treatment. To date, no published studies have compared endocannabinoid concentrations in Alzheimer's disease (AD) and healthy cognitive aging. In order to explore the relationship between endocannabinoid physiology and Alzheimer's disease, we measured plasma concentrations of 2-arachidonyl glycerol (2-AG) and *N*-arachidonoyl ethanolamine (AEA) in a cohort of well characterized subjects with probable AD (N=19) and elderly controls (N=12). Plasma concentrations were measured in donated samples by high performance liquid chromatography and mass spectrometry. In AD, the mean plasma concentration of 2-AG was 15.9 pmol/ml with 95% CI (10.48,21.25), while the mean plasma concentration of AEA was 1.57 pmol/ml with 95% CI(1.19,1.96). In elderly controls, the mean plasma concentration of 2-AG was 10.9 pmol/ml with 95% CI (4.17, 17.72), while the mean plasma concentration of AEA was 1.87 with 95%CI (1.39,2.35). Further investigation is required to determine whether endocannabinoid concentrations either in plasma or cerebrospinal fluid may be a biomarker for inflammatory AD pathology.

11. VASODILATOR EFFECT OF THE CB_2 RECEPTOR AGONIST JWH133 IN NORMAL RAT KNEE JOINTS IS ATTENUATED DURING ACUTE AND CHRONIC ARTHRITIS

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Aims: This study examined the vasoactivity of the CB_2 receptor agonist JWH133 on rat knee joint blood flow. The effect of joint inflammation on these vasomotor responses was also assessed.

Methods: Male Wistar rats (242-495g) were deeply anaesthetised with urethane (2 mg kg^{-1} *i.p.*) and placed under a laser Doppler perfusion imager to measure non-invasively blood flow in the antero-medial aspect of the knee joint capsule. Scans were taken before and at regular intervals following local topical administration of JWH133 (10^{-14} – 10^{-8} mol). Blood flow assessment was carried out in normal, acutely inflamed (2% kaolin, 2% carrageenan intra-articular; 24hr recovery), and chronically inflamed (Freund's complete adjuvant intra-articular; 7day recovery) knees.

Results: In normal knees, JWH133 caused a dose-dependent increase in knee joint blood flow which was maximal 1.5min after administration of the cannabinoid. The selective CB_2 receptor antagonist AM630 successfully blocked this hyperaemic response. The vasodilatatory effect of JWH133 was significantly attenuated in both acutely inflamed and chronically arthritic joints ($P<0.0001$; $n = 8$-12).

Conclusions: Activation of CB_2 receptors in normal knee joints causes synovial hyperaemia. In acute and chronic models of arthritis, the vasodilatatory effect of JWH133 is reduced and this could be due to either downregulation or desensitization of CB_2 receptors by the inflammatory process.

Funding: This work was funded by the Arthritis Society of Canada and the Alberta Heritage Foundation for Medical Research.

12. ARE PARRY-ROMBERG'S SYNDROME PATIENTS DEFICIENT IN ENDOCANNABINOID ACTIVITY?

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Aims: The aim of this abstract is to initiate collaborative research to determine the endocannabinoid status of patients suffering from Parry-Romberg's Syndrome, and whether or not cannabinoid agonists can slow or halt the progression of the disease.

What is Parry-Romberg? PRS is a rare disorder characterized by slowly progressive atrophy of the skin and soft tissues of half of the face (hemifacial atrophy). It is more common in females. Initial facial changes usually involve the tissues above the upper jaw or between the nose and the upper corner of the lip and subsequently progress to the angle of the mouth, areas around the eye, the brow, the ear, and the neck. The deterioration may also affect the tongue, the soft and fleshy part of the roof of the mouth, and the gums. The eye and cheek of the affected side may become sunken. PRS is also accompanied by neurological abnormalities including seizures and episodes of trigeminal neuralgia. The onset of the disease usually begins between the ages of 5 and 15 years, progression often lasts from 2 to 10 years. Muscles in the face may atrophy and there may be bone loss in the facial bones. Problems with the retina and optic nerve may occur when the disease surrounds the eye.
(http://www.ninds.nih.gov/disorders/parry_romberg/parry_romberg.htm)

Is there a cannabinoid connection? Very little is known about the underlying biochemical mechanisms of Romberg's Syndrome despite the dramatic disfiguration and suffering it causes to young people. Because cannabinoids are known to have a positive impact on pain, seizures, and stroke, as well as regulating skin, muscle, fat, nervous, and bone tissue, a possible relationship with the endocannabinoid system was suspected.

Methods: Romberg's patients were contacted through The Romberg's Connection, an international support group made up of over 450 individuals and families whose lives are affected by Romberg's Syndrome. They were asked if the use of marijuana had any affect on their illness.

Results: Two patients cautiously contacted me. One patient used medical cannabis regularly through out her disease progression. She has the mildest disfiguration of any Romberg's patient that she has encountered. A second patient also regularly used medical cannabis for a few years, and had no new Romberg's symptoms during that time. However, she stopped taking cannabis as a result of pregnancy and now appears to be developing some cranial atrophy. Both patients found that cannabis relieved the headaches associated with this illness.

Conclusions: Romberg's Syndrome is a rare, under-researched disease. Many of the symptoms are consistent with a possible involvement of the endocannabinoid system. Patients are eager for more research. CB_2 specific therapies would be ideal since the use of CB_1 agonists in young children would be troublesome due to their psychoactive properties. The known involvement of CB_2 activities in controlling neurological, skeletal, skin and bone biochemistry, all of which are involved in PRS, coupled with limited anecdotal evidence, suggests that cannabinoid based research into Parry-Romberg's Syndrome should be initiated.

13. EVIDENCE OF WIDESPREAD NEURONAL EXPRESSION OF CB_2 CANNABINOID RECEPTOR mRNA AND PROTEIN IN RAT CENTRAL NERVOUS SYSTEM

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Aims: Several recent studies have reported evidence that the CB_2 cannabinoid receptor is expressed in the central nervous system (CNS). Here we have used a variety of techniques to analyse in detail the expression and localisation of CB_2 in the rat CNS and spleen.

Methods and Results: Using RT-PCR with primers complementary to sequences in exons 1 and 3 of the rat *cb2* gene, mRNA expression was detected in the spleen and in all brain regions examined. *In situ* hybridisation using two non-overlapping DIG-labelled cRNA probes and two ^{35}S-labeled oligonucleotides revealed *cb2* expression predominantly in the marginal zone of spleen white pulp, whilst in brain low-level but widespread neuronal *cb2* mRNA expression was observed.

To investigate CB_2 protein expression, novel antibodies were generated against a peptide antigen corresponding to the C-terminal 14 amino-acid residues of the rat CB_2 receptor. Western blot analysis of HEK-293 cells transfected with HA-tagged-*cb2* revealed the same pattern of immunostained bands when probing with anti-CB_2 or anti-HA antibodies. Moreover, the most intensely stained band corresponded to the expected molecular mass for CB_2 (~45 kDa). This band was also detected in both spleen and brain homogenates, but with lower abundance in brain than in spleen. Immunohistochemistry of spleen sections revealed staining in the marginal zone, consistent with *cb2* mRNA expression. In the brain, the distribution of CB_2-immunoreactivity corresponded with *cb2* mRNA expression, showing low-level but widespread neuronal localisation. For example, both *cb2* mRNA and CB2 protein were detected in the cell body and/or dendrites of neurons in the olfactory bulb (mitral cells), cerebellum (Purkinje cells) hippocampus and neocortex (pyramidal cells).

Conclusions: The matching distribution of CB_2 mRNA and protein supports the existence of CB_2 receptors in the CNS in the normal brain. Low but widespread neuronal expression of CB_2 suggests a role for endocannabinoid modulation of neural function via CB_2 in many CNS systems. Furthermore, the methods reported here will be useful for future analysis of changes in CB_2 expression in the CNS that are thought to occur in association with a variety of neuropathological conditions (e.g. neuropathic pain).

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14. GLIOMA EXPRESSION OF CB2 RECEPTORS IS NOT SUFFICIENT FOR CANNABINOID-INDUCED CYTOTOXICITY *IN VITRO*

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Aims: Evidence suggests that cannabinoids activating CB_2 receptors may constitute powerful anti-cancer agents devoid of psychotropic effects. While such cannabinoid agonists exhibit clear cytoxic effects on cannabinoid receptor-expressing glioma tissues, their heterogeneity masks the precise molecular mechanism underlying this action. In the current study we employed a genetic approach to investigate the cytotoxic effects of various cannabinoids on a murine glioma cell line devoid of cannabinoid receptor expression.

Methods: The murine glioma line, delayed brain tumor (DBT), lacks cannabinoid receptors CB_1, CB_2 and GPR55 (as shown by Q-PCR). DBTs were transfected with a construct containing full-length murine CB_2 receptors and an IRES/EGFP sequence, allowing for the simultaneous yet independent expression of both the gene of interest and the fluorogenic marker. FACS was then used to enrich bulk transfectant populations and isolate single-cell clones for expansion. Q-PCR and radio-ligand binding was used to confirm and quantify cannabinoid receptor expression by each clone. CB_2-expressing clones were assayed for functional coupling of receptors to MAPK using western blot analysis and the colorimetric proliferation reagent WST-1 was used to evaluate the sensitivity of such clones to various cannabinoids.

Results: We focused on three DBT clones stably expressing various levels of CB_2 receptor (as found by Q-PCR and radioligand binding assay) and found that CP55940 (1 μM) systematically increased ERK1/2 phosphorylation, although with different kinetic profiles in each clone. In all cases this activation was blocked with pre-treatment of the CB_2-selective antagonist SR144528 (1 μM), while the CB_1-selective antagonist SR141716A (3 μM) had no effect. Although these DBT clones express functional CB_2 receptors, they remained insensitive to various cannabinoid agonists after five days of treatment using a range of administration regimens.

Conclusions: These data indicate that while DBT clones express functional CB_2 receptors, such expression is not sufficient for cannabinoid-induced cytotoxicity.

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15. 2-ARACHIDONOYLGLYCEROL INHIBITS PHORBOL 12-MYRISTATE 13-ACETATE-INDUCED TUMOR NECROSIS FACTOR ALPHA PRODUCTION IN HL-60 CELLS VIA THE CB_2R

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2-Arachidonoylglycerol (2-AG), is an endogenous ligand for both, the central cannabinoid (CB_1R) receptor and the peripheral cannabinoid (CB_2R) receptor. Recent studies have indicated the importance of 2-AG in immunomodulation, with emphasis in its physiological role in cytokine production. However, the molecular mechanism underlying the regulation of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) by 2-AG, has not been well elucidated. This study explores the role of 2-AG in the regulation of Phorbol 12-myristate 13-acetate (PMA) induced TNF-α production. PMA activates protein kinase C, and is known to induce the production of TNF-α in immune cells.

We used end-point PCR as well as real-time PCR to determine TNF-α mRNA expression. To determine TNF-α protein secretion, we used ELISA. The role of the classical mitogen-activated protein kinase (MAPK) pathway was determined by Western Blot.

In this study, we have demonstrated that PMA (100nM)-induced TNF-α production in the human promyelocytic cell line, HL60, is regulated by the classical MAPK pathway and the transcription factor NF-κB. A time course study demonstrated that 2-AG (1 M) exerts an inhibitory effect on PMA-induced TNF-α transcription and secretion in serum-starved HL60 cells when 2-AG is added 30 minutes after PMA. Through the use of the CB_2R antagonist AM630 (10-1000 nM), we determined that the modulation of PMA-induced TNF-α production in HL60 cells by 2-AG, is indeed through CB_2R. 2-AG has been previously shown to induce the MAPK activation by the phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2). However, 2-AG did not affect PMA-induced ERK1/2 phosphorylation in this study, hence suggesting the participation of other signaling cascades.

Together, these findings indicate that 2-AG is a key modulator of inflammation. Further studies will be extended to confirm whether specific transcription factor activation is regulated by 2-AG.

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16. UPREGULATION OF CANNABINOID RECEPTOR 2 IN CHRONICALLY INFLAMED BLADDER OF RATS

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Aims: Painful bladder syndrome is a chronic disorder associated with severe pelvic pain and bladder inflammation for which there is currently no effective treatment. Cannabinoids have been shown to have anti-inflammatory and analgesic properties in a number of tissues and have been reported to decrease the motility of normal and inflamed bladder in rats, suggesting that cannabinoid receptors (CBs) may be expressed in the bladder. The purpose of this investigation is to confirm the presence of both CB_1 and CB_2 in rat bladder and to determine their expression under conditions of chronic inflammation.

Methods: Chronic inflammation of the bladder was induced in rats via intravesical instillation of acrolein for 30 minutes, repeated three times at intervals of 72 hours. Animals were sacrificed after the last treatment, and bladder, DRG (L5,L6,S1) and spinal cord (L3-S1) were isolated. Real time PCR and Western blot analyses were performed on animals sacrificed 2 and 72 hours, respectively, after the final treatment.

Results: Both CB_1 and CB_2 protein and mRNA were detected in the bladder, DRG and spinal cord. CB_2 protein and mRNA were both up-regulated in the chronically inflamed bladder compared with controls, but CB_1 mRNA and protein were not. Neither CB_1 nor CB_2 showed an increase in mRNA or protein expression in the DRG or spinal cord of animals in which chronic inflammation was induced.

Conclusions: Our findings confirm that CBs are expressed in rat bladder. Further, the specific up-regulation of CB_2 in chronically inflamed bladder tissue suggests that this receptor may be a valuable target for development of treatment for painful bladder syndrome. Future studies will determine which cells express increased CB_2 and whether treatment with CB_2 agonists can diminish inflammation and/or reduce pain in this model of chronic bladder inflammation.

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17. ENDOCANNABINOIDS INCREASE MATRIX METALLOPROTEINASE-9 IN HUMAN BRONCHIAL EPITHELIAL CELLS VIA CANNABINOID RECEPTOR SIGNALING AND p42/p44 MAPK

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Background: Non-small cell lung cancer (NSCLC) cells often elevate endocannabinoid production. Recent work has emphasized the role of surrounding tissue in tumor progression; therefore, we have assessed the effect of tumor-derived endocannabinoids on nearby lung epithelium using an *in vitro* model. In a non-transformed human bronchial epithelial cell (HBEC) line, cannabinoids concomitantly induce matrix metalloproteinase-9 (MMP9), cyclooxygenase-2 (COX-2) and its enzymatic product, prostaglandin E2 (PGE2). In lung cancer models, PGE2 has been linked to enhanced secretion of matrix metalloproteinase enzymes, which promote tumor invasion and metastasis by degrading the extracellular matrix. Additionally, in others systems, signaling through p42/p44 MAPK can elevate MMP9 secretion. Here we report on the relative contribution of cannabinoid receptors, CB_1 and CB_2, PGE2, and p42/p44 MAPK in MMP9 induction by cannabinoids in HBECs. **Aim:** Identify the signal transduction pathway responsible for MMP9 elevation in HBECs exposed to tumor-derived cannabinoids.

Methods: HBEC cells were cultured in serum free media and treated with one of three cannabinnoids (anandamide, 2-AG, or methanadamide) for 24 h. Culture supernatants were collected for PGE2 and MMP9 determination, and cell lysates were collected for western and RT-PCR analysis. To determine the role of CB_1 and CB_2 cannabinoid receptors in MMP9 induction, HBEC cells were incubated with the CB_1 antagonist, SR141716, or the CB_2 antagonist, SR144528, for 1 hr before addition of anandamide. The COX2 inhibitor, SC52836, was employed to evaluate the role of increased COX2/PGE2 in anandamide-mediated increased MMP9 production.

Results: All tested cannabinoids, anandamide, 2-AG, and methanandamide, increased PGE2 production to varying degrees, however only anandamide and methanadamide enhanced MMP9 secretion. Furthermore, pharmacologic inhibition of COX2 had no effect on MMP9 secretion in HBECs exposed to anandamide. Real time PCR indicates that anandamide up-regulates the CB_2 receptor in HBECs, and CB_1 and CB_2 receptor antagonists prevent MMP9 induction by anandamide. In addition, the p42/44 inhibitor, PD98059, abrogates anandamide induction of MMP9.

Conclusions: While, certain endocannabinoids concomitantly increase PGE2 and MMP9 production, and PGE2 has been linked to MMP9 expression in other models, our results demonstrate that PGE2 is not responsible for increased in MMP9 secretion in HBECs exposed to anandamide. Instead, anandamide elevates MMP9 through cannabinoid receptors, via p42/p44 MAPK. Our data also supports the existence of a possible feedback loop in which exposure of HBECs to cannabinoids increases CB_2 receptor expression. Both MMP9 and COX-2 are increased in NSCLC and these molecules have been targeted in anti-cancer strategies. Our results indicate that these two enzymes may be independently regulated in lung epithelial cells. This information may be useful for designing novel strategies for combined therapy of NSCLC, with COX-2 inhibitors or other agents. **Funding:** This work is supported by National Institutes of Health Grants KO1 5K01DA16339-3 and the UCLA SPORE in Lung Cancer P50 90388.

18. CB_2 RECEPTORS MAY PLAY A ROLE IN FEVER INDUCED BY THE ENDOTOXIN LIPOPOLYSACCARIDE

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Cannabinoids have been reported to modulate the actions of lipopolysaccaride (LPS), where both cannabinoid agonists and antagonists attenuate the neuroinflammmatory process, in particular cytokine release (Roche et al., J Neuroimmunol. 181:57-67). CB_1 receptor activation has been reported to attenuate the LPS-fever response in rats but the role of the CB_2 receptor is unclear (Benamar *et al,* JPET; 320:1127-1133, 2006). The aim of the present study was to clarify the role of CB_2 receptor in the febrile response to LPS by examining temperature responses to LPS in CB_2 knockout mice.

Wild type and CB_2 knockout mice (C57Bl6 background) were implanted with temperature data loggers (SubCue Dataloggers, Calgary.). Animals were left for 7 days to recover, then were acclimatized to 27° C for 18 hours prior to experiment. Animals were injected with either saline or LPS (100µg/kg, i.p.) and temperature was monitored for a further 7 hours.

Circadian temperature rhythms were not significantly different between wildtype and CB_2 knockout mice at either 21°C or 27°C. Both wildtype and knockout mice treated with saline had no change in core body temperature compared with baseline. Wildtype mice treated with LPS displayed a typical biphasic fever response, with an initial peak in core body temperature at around 60 mins after injection and subsequent second peak that remained elevated for up to 5 hours. In contrast, CB_2 knockout mice had no febrile response to LPS in that their temperature responses were not significantly different to the saline controls or to their own baseline temperatures.

Our findings that CB_2 knockout mice are unable to mount a febrile response to an LPS challenge suggest that CB_2 receptors play an important role in the febrile response, most likely by modulation of peripheral immune responses to LPS.

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19. TAGGING CB_2 RECEPTORS WITH GFP/CFP

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Aims: Recent studies have shown that CB_2 receptors are dynamically regulated in various pathological conditions, like contact skin hypersensitivity and neuropathic pain. Unfortunately, it is difficult to assess CB_2 receptor expression by immunostaining, because we still often observe immunostaining in tissues from CB_2 knock-out mice. To overcome this problem we tried to generate mice with fluorescently tagged CB_2 receptors.

Methods: We generated various cell lines expressing murine CB_2, CB_2eCFP and CB_2eGFP receptors. The cellular distribution and localization of the fusion proteins was analyzed by fluorescence microscopy. We also studied agonist dependent Erk phosphorylation using CB_2 and CB_2-eCFP cell lines.

Mice with CFP and GFP tagged CB_2 receptors were generated by a knock-in strategy.

Results: We found that the fusion protein was localized in the cell membrane as well as in intracellular compartments. This pattern was strikingly similar to the one observed with a similar CB_1-eGFP fusion protein, indicating that the fusion does not change cellular localization.

To determine whether downstream signalling pathways were affected by the C-terminal fusion, we treated CB_2 and CB_2-eCFP expressing CHO cells with increasing concentrations of the CB_2 specific agonist HU-308. We observed a similar pattern of phosphorylation, indicating that the downstream signalling was not altered.

Having established that fluorescence tagging does not affect functionality of CB_2 receptor, we next generated CB_2-eCFP and CB_2-eGFP knock in mice. Unfortunately, we observed only a weak mRNA expression for CB_2-eCFP in spleen, lymph nodes and thymus, and a very low CB_2-eCFP protein expression by immunoprecipitation. We found no CB_2-eCFP expression in peritoneal and bone marrow derived macrophages, dendritic cells, splenocytes, and lymph node cells by FACS analysis, and also no specific fluorescence in tissue sections of spleen, lymph node, spinal cord and thymus.

Conclusions: The in-vitro experiments indicate that the functionality of CB_2 receptor was not altered by eCFP fusion. Thus, the tagging strategy should to be useful for the detection of CB_2 receptors in living cells or tissues.

However, the very weak expression of CB_2-eCFP receptors in knock-in mice indicates that the fusion affects expression and/or stability of the modified protein. We have also generated CB_2-eGFP knock-in mice, which we are currently analyzing.

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20. COMPARATIVE ANALYSIS OF CB_2 TRANSCRIPT EXONAL USAGE IN MOUSE AND HUMAN B CELLS

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Aims: Studies have shown that the peripheral cannabinoid receptor (CB_2) message expression is most abundant in mouse and human B cells. Searching of genomic databases revealed that the mouse *cnr2* gene is predicted to produce two transcripts employing different first exons, whereas the human *cnr2* gene is predicted to produce one CB_2 transcript utilizing one first exon. However, from the database search we could not determine this exonal usage was representative of B cells. Therefore, we wanted to explore CB_2 transcript usage at basal activity in B cells from mouse and human.

Methods: We isolated B cells from mouse splenocytes and human PBMCs using either the mouse or human EasySep B cell enrichment kit (Stem Cell Technologies Inc). B cell enrichment was determined by FACS analysis using CD19-PE and CD3-PerCP antibodies (BD Pharmingen). Total RNA was extracted from the B cells using Tri-Reagent (Sigma). The RNA was then used for RT-PCR employing the SMART RACE technique (Clontech). All RACE products were gelpurified using the Perfectprep Gel Cleanup kit (Eppendorf) and sent for DNA sequencing to the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center & Research Institute.

Results: The FACS analysis demonstrated that the B cell populations were enriched by 95 to 98% from both the mouse splenocytes and human PBMCs. Sequencing of the RACE products showed that mouse B cells express three CB_2 transcripts utilizing two different first exons, whereas the human B cells express one CB_2 transcript utilizing one first exon. Alignment of the sequenced RACE products to either the mouse or human genome, along with the GenBank submitted mRNA sequences, revealed that the transcripts we isolated contained previously unidentified transcriptional start sites (TSS).

Conclusions: The data show, at basal activity, that mouse splenic B cells express three c*nr2* gene transcripts utilizing two first exons, whereas human B cells express one *cnr2* gene transcript. Furthermore, the data illustrate for the first time multiple putative TSS utilized for the expression of CB_2 in mouse B cells and one putative TSS for CB_2 message expression in human B cells. Future studies will examine the CB_2 exonal usage in B cells of mice under various conditions of activation.

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21. THE CB_2 CANNABINOID RECEPTOR AGONIST JWH-015 MODULATES CHEMOKINE RECEPTOR

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Aims: Recruitment of leukocytes in response to inflammatory stimuli is crucial in the pathogenesis of inflammatory disorders such as atherosclerosis. We have previously reported that the cannabinoid delta-9-Tetrahydrocannabinol (THC) inhibits atherosclerosis progression in a mouse model via pleiotropic immunomodulatory actions. In vitro, THC inhibited macrophage chemotaxis, a crucial step for the development of atherosclerosis, in a CB_2-dependent manner. The aim of this study was to investigate if cannabinoids would also modulate the inflammatory and chemotactic response of human vascular cells.

Methods: Human monocytes were isolated by Ficoll-Percoll density gradient centrifugation, and endothelial cells (ECs) were obtained by collagenase treatment of saphenous veins. The cells were stimulated with IFN- in the presence of various concentrations (0, 5, 10, 20 M) of JWH-015. MCP-1 and MIP-1 secretion was measured in cell culture supernatants after 4-12h of stimulation via ELISA. Cannabinoid receptor CB_2 and chemokine receptor expression were determined via flow cytometry and quantitative real time PCR.

Results: We found high CB_2 cell surface expression on human monocytes, whereas no CB_2 expression was detected on vascular endothelial cells. IFN- induced the secretion of MCP-1 and MIP-1 in monocytes, whereas surface expression of CCR1 and CCR2 was reduced. This might be a secondary effect of induced MCP-1 and MIP-1 secretion, since chemokine binding is known to down-regulate chemokine receptor expression. Indeed, co-incubation with a neutralizing anti-MCP-1 antibody reversed the downregulation of CCR2 expression. JWH-015 treatment did not modulate the IFN- mediated effects on MCP-1 induction and chemokine receptor downregulation. However, we found that JWH-015 treatment alone reduced chemokine receptor expression, without inducing enhanced chemokine production.

Conclusion: Activation of CB_2 receptors with selective receptor agonists modulates chemokine receptor expression in human monocytes. This might have a therapeutic benefit to reduce activated monocyte recruitment in inflammatory disorders such as atherosclerosis.

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22. THE CANNABINOID-2 (CB_2) RECEPTOR MEDIATES PROTECTION AGAINST TNBS COLITIS IN MICE

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Background: The endocannabinoid-system has been reported to be involved in intestinal inflammation. Activation of cannabinoid-1 (CB_1) receptors results in attenuation of experimental colitis. However, little is known about the possible influence of the CB_2 receptor in intestinal inflammation.

Aim: Our aim was to describe the effects of a CB_2 receptor agonist (JWH 133) and a CB_2 receptor antagonist (AM630) in experimentally induced, trinitrobenzene sulfonic acid (TNBS), colitis in mice.

Methods: Colitis was induced in male C57BL/6 mice by intra-rectal installation of 100 µl 4% TNBS in 30% ethanol. Weight changes were observed for 3 days. At day 3 the mice were sacrificed and the colon was removed for macroscopic scoring and the evaluation of myeloperoxidase (MPO) activity. 6-10 mice in each group were treated with intraperitoneal injections of the CB_2 receptor agonist JWH 133 (20mg/kg) once daily or twice daily, the CB_2 antagonist AM630 (10mg/kg) once daily or vehicle.

Results: Intrarectal installation of TNBS caused marked colonic inflammation. TNBS treated mice had a macroscopic score of 7.2 ± 2.2 compared to 1.2 ± 1.1 in the vehicle (ethanol) treated control mice. TNBS treated mice showed a MPO level of 5.5 ± 2.1U/mg compared to 1.2 ± 1.1 U/mg in the vehicle control mice. The CB_2 receptor agonist JWH 133 reduced the macroscopic score and MPO levels in a dose dependent manner (macroscopic score: 5.4 ± 2.8 once daily; 5.4 ± 2.2 twice daily; MPO: 1.2 ± 0.8 U/mg and 0.6 ± 1.0 U/mg). In contrast, the CB_2 receptor antagonist AM630 caused no significant change in inflammation (macroscopic score: 9.2 ± 1.7; MPO: 4.9 ± 1.7). Body weight changes and survival rates were not significantly different amongst treatment groups.

Conclusion: This study provides evidence that activation of the CB_2 receptor reduces colonic inflammation under experimental conditions suggesting that the CB_2 receptor may be a possible therapeutic target in inflammatory bowel disease.

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23. EFFECTS OF CANNABINOIDS ON MOUSE SPLENOCYTE PROLIFERATION AND CYTOKINE SECRETION

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Aims: Cannabinoids have been shown by us to alter cell proliferation *in vitro*. Similarly, cannabinoids influence T helper cell biasing effects by modulating Th1 or Th2 cytokines. The involvement of the cannabinoid receptors (CB_1R and CB_2R) in these events is mostly unknown. The present study was designed to address the effects of cannabinoids (THC and CP-55940) on cell proliferation and cytokine secretion in immune cells of wild type ($CB_2R^{+/+}$) and CB_2 knockout ($CB_2R^{-/-}$) C57BL/6 mice to determine cannabinoid specific events and involvement of CB_2R.

Methods: Eight to 12 week-old C57BL/6 were each injected intraperitoneally with a single dose 15mg/kg of THC, 0.4 mg/kg of CP-55940, or vehicle (saline, ethanol, cremophore; 18:1:1). Splenocytes were stimulated with mitogens *in vitro*. Bromodeoxyuridine incorporation was used to determine splenocyte proliferation. Secreted cytokine levels were determined by enzyme-linked immunosorbent assays (ELISA).

Results: THC did not alter cellular proliferation in the presence or absence of the T-cell mitogens concanavalin A (Con A, 2.5 g/mL), or by costimulation with anti-CD3/CD28 antibody (5 g/mL and 0.5 g/mL, respectively), nor in the presence of the B cell mitogen lipopolysaccharide (LPS, 10 g/mL). THC injection showed a transitory inhibitory effect on both Con-A and anti-CD3/CD28 -induced Interleukin-2 (IL-2) and Interferon- (IFN-). After an initial decrease at 2 hrs, IL-2 and IFN- secretion of the $CB_2R^{+/+}$ splenocytes returned to control levels at 24 hr after THC treatment. CP-55940 reduced ConA and anti-CD3/CD28-induced cell proliferation of $CB_2R^{+/+}$ splenocytes but not of $CB_2R^{-/-}$ cells. In addition, decrease of ConA-induced IFN- secretion in $CB_2R^{+/+}$ splenocytes by CP-55940 treatment was not observed in the ConA-stimulated $CB_2R^{-/-}$ splenocytes.

Conclusions: These data suggest that THC and CP-55940 alter splenocyte proliferation and cytokine secretion differently. While THC had no effect on $CB_2R^{+/+}$ splenocytes proliferation, CP-55940 inhibited $CB_2R^{+/+}$ cell proliferation. Inhibition of cell proliferation by CP-55940 was mediated by CB_2R, as this inhibition was not seen in $CB_2R^{-/-}$ cells. While a 2 hr *in vivo* exposure to THC reduced IL-2 and IFN- secretion, an effect that was no longer observed 24 hr after THC injection, CP-55940 was able to inhibit IFN- secretion 24 hr post CP-55940 injection. Furthermore, the effect of CP-55940 on IFN- secretion was mediated by CB_2R, since CP-55940 was unable to alter $CB_2R^{-/-}$ cells. The study of the involvement of CB_2R in the THC effect is underway.

NOTES