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

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

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

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THE THERAPEUTIC HANDSHAKE: CRAFTING MEDICINES FOR COMPLEX MALADIES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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CONTEXT. Endocannabinoids induce a profile of pro- and anti-inflammatory effects that are related, in part, to their metabolism. Our research group has demonstrated that the endocannabinoid 2-arachidonoyl-glycerol (2-AG) activates human neutrophil and eosinophil functions through its metabolites. Interestingly, endocannabinoids are substrates for the cyclooxygenase-2 and can be oxidized into prostamides (PG-EAs) and glyceryl-prostaglandins (PG-Gs). Given the structural resemblance between these metabolites and PGE_2, we postulated that PGE_2-G and PGE_2-EA, like PGE_2, would inhibit human neutrophil functions.

AIM. To determine the impact of PGE_2-G and PGE_2-EA on human neutrophils functions and the cellular mechanisms involved.

RESULTS. PGE_2-G, but not PGE_2-EA inhibited neutrophil functions such as leukotriene biosynthesis, chemotaxis, the killing of pathogens and the respiratory burst. The effects of PGE_2-G were mimicked by PGE_2 and were prevented by the EP_2 receptor antagonist AH6809. Given PGE_2-G does not bind to the EP_2 receptor, we verified if the inhibitory effect we observed was the consequence of PGE_2-G hydrolysis into PGE_2. Using deuterated PGE_2-G, we found that the latter had a half life of ~60 minutes in our neutrophil suspensions. The hydrolysis of PGE_2-G was inhibited by potassium fluoride but not MAFP or JZL-184, indicating that a serine hydrolase other than MAG lipase was involved in the hydrolysis of PGE_2-G into PGE_2. We also observed that PGD_2-G, but not $PGF_{2\alpha}$-G, also inhibits leukotriene biosynthesis by neutrophils. Importantly, PGD_2- and PGE_2-serinol amide had no effect. Finally, all the inhibitory effects of PGE_2-G on human neutrophil functions were prevented by the cAMP-dependent protein kinase inhibitor H-89, underscoring the important role of cAMP in this process.

CONCLUSIONS. PGD_2-G and PGE_2-G inhibit human neutrophil functions through their hydrolysis into PGD_2 and PGE_2, respectively. The inhibitory effects of the COX-2 metabolites PGD_2-G and PGE_2-G on human neutrophil functions partially explains the immunosuppressive role of endocannabinoids *in vivo*.

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

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

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

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

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

THE EFFECT OF CANNABINOIDS ON HUMAN BRONCHIAL EPITHELIAL CELL PERMEABILITY

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

Reference

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INACTIVATION OF DIACYLGLYCEROL LIPASE IN INFLAMMATORY DISEASE

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

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

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

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

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

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

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

Acknowledgments: Funded by Science Foundation Ireland

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ENDOCANNABINOIDS DIFFERENTIALLY MODULATE NLRP3 INFLAMMASOME ACTIVATION IN PRIMARY HUMAN MACROPHAGES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

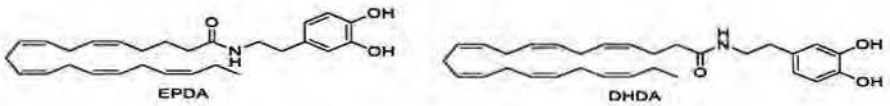

Fig. 1. Structure of *N*-eicosapentaenoyldopamine (EPDA) and *N*-docosahexaenoyldopamine (DHDA)

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

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

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

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

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

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

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COLITIS ALTERS CENTRAL ENDOCANNABINOID CONTENT

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

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

MONOGLYCERIDE LIPASE DEFICIENCY CAUSES INTESTINAL CANNABINOID RECEPTOR DESENSITIZATION

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

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

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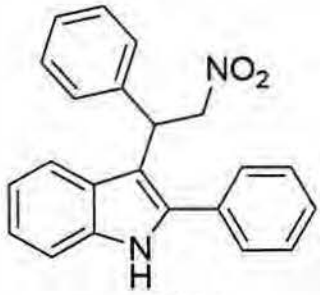
Glaucoma is a multifactorial disease involving cupping of the optic nerve and selective loss of retinal ganglion cells (RGCs). The most important modifiable risk factor in glaucoma is intraocular pressure (IOP), and reducing IOP is the main therapeutic goal of glaucoma therapies. However, despite IOP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

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



GAT211

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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INVESTIGATION OF THE ROLE OF THE TRPV1 CHANNEL IN ENDOTHELIAL INFLAMMATION

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

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

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ENDOCANNABINOID LEVELS AND FUNCTIONAL DISABILITY STATUS IN PATIENTS WITH PAINFUL OSTEOARTHRITIS

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

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

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

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

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

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

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

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

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

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

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

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

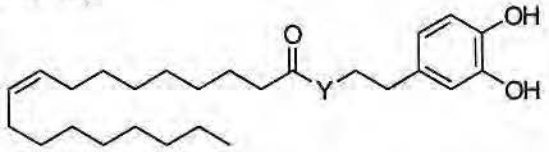

Figure 1: Structure of Hydroxytyrosyl Oleate (Y = O) and *N*-Oleoyldopamine (Y = NH).

Hydroxytyrosol was subjected to lipase-catalyzed acylation with oleic acid methyl ester. The resulting hydroxytyrosyl oleate was evaluated for its ability to reduce the release of nitric oxide (NO) by lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The inhibitory effect was further assessed at the gene expression level using quantitative RT-PCR to determine inducible NO synthase (iNOS) and the inflammatory cytokine interleukin-1β (IL1β) mRNA expression.
At the tested concentrations (0.5-5 μM), hydroxytyrosyl oleate reduced the production of NO in a concentration-dependent way, whereas its parent compounds hydroxytyrosol and oleic acid were ineffective. Inhibition was also found to take place at a transcriptional level, as gene expression of iNOS and IL1β was inhibited by hydroxytyrosyl oleate.
We conclude that lipophylization of hydroxytyrosol with an oleic acid moiety led to the formation of a compound that may be more effective than hydroxytyrosol in the modulation of inflammation.
We are currently investigating the mechanisms of action of hydroxytyrosyl oleate and whether it can be formed *in vivo*.

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ATTENUATING NEUROPATHIC PAIN THROUGH DUAL INHIBITION OF CYCLOOXYGENASE AND MONOACYLGLYCEROL LIPASE

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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LONG-TERM EFFECTS OF ADOLESCENT THC EXPOSURE ON ADULTHOOD PSYCHOPATHOLOGY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ROLE OF THE ENDOCANNABINOID SYSTEM IN AUTOPHAGY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 1 IC_{50} values of phytocannabinoids in isolation

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

NT= not tested

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

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

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

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

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

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

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

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

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

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ALLOSTERIC MODULATION AND BIASED SIGNALLING AT CB1 CANNABINOID RECEPTORS

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

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

PROFILING THE ACTIVITY OF GPR55 ANTAGONISTS AGAINST RECOMBINANT AND ENDOGENOUS GPR55

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

LORATADINE ANALOGUES AS MAGL INHIBITORS

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

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

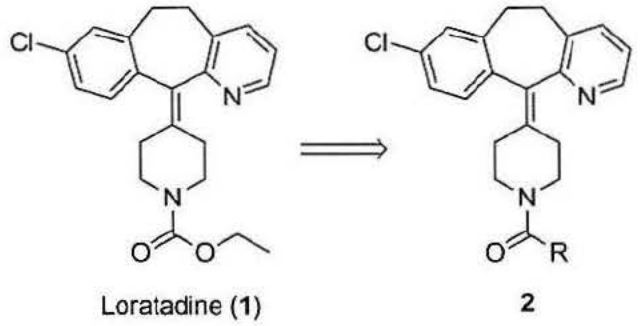

Fig. 1. Loratidine (1) and a general structure of loratadine-based MAGL inhibitors (2).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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NEURONAL CIRCUITS UNDERLYING CANNABINOID WITHDRAWAL

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

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

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

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

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

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

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

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

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

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

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

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

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INTEGRAL FRAMES: INTERDISCIPLINARY TOOLS SUPPORTING CANNABIS RESEARCH

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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SYNTHESIS AND CHARACTERIZATION OF A NANOMICELLE CANNABINOID FORMULATION

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

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

INHIBITION OF ENDOCANNABINOID METABOLISM BY THE METABOLITES OF IBUPROFEN AND FLURBIPROFEN

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

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

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

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

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DIETARY EFFECTS ON CIRCULATING OXYLIPINS AND ENDOCANNABINOIDS

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

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

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

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

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

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

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

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

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

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

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

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

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

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EXPRESSION OF ENZYMES INVOLVED IN ENDOCANNABINOID METABOLISM BY HUMAN LEUKOCYTES

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

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

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

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

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SR141716A-INDUCED HYPERACTIVITY IS NOT REVERSED BY RITALIN

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

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CANNABINOID CB1 RECEPTOR CALIBRATES EXCITATORY SYNAPTIC BALANCE IN THE MOUSE HIPPOCAMPUS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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CANNABINOID EFFECTS ON PREFRONTAL ACTIVATION DURING REGULATION OF NEGATIVE AFFECT

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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AN INVESTIGATION OF PANX 1 MEMBRANE CHANNELS IN CANNABINOID MEDIATED NEUROPROTECTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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THE SATIVA-INDICA DILEMMA

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

Differentiation between *sativa* and *indica* types of cannabis is currently the most common way to classify different strains and their physiological effects among breeders, recreational users and patients. Although *Cannabis sativa* L. is a monotypic species according to current taxonomic consensus, many scientific discussions about the existence of multiple (sub)species have taken place over the centuries [1]. It is unclear whether this classification reflects any relevant differences in chemical composition [2].

Supposing there were originally two main gene pool centres of *sativa* and *indica*, some distinctive characteristics can be expected to have remained on the chemical and genetic level. In our study, we focused on chemical profiling of 8 main cannabinoids and 40 terpenoids by GC-MS. Additionally, samples were analyzed for genetic variations by the SNP-LINE method. Based on principal component analysis (PCA) of the chemical and genetic data, we were able to identify relevant markers for distinguishing *sativa* from *indica* types of cannabis. The study indicates the usefulness of a PCA approach for chemotaxonomic classification of Cannabis varieties.

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EFFECTS OF EXTRA VIRGIN OIL PHENOLIC COMPOUNDS ON CB_1 GENE EXPRESSION: A ROLE FOR EPIGENETIC MECHANISMS

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Extra virgin olive oil (EVOO) represents the typical lipid source of the Mediterranean diet, a dietary habit that has been associated with a significant reduction of cancer risk in Mediterranean populations. The aim of our study was to investigate the ability of EVOO and its phenolic compounds to modulate gene expression and epigenetic regulation of elements of the endocannabinoid system (ECS), in colon cancer (Caco-2) cells, as well as to explore the underlying mechanisms responsible for its potential beneficial effects. Moreover, we investigated the short- and long-term influence of dietary EVOO on ECS components in rat colon mucosa.

Gene expression and promoter methylation was determined by qPCR analysis and pyrosequencing respectively, in colonic epithelial cells treated with EVOO or total phenolic extracts (TPE). Additionally, adult female Sprague Dawley rats were fed with either a standard diet or with EVOO supplement, and the expression of ECS elements was assessed in rat colonic epithelium after 2hr and 10 days.

We found a selective increase in CB_1 gene expression in Caco-2 cells exposed to EVOO (100ppm) or TPE (10-50 μM) for 24 hr, compared to untreated cells. The stimulatory effect of EVOO and TPE on CB_1 expression inversely correlated to levels of DNA methylation at *CNR1* promoter and was associated with DNA methyltransferase (DNMT) 3a down-regulation. We also found that EVOO and TPE inhibited proliferation of Caco-2 cells and arrested their cycle, and that animals receiving dietary EVOO supplementation for 10 days displayed a significant increase in CB_1 gene expression levels in colon. Finally, we found that CpG methylation of the rat *Cnr1* gene was reduced after acute EVOO administration, but remained unaltered after chronic EVOO administration compared to standard diet.

In conclusion, our findings that CB_1 gene expression can be modulated by EVOO or its phenolic compounds *via* epigenetic mechanisms, both *in vitro* and *in vivo*, may provide a new therapeutic strategy for treatment and/or prevention of colon cancer.

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DEVELOPMENT OF A FLOW CYTOMETRY-BASED BRET ASSAY UTILISING GENETICALLY-ENCODED BIOSENSORS FOR MIXED CELL POPULATIONS: APPLICATION TO CANNABINOID CB1 RECEPTORS

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Genetically-encoded bioluminescent resonance energy transfer (BRET)-based biosensors have been invaluable tools in the quantitative and qualitative analysis of intracellular signalling processes. BRET biosensors are typically transiently transfected into cultured cells, and the resulting signal detected using a plate reader. These plate readers integrate the results from large samples of cells – typically 30,000-100,000 cells per 96-well. Here, we report the development process of a high throughput assay for BRET biosensors in single cells with the primary aim of developing a method to differentiate and selectively analyse subpopulations of cultured cells. Specifically, we are developing a flow cytometry (FCM) method for single-cell cAMP measurement, using the genetically-encoded BRET-based biosensor CAMYEL (Jiang, et al. JBC. 2007;282(14):10576-84).

FRET by FCM has previously been demonstrated (e.g. Banning, et al. PLoS ONE. 2010;5(2):e9344), but to date there have been no published examples of FCM BRET detection, although this approach would help overcome the spectral overlap of FRET-pair fluorophores. The BRET-based biosensor assay was optimised using a Becton Dickinson LSRII flow cytometer, with the 488 nm laser occluded to prevent laser-excitation of the BRET acceptor. As the 488 nm laser typically provides the forward and side-scatter data used to identify cells, an antibody-based cell-surface marker was utilised to identify and selectively gate for cells. We found that, while the Rluc and YFP BRET pair had insufficient energy for detection, with a sufficiently slow flow rate, the high emission of the variants Rluc8 and Venus provided satisfactory signal for detection of both wavelengths. Site directed mutagenesis was therefore employed to modify CAMYEL to optimise detection, and results with the optimised biosensor will be presented.

This technique is well-suited for mixed cell cultures, and can provide detailed data from as little as a few thousand cells. BRET by FCM is also useful for monitoring interactions from a range of interacting proteins, such as dimer partners. Here we present the proof-of-principle data from a transfected HEK cell population expressing the human cannabinoid CB1 receptor. In conclusion, while it is technically challenging to establish a suitable single cell detection method, such a high throughput approach would be a valuable to analysing cell signalling biology and for drug discovery.

ADOLESCENT THC EXPOSURE INDUCES EPIGENETIC CHANGES AND ALTERS GENE EXPRESSION IN THE RAT PREFRONTAL CORTEX

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We recently demonstrated that adolescent female rats treated with the psychoactive ingredient of marijuana delta-9-tetrahydrocannabinol (THC), develop a depressive/psychotic-like phenotype in adulthood. Interestingly, adolescent, but not adult, chronic THC exposure leads to this phenotype, suggesting that adolescence may represent a more vulnerable period for the adverse effect of THC exposure. However the neurobiology of this vulnerability is not clear. Recently, several papers support an involvement of epigenetic mechanisms in the pathogenesis of psychiatric illnesses. Thus we check for the presence of epigenetic alterations induced by adolescent THC exposure (e.g. H3 tri- and di-methylation or acetylation) in the prefrontal cortex (PFC) of adolescent THC-treated animals. To this aim, adolescent female rats were treated with increasing doses of THC twice a day from PND 35 to 45. Two and 24 hours after the last THC injection, epigenetic modifications were investigated by immunoblotting.

In the PFC of THC-treated animals we observed increased tri-methylation of Lysin27 on the histone H3 (H3K27me3, associated with transcriptional repression) 2 hours after the end of the THC treatment. Moreover, 24 hours later, this increase was still present together with increased di-methylation of Lysin9 and acetylation of Lysin14 on histone H3 (H3K9me2 and H3K14Ac, associated with transcriptional repression and activation respectively).

Since histone modifications impact transcriptional activity, the second aim of this work was to investigate the effect of adolescent THC exposure on gene expression in the PFC. Adolescence is characterized by intense processes of neuronal refinement in which the endocannabinoid system (ECS) seems to play a crucial role, thus we focused our attention on genes closely related to the ECS or involved in synaptic plasticity. To this aim, adolescent female rats were treated with increasing doses of THC twice a day from PND 35 to 45. Real-Time PCR array analysis was performed in the PFC 2, 24 and 48 hours after the last THC injection. Besides genes related to the ECS, we investigated 34 genes involved in synaptic plasticity (e.g. belonging to the glutamatergic and gabaergic system as well as coding for proteins or pathways related to plasticity).

Two and 24 hours after the THC treatment mRNA levels mainly decreased whereas they returned to control levels or even increased 48 hours after the end of the treatment. These alterations might play a role in the development of the depressive/psychotic-like disorder induced by adolescent THC exposure.

As a whole, these data suggest that adolescent THC treatment impairs the steady state expression of a set of genes involved in brain plasticity. These alterations might play a role in the development of the depressive/psychotic-like disorder induced by adolescent THC exposure.

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SELECTIVE ACTIVATION OF CB_2RS ELIMINATES AGONIST-INDUCED CA^{2+} SIGNALS IN PANCREATIC ACINAR CELLS: A NOVEL CELLULAR MECHANISM FOR ACUTE PANCREATITIS THERAPEUTICS

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Acute pancreatitis is an inflammatory disease of the pancreas, which has several causes and symptoms which require immediate medical attention. Acute pancreatitis occurs when pancreatic pro-enzymes (especially trypsin), secreted from pancreatic acinar cells, are activated in the pancreas instead of the small intestine, causing autodigestion. In clinical practice, there are still no efficient drugs that specifically treat acute pancreatitis. Emerging lines of evidence demonstrate that the primary event initiating the process of acute pancreatitis is the excessive release of Ca^{2+} from intracellular Ca^{2+} pools. This provides a promising therapeutic strategy, as the blockade of intracellular Ca^{2+} signals in pancreatic acinar cells may provide protection against Ca^{2+} overload, intracellular protease activation and necrosis, which are major triggers of acute pancreatitis. The cannabinoid receptor type 2 (CB_2R) is a G protein-coupled receptor from the cannabinoid receptor family. CB_2Rs are predominantly expressed in peripheral system, especially in immunal cells, suggesting that they are the major targets in mediating the effects of cannabinoids on the immune system. Thus, CB_2R agonists are commonly used as a therapeutic drug for the treatment of inflammation and pain.

A large body of evidence suggests that CB_1 and CB_2 receptors are expressed in the pancreatic gland and modulate pancreatic cell function, alter insulin release, and play important roles in the regulation of body metabolism. In addition, emerging data demonstrate that the activation of CB_2Rs in pancreatic acinar prevents acinar cell pathogenesis in acute pancreatitis animal models. Whether or not the activation of CB_2Rs modulates intracellular Ca^{2+} signals in pancreatic acinar cells is unknown. Also, whether the pancreatitis inducer L-arginine enhances Ca^{2+} oscillations, and if a CB_2R agonist eliminates L-arginine-induced enhancement of agonist-induced Ca^{2+} oscillations in pancreatic acinar cells remains elusive. In the present study, we address these important questions by using patch-clamp and confocal Ca^{2+} imaging approaches combined with qRT-PCR and immunohistochemical staining in wild type (WT), CB1 knockout (KO) and CB2 KO mice. We found that selective activation of CB_2Rs, by a selective agonist GW13542, significantly reduced ACh (10-30 nM)-induced Ca^{2+} oscillations in acutely-dissociated single pancreatic acinar cells. The inhibitory effect of GW13542 could be eliminated by a selective CB_2R antagonist, AM630, or was absent in CB_2R, but not CB_1R, KO mice. Next, we showed that intracellular application of GW13542 failed to inhibit Ca^{2+} oscillations and intracellular application of AM630 also failed to prevent bath-applied GW13542-induced inhibition in Ca^{2+} oscillations. Together, these results suggest that GW13542 eliminates ACh-induced intracellular Ca2+ signals through CB_2Rs on the acinar cell surface. Finally, we demonstrated that L-arginine enhanced ACh-induced Ca^{2+} oscillations, while GW13543 eliminated this enhanced effect. Collectively, our study provides a novel cellular mechanism that the CB_2R agonist GW13542 eliminates intracellular Ca^{2+} signaling in pancreatic acinar cells, which may provide a new therapeutic strategy for treating acute pancreatitis.

A NEW ANTI MOUSE CB2 ANTIBODY SENSITIVE TO RECEPTOR CONFORMATION

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A new anti mouse CB2 antibody was generated by immunizing mice with living HEK293 cells expressing the N-terminal 34 amino acids of mouse CB2 fused to mouse glycophorin A and directed to the cell surface with a bee venom melittin signal sequence. The AB 1/45 was identified by immunofluorescence using recombinant CHO cells to be selective for mouse CB2 since it did not cross react with either human CB2 nor CB1. Using the same recombinant mouse CB2 expressing CHO cells, the antibody exhibited a bright and specific signal detectable by flow cytometry and was useful to immunoprecipitate CB2. The CB2-specific signal observed via flow cytometry is lost however by the addition of CB2 ligands. Irrespective of the CB2 ligand function (Agonists: HU-910, WIN 55.212-2, 2AG; n=3; Inverse agonists: SR-144528, NE40), we found greater than 88% displacement of the fluorescent signal at an EC50 similar to the respective K_i ($r^2 = 0.93$).

A first hypothesis was that this ligand-mediated signal loss is a consequence of CB2 receptor internalization. However, ligand-mediated antibody displacement at 4°C was observed at comparable potency and kinetics (less than a minute) as compared to the initial experiment at 37°C, thus excluding the involvement of receptor internalization. We thus rather hypothesized, that AB1/45 is able to bind in a conformation-sensitive manner distinguishing bound and unbound CB2. This was confirmed by immunohistochemistry using a directly fluorophore labeled AB 1/45. The ligands from above were fully efficacious in displacing AB 1/45 with the exception of AM630 which only partly displaced AB 1/45 by 47% while the EC50 of 14nM still resembled the K_i. In contrast, Anandamide (AEA) increased rather than decreased AB1/45 binding up to 10μM. As so far the only anomalous ligand identified was AEA, it can be assumed that AEA results in a different conformational change to CB2.

We then determined the effect of AB 1/45 on CB2 function in a cAMP assay. After forskolin-induced activation, AB1/45 was able to act as an inverse agonist with an EC50 of 218nM. It remains to be shown if AB1/45 is displaced by the high affinity ligands or is rather recognizing a specific bound CB2 conformation. Interestingly, AB 1/45 is unable to detect CB2 on leukocytes isolated from mouse blood. This could be a result of a ligand bound state, or a masked epitope by glycosylation or interacting proteins of native endogenous mouse CB2 in leukocytes compared toCB2 expressed in CHO cells. In summary by using a whole cell immunization protocol based on a hybrid N-terminal CB2 antigen, we discovered a mouse CB2 selective antibody able to detect ligand free CB2 receptors in transfected CHO cells serving as a tool to unravel the difference between endogenous CB2 versus recombinant mouse CB2 receptors.

2-ARACHIDONOYLGLYCEROL PROMOTES LEUKOCYTE RECRUITMENT THROUGH CB_1 AND CB_2 RECEPTORS

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Accumulated evidence points to a key role for endocannabinoids in cell migration, and here we sought to characterize the role of these substances in early events that modulate communication between endothelial cells and leukocytes.

We found that 2-arachidonoylglycerol (2-AG) was able to initiate and complete the leukocyte adhesion cascade, by modulating the expression of selectins. A short exposure of primary human umbilical vein endothelial cells (HUVECs) to 2-AG was sufficient to prime them towards an activated state: within 1 hour of treatment, endothelial cells showed time-dependent plasma membrane expression of P- and E-selectins, which both trigger the initial steps (*i.e.*, capture and rolling) of leukocyte adhesion. The effect of 2-AG was mediated by CB_1 and CB_2 receptors and was long lasting, because endothelial cells incubated with 2-AG for 1 hour released the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) for up to 24 hours. Consistently, TNF-α-containing medium was able to promote leukocyte recruitment: human Jurkat T cells grown in conditioned medium derived from 2-AG-treated HUVECs showed enhanced L-selectin and P-selectin glycoprotein ligand-1 (PSGL1) expression, as well as increased efficiency of adhesion and trans-migration.

In conclusion, our *in vitro* data indicate that 2-AG, by acting on endothelial cells, might indirectly promote leukocyte recruitment, thus representing a potential therapeutic target for treatment of diseases where impaired endothelium/leukocyte interactions take place.

CANNABINOID 2 RECEPTOR MODULATION IN EXPERIMENTAL SEPSIS

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Introduction: Sepsis results from a dysregulated immune response to an infection. Currently available medical treatments are of limited efficacy with regard to improvement of survival. The endocannabinoid system (ECS) opens a novel avenue in sepsis therapy due to its unique characteristics. Cannabinoid 2 (CB_2) receptors expressed on the surface of immune cells provide a direct approach to the modulation of the systemic immune response in sepsis. The present study explored the effects of CB_2 receptor modulation in two mouse models of acute sepsis (endotoxemia and abdominal fecal peritonitis).

Methods: Endotoxemia was induced by intravenous administration of lipopolysaccharide (LPS, 5 mg/kg; from *E. coli,* serotype: O26:B6). Six groups of mice were used to assess leukocyte activation within the intestinal microcirculation as well as functional capillary density (FCD) by intravital microscopy. The treatment compounds tested were: a specific CB_2 receptor agonist, HU308, a CB_2 receptor antagonist/inverse agonist, AM630, and the cannabinoid degradation enzyme inhibitors URB597 (fatty acid amide hydrolase inhibitor), and JZL184 (monoacylglycerol lipase inhibitor). Abdominal fecal peritonitis was induced by implanting a stent into the ascending colon and allowing fecal matter to pour out into the abdominal cavity (CASP). Four groups of mice were used to asses the same microcirculatory parameters mentioned previously, with JZL184 as the treatment compound.

Results: In the endotoxemia model, administration of the CB_2 receptor agonist, HU308, reduced significantly the number of adhering leukocytes in submucosal venules, but did not restore muscular and mucosal villi FCD in endotoxemic mice. The CB_2 receptor antagonist, AM630, did not exacerbate leukocyte activation within the intestinal microcirculation, but further reduced muscular and mucosal FCD of the intestinal wall. The cannabinoid degradation enzyme inhibitors URB597 and JZL184 both significantly reduced the number of adhering leukocytes in submucosal venules. Furthermore, JZL184 administration completely restored muscular FCD. In the CASP model, JZL184 reduced significantly the number of adherent leukocytes in the submucosal venules. Rolling behaviour of leukocytes also showed a trend to normalization by JZL184 treatment.

Conclusions: CB_2 receptor activation by a specific agonist or cannabinoid degradation enzyme inhibition was effective in reduction of leukocyte activation within the intestinal microcirculation. The CB_2 receptor pathway seems to be involved in the inflammatory cascade elicited early during sepsis. Therefore, modulation of the CB_2 receptor pathway might offer new therapeutic options for the manipulation of the immune system in sepsis.

CB2R MODULATION OF LPS-INDUCED TOLL-LIKE RECEPTOR 4 SIGNALLING IN OCULAR INFLAMMATION

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Introduction: Uveitis is an ocular inflammatory disease that can lead to impaired vision. Experimental endotoxin-induced uveitis (EIU) can be generated with an intravitreal (IVT) injection of lipopolysaccharide (LPS), a component of gram-negative bacteria. LPS activates the Toll-like receptor 4 (TLR4) and induces the release of inflammatory mediators that may cause tissue damage. TLR4 can signal by both the MyD88-dependent pathway and the MyD88-independent pathway (TRAM/TRIF pathway). In the eye, cannabinoids acting at CB2 receptors (CB2R) are anti-inflammatory and can reduce pro-inflammatory cytokines, although the signaling mechanisms giving rise to these effects remain to be clarified. Therefore, this study examined the effects of CB2R modulation on TLR4 signaling in EIU.

Methods: Five groups of Lewis rats (n= 8-10 per group) were studied: control (saline, IVT), EIU (100 ng LPS, IVT), EIU + CB2R agonist (HU308; 1.5 μg/μL eye drop), EIU + CB2R antagonist (AM630; 2.5 mg/kg, i.v.), EIU + CB2R agonist + CB2R antagonist. Drug treatments were given 15 min after EIU induction. Tissue was harvested and RNA was extracted. Quantitative PCR was conducted using LightCyclerR system and software with SYBR Green I. All qRT-PCR data was normalised to the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA expression levels of different molecules in both downstream signalling pathways of the TLR4 were investigated: NF-κB (nuclear factor kappa B), activator protein-1 (AP-1), receptor-interacting protein 1 (RIP1), interferon regulatory transcription factor 3 (IRF3). The changes in mRNA levels were also investigated for the CB2R.

Results: A significant increase of NF-κB mRNA occurred in LPS-treated eyes compared to control ($P<0.001$). This increase in NF-κB mRNA was abrogated by administration of the CB2R agonist, HU308. The CB2R antagonist, AM630, significantly increased NF-κB mRNA compared to LPS ($P<0.05$), while the combination treatment of AM630 and HU308 decreased these levels to that comparable to control. AP-1 mRNA was significantly increased in LPS animals compared to control ($P<0.001$); this increase was attenuated by treatment of HU308. AM630 had no effect on altering AP-1 mRNA levels, although treatment with both AM630 and HU308 resulted in a significant decreased ($P<0.001$) of these levels. There were no significant changes in the mRNA levels of IRF3 and RIP1 amongst any of the experimental groups studied ($P>0.05$). CB2R mRNA expression was significantly increased in LPS in comparison to control ($P<0.05$).

Conclusion: These data demonstrate that CB2R are capable to modulate (ocular) inflammation via TLR4 signalling. CB2R agonist treatment decreases mRNA levels of NF-κB and AP-1. However, as CB2R activation does not modulate RIP-1 or IRF3 mRNA expression, these data suggest that the anti-inflammatory actions of CB2R activation may be primarily mediated by actions on the MYD88-dependent pathway and are independent of TRAM/TRIF signalling.

CANNABINOID RECEPTOR 2: A NOVEL IMMUNOSUPPRESIVE TARGET IN EXPERIMENTAL PROLIFERATIVE VITREORETINOPATHY

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Introduction: Proliferative vitreoretinopathy (PVR) is a sight- threatening complication of retinal detachment, ocular trauma or inflammation. Cannabinoid receptor 2 (CB_2) is expressed on immune cells and activation of this receptor is anti-inflammatory. In an experimental murine model of PVR, we previously showed that pathological changes in the retina of CB_2 receptor knockout animals were more pronounced than in control C57Bl/6 animals. These changes were characterized by the formation of retinal folds, inflammation of the choroid and a significant increase in activated microglia. We further examined the role of CB_2 in the pathology of PVR, including changes in the expression profiles of pro-inflammatory cytokines, prostaglandins and related lipids in both, $CB_2^{-/-}$ and C57BL/6 animals.

Methods: PVR was induced in $CB_2R^{-/-}$ and C57BL/6 mice by intravitreal dispase injection (0.1U; 2μl). At 7 days post-dispase injection, the morphology of the eyes was examined using light microscopy and scored using a clinical grading system. Animals were then sacrificed, eyes enucleated and the levels of prostaglandins and related lipids were analyzed by tandem mass spectrometry. In addition, the pro-inflammatory cytokines expression was evaluated by ELISA.

Results: Dispase injection (0.1U; 2 μl) resulted in pronounced damage to ocular tissues in $CB_2^{-/-}$ animals. As previously shown, there was a mild or absent pathology observed in C57BL/6 animals. The mass spectrometry analysis revealed elevated levels of prostaglandins and related lipids in $CB_2^{-/-}$ animals, as compared to controls. In addition, changes in the levels of pro-inflammatory cytokines were also observed in $CB_2^{-/-}$ animals.

Conclusion: Mice lacking CB_2 had increased susceptibility to dispase-induced retinal damage. The retinal pathology in $CB2^{-/-}$ mice was associated with elevated levels of prostaglandins and neurotoxic lipids, as well as selective cytokines. This data is supportive of an immunosuppressive role for CB_2 in inflammatory ocular conditions and suggests that drugs that activate CB_2 may be useful in the treatment of ocular inflammatory disease.

ASSESSMENT OF CB2 IN HUMAN BRAIN TISSUE USING *IN SITU* HYBRIDISATION

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Background and Aim. In the past 5 years, there has been increasing debate around the presence or absence of the cannabinoid receptor-2 (CB2) in the healthy central nervous system. Most of the pro-voice is from research using rodent brain tissue and antibody based applications. Prior to this, much of the cannabinoid field was in reasonable agreement that CB2 was largely absent from the CNS, but can be elevated during neuroinflammation (due to leukocyte infiltration). The aim of this research is to investigate CB2 expression at the mRNA level and protein level using a combination of in situ hybridisation using healthy or normal appearing brain tissue.

Methods. Complementary RNA probes (riboprobes) were synthesised for CB2 and markers of various cell types including endothelial cells (CD34), pericytes (SMA), microglia (IBA-1), neurons (NeuN) and CB1 as a control probe. Cell-lines were generated expressing the target gene for each probe set and grown as an artificial tissue for the purposes of ISH sectioning to create positive controls. Human brain tissue from the Neurological Foundation Brain Bank was used in this study.

Results. Our histological data indicates the absence of CB2 protein expression in neuronal populations and glial (astrocytes and microglia) cells. Some positive antibody staining has been observed within structures of the blood-brain barrier, at this time thought to be the endothelial cells. The specificity of *in situ* riboprobes were rigorously validated using positive control cells grown in 3D-Alvetex scaffolds to generate artificial tissue layers. *In situ* hybridisation for CB2 probes reveals a very low to negligible signal from the human brain tissue regions investigated to date. In contrast, signals from CB1 and NeuN probes were very strong, validating the effectiveness of the *in situ* method.

Conclusions and Future Developments- Based on our current histological and *in situ* data we conclude that CB2 is <u>not abundantly</u> expressed in the healthy human CNS tissue. Future experiments will involve a greater range of human brain regions and development of colorimetric *in situ* for anatomical localisation of CB2 signals. Extension of the approach using neurological tissue with pronounced inflammation is an important future goal.

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DIFFERENTIAL EXPRESSION AND FUNCTION OF THE CB2 RECEPTOR IN DISTINCT SUBSETS OF HUMAN MONOCYTES AND MACROPHAGES FROM BLOOD

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Background and Aim. CB2 receptor agonists have anti-inflammatory properties in rodent models of stroke and multiple sclerosis, and appear to improve the neurological deficits caused in part by the detrimental neuroinflammation in these debilitating models. The aim of this research is to better understand the function of the CB2 receptor expressed by specific human immune cells circulating in blood. We are particularly interested in CB2 expression and function of human monocyte subsets as these cells have key roles in most forms of neuroinflammation.

Methods. Flow-cytometry was conducted to compare CB2 expression across the distinct monocyte subsets including the classical $CD14^+$ monocytes (represents ~80% of monocytes), intermediate $CD14^+/CD16^+$ (~10% of monocytes) and the non-classical $CD16^{++}/CD14^{low}$ monocytes (~10%) to reveal distinct expression patterns for CB2. Functional assays (including cytometric bead array and phagocytosis of fluorescent particles) were conducted to determine the role of the CB2 receptor in regulating secretion of a key panel of inflammatory cytokines and influence of CB2 on phagocytic activity of different monocyte subsets.

Results. All monocyte subsets expressed CB2, with the non-classical $CD16^{++}$ subset having 10-fold higher levels than the $CD14^+$ monocytes. However, CB2 agonists did not have a major effect on any of the major monocyte-secreted chemokines or cytokines measured in our CBA panel. In contrast, CB2 substantially suppressed the phagocytic activity of monocyte-derived macrophages at agonist concentrations within 10nM to 100nM.

Conclusions- The differential expression level of CB2 across human monocyte subsets is novel, but more importantly suggests functions associated with specific phenotypes or states of activation. Intriguingly, we observed pronounced effects of CB2 agonists on macrophage phagocytosis, but not on monocyte cytokine secretion. Cytokine/chemokine communication is a major component of the inflammatory response and is surprising that CB2 had no major influence. These data may suggest that CB2 is involved in regulating a more mature macrophage phenotype than the monocyte phenotypes observed in blood.

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NEW MOUSE MODEL FOR THE STUDY OF CB_2 CANNABINOID RECEPTORS

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The study of cannabinoid CB_2 receptors has been limited by several factors. Among others, the questions on the specificity of anti-CB_2 antibodies and the expression of a truncated form of the protein in CB_2-knockout mice have been of most relevance. We here report the design and generation of a new transgenic mouse line that may help to unveil the precise pathophysiological roles of cannabinoid CB_2 receptors. The mouse model was generated by inserting an eGFP reporter gene preceded by an IRES sequence in the 3' UTR of the *Cb2* mouse gene. This approach results in the expression of the reporter gene under the control of the endogenous mouse *Cb2* promoter and transcript from the same bicistronic mRNA as the CB_2 protein. In addition, the whole exon 3, including the 3' UTR and the knocked-in reporter was flanked by *loxP* sites, allowing the conditional inactivation of the *Cb2* gene. The mouse model ($CB_2^{eGFP/f/f}$) was generated by homologous recombination in embryonic stem cells, in the C57BL/6J genetic background. The presence of GFP was determined in circulating cells by flow cytometry, revealing a relative abundance concordant with that expected for CB_2 receptors (CD4<CD8<macrophages<B cells). In addition, GFP was detected in cortical areas of the spleen and in the epithelial layer of the bladder. In the intact, healthy CNS, the expression of GFP was undetectable. We performed a hemilateral administration of lipopolysaccharide (LPS) in the striatum of $CB_2^{eGFP/f/f}$ mice. After one week of survival, mice were anesthetized, perfused and their brains used for morphohistological analysis. A dramatic increase in GFP expression was evident in the lesioned cortex, corpus callosum and striatum, but was unchanged in the non-lesioned side. In a second study, electric foot shock was used to produce pain. Twenty four hours later, significant GFP fluorescence was detected in the vicinity of blood vessels and in the amygdala. We present a novel mouse model that may be useful to characterize the role of cannabinoid CB_2 receptors in a wide variety of pathophysiological processes.

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NON-PSYCHOACTIVE PHYTOCANNABINOIDS FOR THE TREATMENT OF CANCER ANOREXIA-CACHEXIA SYNDROME: EVIDENCE FOR THE THERAPEUTIC POTENTIAL OF CANNABIGEROL

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Cancer anorexia-cachexia syndrome (CACS) is a multifactorial wasting syndrome for which no standard of care treatment currently exists. Thus, there is an unmet clinical need for compounds which stimulate food intake, attenuate weight loss and improve physical performance. Clinical trials of Δ^9-THC, the main psychoactive phytocannabinoid (pCB), have shown limited clinical efficacy, likely due to dose-limiting psychoactive side-effects (Pacher & Kunos, FEBS J. 280 (2013) 1918-43). Recent pre-clinical studies indicate that non-psychoactive pCBs can stimulate feeding behaviours (Farrimond et al., Psychopharmacology. 223 (2012) 117-29) and may thus have therapeutic potential in the treatment of CACS. In the present study we investigated the non-psychoactive pCB cannabigerol (CBG), in both pure isolate and botanical drug substance (BDS) forms, to determine neuromotor tolerability profiles and ability to stimulate feeding.

Tolerability Profile: Male Lister-hooded rats (200-225g, n=12) were administered CBG pure or BDS (0, 30, 60, 120 mg/kg; *p.o.*) using a counterbalanced, repeated measures design. One hour later animals started a battery of neuromotor tolerability tests: (1) an open field to test gross locomotor activity, (2) a static beam to assess balance and motor control, and (3) grip strength to measure muscular strength. Neither pure CBG nor CBG-BDS elicited significant effects on line cross measures in the open field (p=0.716 & p=0.175) or pass rate on the static beam (p=0.30 & p=0.112). Pure CBG also had no effect on forelimb grip strength (p=0.643), however there was a significant effect of CBG-BDS (p=0.029) with attenuation of grip strength at both 30 and 120 mg/kg (planned comparisons: p=0.013 & p=0.02 respectively).

Feeding Behaviour: Male Lister-hooded rats (200-225g, n=16) were tested using a well-established pre-feed paradigm for the investigation of hyperphagia (Williams et al., Physiol Behav. 65 (1998) 343-6). Using a counterbalanced, repeated measures design, animals were administered CBG pure or BDS (0, 30, 60, 120, 240 mg/kg; *p.o.*) and feeding behaviour was assessed for 2 hours. Both CBG pure and BDS showed significant increases in total food consumed during the test (p=0.006 & p=0.028), resulting from highly significant reductions in the latency to begin feeding (p=0.021 & p=0.009) and increases in the amount of food consumed within meals (p=0.007 & p=0.044).

Both CBG pure and BDS demonstrated favourable tolerability profiles with no impairments in nearly all measures. However, CBG-BDS did attenuate performance in the grip strength test which may indicate a potential clinical concern and requires further investigation. Both CBG pure and BDS significantly stimulated both the appetitive (latency) and consummatory (meal size) components of feeding behaviour, with the more robust effect elicited by pure CBG. Based on these data pure CBG (120-240 mg/kg) will be investigated for efficacy in pre-clinical models of tumour-induced wasting and chemotherapy-induced nausea and vomiting.

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RELEASE OF *N*-ACYLETHANOLAMINES BY BIOMATERIALS OF CLINICAL RELEVANCE

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Tissue engineering is considered an attractive field of research, in virtue of its promising potential for recovery and replacement of organ subunits. Targeting tissue regeneration or replacement, a good deal of the literature currently focuses on developing 3D scaffolds using synthetic polymers to reproduce the extracellular matrix (ECM) and provide a good substrate for cell adhesion, proliferation and differentiation. Electrospinning is one of the approaches that allow the fabrication of several synthetic materials into fibrous and porous structures in the micro- and nanometer scale. Moreover, electrospun materials can be functionalized with drugs for localized delivery.

Here, we report the convenient incorporation of *N*-acylethanolamines (NAEs) into an electrospun polyesther scaffold for their sustained release in vitro.
Scaffolds were prepared starting from a 8 wt% poly(ε-caprolactone) (PCL) solution containing [^{14}C]-AEA (0.1% w/w vs. PCL), that was electrospun at 15 kV onto a grounded target placed at 15 cm, with a feed rate of 1.5 mL/h. Scaffold morphology were characterized by Field Emission Scanning Electron Microscopy (FE-SEM). A time-dependence of release of AEA by the scaffold was assessed by measuring the amount of radioactivity associated with the eluates for up to 14 days at 37°C in PBS. Additionally, FAAH enzymatic assay performed in rat brain membrane preparation confirmed that AEA released by the scaffold was still substrate for its main hydrolytic enzyme.

In conclusion, we reported data indicating that electrospun PLC scaffold might be functionalized with AEA without interfering with its chemical structure and might be a good platform for AEA release. Moreover, the reported data encourage the use of biodegradable electrospun polymers as suitable delivery system for AEA long-term administration on 3D cultured cells.

CANNABIDIOL ATTENUATES SCHIZOPHRENIA-LIKE EFFECTS AND CHANGES ON PARVALBUMIN EXPRESSION IN THE MEDIAL PREFRONTAL CORTEX INDUCED BY ANTAGONISM OF NMDA RECEPTORS IN MICE

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Preclinical and clinical data suggest that cannabidiol (CBD), a major non-psychotomimetic constituent of Cannabis sativa, has antipsychotic-like effects. However, the antipsychotic properties of repeated treatment with CBD in laboratory animals have been poorly investigated. Thus, we have evaluated if the repeated treatment with CBD would attenuate the behavioral changes induced by chronic administration of MK-801, an NMDA receptor antagonist, since this treatment is considered a valid animal model to induce psychotic-like signs. In addition, we also evaluated changes in parvalbumin (PV) expression, a calcium-binding protein expressed in a subclass of GABAergic interneurons. Abnormalities in PV(+) neurons are found in schizophrenic patientsand have been related to a hypofunction of NMDA receptor signaling. Methods: Male C57BL/6J mice (6 weeks of age when experiment began) received daily ip injections of MK-801 (0.1, 0.5 or 1 mg/kg) for 14, 21 or 28 days. Twenty-four h after the last injection, the animals were submitted to the prepulse inhibition test (PPI). After that, we investigated if repeated treatment with CBD (15, 30 and 60 mg/kg) would attenuate the impairment induced by chronic treatment (28 days) with MK-801 (1 mg/kg) in PPI. CBD treatment began on the 6th day after the start of MK-801 administration and continued until the end of the treatment. In a third experiment, we investigated if CBD would attenuate the MK-801-induced behavioral changes in the social interaction (SI) and object recognition (OR) tests. Immediately after the PPI, the animals were perfused to evaluate changes in PV expression in the medial prefrontal cortex (mPFC), striatum, nucleus accumbens and hippocampus by immunohistochemistry. MK-801 administration at the dose of 1 mg/kg for 28 days impaired PPI. CBD (30 and 60 mg/kg) attenuated PPI impairment. Repeated treatment with MK-801 also impaired social interaction and object recognition, effects also attenuated by CBD. MK-801 treatment induced a decrease in PV expression in the mPFC. This change was attenuated by CBD. Moreover, CBD by itself did not change the behavioral responses or PV expression. These results indicate that repeated treatment with CBD was able to reverse the psychotomimetic-like effects and to attenuate PV expression changes in the mPFC observed after chronic administration of a NMDA receptor antagonist. The data support the view that CBD may have antipsychotic properties.

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CHARACTERIZATION OF STRUCTURAL REQUIREMENTS FOR POTENT MECHANISM-BASED INHIBITION OF HUMAN CYP1A1 BY CANNABIDIOL

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Our previous study has shown that cannabidiol (CBD) is a potent mechanism-based inhibitor of human cytochrome P450 1A1 (CYP1A1) (Yamaori et al., Biochem. Pharmacol. 79 (2010) 1691-1698). In this study, we investigated the characterization of structural requirements for the inactivating effect of CBD on CYP1A1 using nine CBD-related compounds. A preincubation of olivetol, which corresponds to pentylresorcinol moiety of CBD, exhibited time- and NADPH-dependent enhancement of the inhibition for ethoxyresorfin *O*-deethylase activity of human recombinant CYP1A1. The lack of both phenolic hydroxyl groups (pentylbenzene) or the pentyl side chain (resorcinol) in olivetol abolished the metabolism-dependent CYP1A1 inhibition. These results indicate that the pentylresorcinol structure in CBD plays an important role in CYP1A1 inactivation. In addition, orcinol (methylresorcinol) preserves the ability to inactivate CYP1A1 activity to some extent. Furthermore, the inactivation studies with CBD-2"-monomethylether (CBDM) and CBD-2", 6"-dimethylether (CBDD) revealed that two free phenolic hydroxyl groups in CBD may be required for the potent CYP1A1 inhibition. The substitution of the pentyl side chain of CBD to a propyl group (cannabidivarin, CBDV) did not significantly affect the ability to inactivate CYP1A1 activity. The preincubation of CBD-hydroxyquinone marginally enhanced CYP1A1 inhibition. The kinetic studies demonstrated that olivetol, CBDM, CBDD, CBDV, and orcinol , as well as CBD (k_{inact} = 0.215 min^{-1}), inactivated CYP1A1 activity; their k_{inact} values were 0.154, 0.0638, 0.0643, 0.226, and 0.0353 min^{-1}, respectively. The present study has indicated that the CYP1A1 inhibition of CBD is chemically based primarily on the presence of the methylresorcinol partial structure.

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IN VIVO CANNABIDIOL TREATMENT ENHANCES VASORELAXATION TO ACETYLCHOLINE AND SODIUM NITROPRUSSIDE IN ARTERIES FROM ZUCKER DIABETIC RATS

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In vitro incubation of aortae or femoral arteries with cannabidiol (CBD) enhances endothelial function in a model of type 2 diabetes (Zucker Diabetic Fatty (ZDF) rats) (Stanley & Wheal *et al.* 2013). The aim of the present study was to determine if chronic *in vivo* treatment with CBD would have the same results.

Male ZDF rats (n=12) were treated for 7 days (daily i.p. injection) with either 10mg/kg CBD or vehicle (3 ethanol: 1 Tween 80: 16 Saline). Following the treatment period, the rats were killed by a Schedule 1 method. Thoracic aortae and femoral arteries were dissected into rings and mounted on fixed hooks, and mesenteric arteries were mounted on wires in a multi-channel myograph. The arteries were bathed in warmed (37°C) and gassed (95% O_2/5% CO_2) modified Krebs'-Henseleit solution in the myograph, with the femoral and mesenteric arteries set to a resting tension of 4.9mN, and thoracic aortic rings set to 9.81mN. After an equilibration period, the arteries were contracted with methoxamine, then cumulative concentration-response curves to the endothelium-dependent vasorelaxant acetylcholine (ACh, 1nM-30μM) or the nitric oxide donor sodium nitroprusside (SNP, 1nM-30μM) were constructed. Comparison of arterial vasorelaxation between CBD-treated and vehicle-treated groups was performed using two-way ANOVA, with data written as mean ± SEM, and $P<0.05$ taken as significant unless otherwise stated.

The weights and post mortem blood glucose levels of ZDF rats were not different after CBD- or vehicle-treatment ($P>0.05$, Students' unpaired t-test). ACh and SNP caused concentration-dependent vasorelaxations in all arteries. Treatment with CBD significantly enhanced vasorelaxation to ACh in femoral (R_{max}: CBD-treated ZDF = 72.4 ± 3.2 % (n=6); Vehicle-treated ZDF = 67.9 ± 4.0 % (n=6)) and mesenteric arteries (R_{max} CBD-treated ZDF = 98.8 ± 2.4 % (n=6); Vehicle-treated ZDF = 90.9 ± 4.3 % (n=6)), but not in aortae except when also in the presence of indomethacin or L-NAME. In mesenteric arteries, the enhanced vasorelaxation was abolished in the presence of indomethacin, but not by L-NAME treatment. CBD also enhanced sodium nitroprusside vasorelaxations in mesenteric arteries taken from ZDF rats (R_{max} CBD = 97.2 ± 4.3 % (n=5); Vehicle = 85.8 ± 14.2 % (n=6), $P<0.0001$).

In conclusion, *in vivo* treatment with CBD enhances endothelium-dependent and -independent vasorelaxations in ZDF rats. In mesenteric arteries, the augmented vasorelaxation to ACh is likely via a cyclooxygenase-mediated mechanism.

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Stanley C. & Wheal AJ. *et al.* (2013) Eur J Pharmacol 720: 376–382.

TEMPORARY THERAPEUTIC WINDOW OF CANNABIDIOL FOR NEUROPROTECTION AFTER BRAIN HYPOXIA-ISCHEMIA IN NEWBORN MICE

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Background: cannabidiol (CBD) administered to newborn rodents 15 min after a hypoxic-ischemic (HI) insult leads to significant and long-term sustained neuroprotection.

Aim: to determine the temporary therapeutic window (TTW) of CBD, that is how long CBD administration can be delayed after HI without losing its neuroprotective effect. Such TTW is stablished in 6 h for the standard therapy, hypothermia.

Methods: 9-day old C57BL6 mice underwent a HI insult by being exposed to 10% oxygen for 90 min after electrocoagulation under anaesthesia of the left carotid artery. Then, 0.1 mL of vehicle (ethanol:solutol:saline 2:1:17) (HV, n=25) or CBD (1 mg/kg) was administered s.c. 15 min, or 1, 3, 6, 12 or 24 h after the end of the HI insult (HC0.15 n=10; HC1, n=6; HC3, n=7; HC6, n=6; HC12, n=9; HC24, n=9, respectively). Seven days later pups were killed, transcardially perfused with formaline 10% and their brains removed and stored in formaline. Then, the ipsilateral hemisphere volume (IHV) loss was calculated from T2W sequences of brain MRI scan. Then, the brains were processed for conventional (Nissl) staining to assess necrotic by a neuropathological score (NPS, from 0=no damage to 5=massive tissue damage). In addition, TUNEL staining was done to assess apoptotic damage. Non-HI mice served as controls (SHM, n=15).

Results: post-HI administration of CBD induced a neuroprotective effect as observed in all items (Table). The best results were obtained with CBD being administered just after the end of the HI insult. However, the neuroprotective effects were still significant even when CBS administration was delayed up to 12 h after the HI insult. CBD-induced reduction of brain damage did not achieve statistical significance when post-HI CBD administration was delayed 24 h.

Item	*SHM*	*HV*	*HC0.15*	*HC1*	*HC3*	*HC6*	*HC12*	*HC24*
IHV (%)	0	*13.1(1.3)*	*5.1 (1)*	*6.4(1.8)*	*6.6(1.8)*	*6.4(0.9)*	*7.5(1.2)*	***10.2(1.7)***
NPS (pts)	0.5(0.2)	*2.8(0.2)*	1.0(0.4)	1.2(0.5)	*1.7(0.4)*	***1.8(0.5)***	***1.5(0.2)***	***2.2(0.5)***
TUNEL (n/field)	0.4(0.1)	*12.2(3.8)*	0.5(0.1)	*1.2(0.1)*	*2(0.3)*	***3(0.4)***	***3.4(0.6)***	***6.2(2.8)***

Italic: p<0.05 vs SHM. Underlined: p<0.05 vs. HV. **Bold: p<0.05 vs HC0.15**

Conclusions: CBD shows a TTW longer than usual for hypothermia. TTW for CBD seems to be between 12 and 24 h after the end of the HI insult. Supported by FIS PS09/01900, Health Trust South East Norway and GWCRI091190-2

THE EFFECT OF CBD, CBG, AND THCV ON UWB1.289, A HUMAN NON-HORMONE DEPENDANT OVARIAN CARCINOMA CELL LINE

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Our recent studies have shown that phytocannabinoids can induce cytotoxicity in hormone- dependant ovarian carcinoma cell lines (Daniel et al., 2013, 2012; Javid et al., 2013). The aim of the present study was to investigate the potential anti-tumour activity of cannabinoid extracts on non-hormone dependant ovarian tumour cells. UWB1.289 cells were grown and maintained in media containing equal proportions of RPMI 1640 and MEGM supplemented with 3% fetal bovine serum at 37°C, 5% CO_2 .The cells were plated in 96-well culture plates at a density of $1x10^4$ cells/well and allowed to adhere at 37°C for 24 hours. The following day, various doses of CBD, THCV, a combination of both CBG plus THCV (1nM-100 μM) or vehicle were added to the cells and further incubated for 24, 48, 72 and 96 hours. Then the supernatant was removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added for 4 hours. The ability of cells to form formazan crystals by active mitochondrial respiration was determined by using a Microplate reader after dissolving the crystals in DMSO. Cytotoxicity was expressed as a relative percentage of the absorbance measured at 540 nm in the control and extract-treated cells. Data were presented as the mean±s.e.mean and analysed using ANOVA followed by Dunnet's t-test; n=4.

μM for CBD, 7.11 μM for THCV and 10.48 μM for CBG in 24 hours, IC50 of 5.68 μM for CBD, 7.5 μM for THCV, 8.86 μM for CBG and in 48 hours, IC50 of 8.767 μM for CBD, 10.11 μM for THCV and 10.34 μM for CBG in 72 hours. IC50 of 3.327 μM for CBD, 5.22 μM for THCV and 5.91 μM for CBG in 96 hours. The application of the vehicle alone did not affect the cells at any time. The data showed that the CBD, THCV and CBG seemed to be inducing a stronger anti-tumour activity in UWB1.289 as compared to the IC50 values of the above pure extracts in hormone-dependant carcinoma cell line, A2780 (Daniel, et al., 2012, 2013). Further experiments are required to investigate the receptor type/subtypes involvement and the mechanism of cell death.

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References: Daniel et al., (2012) Br J Pharmacology;10, issue 4. Daniel et al., (2013) Presentation at ICRS, Vancouver, Canada. Javid et al., (2013). Br J Pharmacology. PA2 in press.

REDUCES INFLAMMATORY AIRWAY IMMUNE CELL ACCUMULATION IN A MOUSE MODEL OF ALLERGIC ASTHMA

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Asthma is a chronic airway disorder characterized by inflammatory immune cell accumulation and airway remodeling leading to bronchial hyperresponsiveness and airway obstruction. Acute-challenge animal models such as the ovalbumin (OVA)-induced allergic asthma, and the staphylococcal enterotoxin B (SEB)–induced asthma, differentially reproduce characteristic immune cell infiltration profiles, a key feature of clinical asthma. In the present study we investigated the effects of CBD on immune cell accumulation and lipid production comparing these two mouse models of airway inflammation.

We detected significant immune cell accumulation in the airways obtained by bronchoalveolar lavage (BAL) of OVA-treated mice. OVA-treated animals showed increased immune cell accumulation (mainly eosinophils) in the BAL, which was significantly reduced by CBD treatment. In addition, CBD treatment modified the immune cell population profile in the BAL of OVA-treated mice, significantly reducing the percent of eosinophils. Analysis of selected lipids using LC/MS/MS revealed significant increases in lung tissue levels of PGE2/PGD2 and linoleoyl glycerol following OVA-induced airway inflammation. SEB-treated animals showed increased BAL immune cell accumulation (mainly lymphocytes). CBD treatment significantly modified the immune cell population profile in the BAL of SEB-treated mice, significantly reducing the percent of lymphocytes and eosinophils. Similarly, lipid analysis revealed significant increases in lung tissue levels of PGE2/PGD2 and linoleoyl glycerol. To summarize, CBD reduces inflammatory immune cell accumulation in a mouse model of allergic asthma.

ACTIVATES GLYCOGEN SYNTHASE KINASE IN NEURONS: EVIDENCE FOR MEDIATION BY CHANGES IN CELLULAR REACTIVE OXYGEN SPECIES

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Glycogen synthase kinase-3 (GSK3) is found throughout the CNS and its over-activity has been linked to bipolar disorder and schizophrenia. Epidemiological studies demonstrate a co-morbidity between *cannabis* use and both schizophrenia and bipolar disorder. The overall hypothesis of this project is that one of the most abundant cannabinoids in *cannabis*, cannabidiol (CBD) affects GSK3 activity and that this contributes to the co-morbidity of *cannabis* use and these disorders. The primary mechanism of regulation of GSK3 occurs via deactivating phosphorylation. Signaling cascades that activate protein kinases PKA, PKB (Akt) and PKC converge and phosphorylate GSK3. Phosphorylation is dynamic; removal of the phosphate occurs via constitutively active protein phosphatases (PP-1 and PP-2), so on-going kinase activity is required to maintain GSK3 in a phosphorylated (and inactive) state.

CBD concentration- and time- dependently decreased GSK3β phosphorylation in cultured rat cerebellar granule neurons (CGN). CBD did not affect either PP-1 or PP-2, suggesting that the mechanism of CBD does involve the removal of phosphates. Phosphorylation of CREB, Akt and ERK1/2 were used to assess the activities of PKA, Akt and PKC, respectively. Twenty-four hour treatment with CBD significantly decreased the phosphorylation of CREB (Ser 133), Akt (Ser 473) and ERK1/2. Thus, the site of action of CBD is up-stream of all of the kinases known to regulate GSK3 phosphorylation.

Previous studies have demonstrated increased production of reactive oxygen species (ROS) activates GSK3 in neuronal and non-neuronal cells; and CBD treatment evokes production of ROS in multiple cell types. Thus, we tested the hypothesis that increased ROS production is required for the effects of CBD to activate GSK3. CBD concentration-dependently produces superoxide measured by dihydroethidium (DHE) staining in CGNs. Pretreatment of the CGN with the antioxidant, N-acetyl-L-cysteine (NAC), significantly inhibits superoxide production and antagonizes CBD-decreased phosphorylation of GSK3, CREB, AKT and ERK1/2, suggesting the activation of these kinases by CBD requires superoxide production. We have begun to explore the molecular target for CBD and find that pertussis-toxin sensitive G proteins are required but that cannabinoid, adenosine and serotonergic receptors are not likely to be involved.

Given the important role of GSK3 in development and neuronal morphology, these data suggest that exposure of susceptible individuals to CBD could produce detrimental effects on the brain through increased neuronal ROS and GSK3 activity.

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PHARMACOLOGICAL PROPERTIES OF PURE CANNABICHROMENE AND CANNABICHROMENE BOTANICAL DRUG SUBSTANCE

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Cannabichromene (CBC) is produced by CBC synthase activity at cannabigerol and is one of the four major cannabinoids in *C.sativa*. Despite its relative abundance, the pharmacology of CBC has not been studied extensively. CBC has, however, been shown to be an inhibitor of endocannabinoid uptake and a potent activator of TRPA1 channels. In addition, it has been found to display anti-inflammatory, analgesic, antibiotic, anti-cancer and antifungal effects, and also antidepressant activity and the ability to reduce THC intoxication in rodents (Russo, 2011).

The tetrad test for pure CBC (1, 10 and 100 mg/kg) was conducted in mice, with pure THC (100 mg/kg) and WIN55,212-2 (10 mg/kg) as comparison and reference substances respectively (n = 10/group). Effects were compared to vehicle via one-way ANOVA with Dunnett's *post hoc* test. Pure CBC did not affect the number of crossings or the number of rears in the Activity Meter Test, rectal temperature, foot-licking in the Hot Plate Test, or the latency to remove forelimbs in the Bar Test. The plasma concentration of CBC was 592.7 ± 138.9 ng/ml in mice that were treated with 100 mg/kg pure CBC (n = 3).

The activity of CBC at CB_1 and CB_2 receptors was investigated with adult male MF1 mouse brain tissue or CHO cells transfected with human CB_1 or CB_2 receptors, using [^{3}H] CP55,940 radioligand displacement assays and [^{35}S] GTPγS binding assays. Pure CBC appears to bind to the CB_1 receptor in the micromolar range, but does not activate the receptor. Pure CBC also appears to bind to the CB_2 receptor in the micromolar range and causes a slight activation of this receptor (16.01% above basal at 10 μM).

The ability of CBC and CBC botanical drug substance (CBC BDS) to displace radioligands from a total of 76 receptors, channels and enzymes was evaluated. Only compounds that displaced a radioligand by ≥ 50% at 10 μM were further tested in order to obtain IC_{50} and K_i values. Pure CBC was found to bind to the following: opioid μ (MOP) receptor (70% displacement, IC_{50} = 68 μM, K_i = 28 μM) and Na^+ channel (site 2) (53% displacement, IC_{50} = 19 μM, K_i = 17 μM). CBC BDS was found to bind to the following: sigma-1 (σ_1) receptor (52% displacement, IC_{50} = 16 μM, K_i = 13 μM), Na^+ channel (site 2) (55% displacement, IC_{50} = 8.5 μM, K_i = 7.7 μM), noradrenaline transporter (54% displacement, IC_{50} = 7.5 μM, K_i = 2.5 μM) and L type Ca^{2+} channel (dihydropyridine site; 56% displacement, IC_{50} = 8.2 μM, K_i = 6.1 μM).

In summary, pure CBC did not induce THC-like responses in the tetrad test, an observation that is consistent with the data showing that CBC does not activate CB_1 receptors. Pure CBC appears to bind to, and behave as a very low efficacy partial agonist, at CB_2 receptors. Pure CBC also binds to and inhibits an opioid receptor and Na^+ channel, whilst CBC BDS binds to and inhibits a σ receptor, the noradrenaline transporter and calcium and sodium channels.

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EVALUATION OF THE GENOTOXICITY OF CANNABIDIOLIC ACID

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The toxicity of cannabinoids such as cannabidiol (CBD) and Δ^9-tetrahydrocannabivarin (THCV) are well studied, as both candidate cannabinoids are currently progressing through clinical development. However, the safety and toxicity of pure cannabidiolic acid (CBDA) and the corresponding CBDA botanical drug substance (BDS) is not well known. Therefore, we report the results of studies evaluating the potential genotoxicity of pure CBDA and its corresponding BDS in standard genotoxicity assays.

Two models of genotoxicity, as recommended by world-wide regulatory authorities, were employed: *in vitro* bacterial reverse mutation assay (Ames test) and *in vivo* rat micronucleus test (RMT). The Ames test is a rapid, reliable and economical method of evaluating the mutagenic potential of a test article by measuring genetic activity in one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system. Concentrations of Pure CBDA and its corresponding BDS were tested in the Ames assays up to a maximum recommended concentration of 5000 μg ACTIVE/plate, plus negative (vehicle) and positive controls.

The RMT is a test to evaluate the potential of chemicals to induce micronuclei (chromosome fragment which are unable to attach to spindles and are left behind in the erythocytes when the main nucleus is extruded) during chromosomal and cell replication. In two separate studies, pure CBDA and its corresponding BDS, formulated in ethanol, were administered to Wistar rats up to 500 mg/kg/day for pure CBDA and up to 350 mg ACTIVE/kg/day for CBDA BDS (over 2 days) at the maximum tolerated dose (MTD).

In the Ames test, there were no increases in the revertant colonies of *Salmonella typhi* following treatment with CBDA or CBDA BDS that was considered of biological significance. In the *in vivo* RMT study, the only clinical observations were transient salivation (pure CBDA) or ataxia and decreased activity (CBDA BDS) at the highest doses employed. These signs returned to normal by the third day. There were no increases in micronucleus frequencies following administration of CBDA or CBDA BDS over the concurrent vehicle control for any of the groups receiving the test articles. CBDA levels measured in plasma were high following treatment, thus confirming systemic exposure.

In conclusion, there was no evidence of genotoxicity of pure CBDA and its corresponding BDS noted in the standard Ames and RMT assays when tested up to the maximum doses. .

PHARMACOLOGICAL PROPERTIES OF PURE CANNABIGEROL AND CANNABIGEROL BOTANICAL DRUG SUBSTANCE

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Cannabigerol (CBG) is the direct precursor in cannabis of the cannabinoids cannabidiol (CBD), tetrahydrocannabinol (THC) and cannabichromene (CBC). Pure CBG has been shown to have weak activity at cannabinoid (CB) receptors and other components of the endocannabinoid system. CBG has also been reported to activate TRPA1, TRPV1 and TRPV2 and potently block TRPM8 channels.

The pharmacology of pure CBG and CBG botanical drug substance (BDS) at CB_1 and CB_2 receptors was compared using displacement and GTPγS binding assays. The cannabinoid composition of CBG BDS used in the binding studies was (w/w) CBGV 0.4%, CBG 0.1% & CBC 0.1%. The study found that both CBG and CBG BDS had very little affinity or activity at CB_1 and CB_2 receptors.

In further work, the ability of CBG and CBG BDS to displace radioligands from a range of receptors, channels and enzymes was evaluated. Only compounds that displaced a radioligand by at least 50% at 10μM were further tested in order to obtain IC_{50} and K_i values. Pure CBG was found to bind to the following: L type Ca^{2+} channels (dihydropyridine site: 75% displacement, IC_{50} = 3.8μM, K_i = 1.3μM; diltiazem site: 57% displacement, IC_{50} = 5.1μM, K_i = 4.7μM) and Na^+ channels (site 2; 97% displacement, IC_{50} = 500nM, K_i = 450nM). CBG BDS was found to be active at the following: melatonin MT_3 receptors (53% displacement; IC_{50} = 9.9μM, K_i = 9.7μM), L type Ca^{2+} channel (dihydropyridine site; 74% displacement, IC_{50} = 2.7μM, K_i = 910nM) and Na^+ channels (site 2; 87%, IC_{50} = 580nM, K_i = 520nM). Furthermore, pure CBG was able to inhibit uptake of dopamine and serotonin into mouse brain synaptosomes, with effects observed in the nanomolar range.

In a model of drug discrimination, rats were trained to discriminate between vehicle and THC prior to the main study. In this initial study, the vehicle percentage response was 17.0 ± 9.5%. When THC (1, 3 or 10 mg/kg p.o.) was administered 90 minutes before the test session, the THC-lever responding responses were 54.2 ± 13.5%, 99.5 ± 0.2% and 95.5 ± 2.6%, respectively (n=12/group). In the CBG study, the vehicle response was 18.3 ± 10.3%. When CBG (1, 3 and 10 mg/kg p.o.) was administered 90 minutes before the test session, no increase in THC-lever responding was observed compared with vehicle controls; 0.3 ± 0.2%, 1.0 ± 0.4% and 25.2 ± 12.2% respectively (n=11/CBG group).

CBG and CBG BDS (3-100 mg/kg) were evaluated in an Irwin assay to determine behavioral and physiological changes induced by active compound compared to vehicle. Parameters tested included spontaneous activity in home cage and novel cage, reactivity, restlessness, touch-escape response, positional passivity, grooming, startle response, body posture, abdominal tone, abnormal gait and piloerection. Animals were treated with CBG, CBG BDS or vehicle (n=4/group) and observed at the following time points: 0.17-1hr, 1-2hr, 2-3hr, 4-5hr & 24-25hr. Pure CBG caused a slight reduction in spontaneous activity and reactivity only at the 1-2 hr time point, which was not observed at the following 2-3hr time point. Pure CBG and CBG BDS did not alter any other measured parameters at doses of up to 100 mg/kg compared to vehicle group.

Overall CBG and CBG BDS do not appear to have affinity for CB receptors but do bind to calcium and sodium channels and do inhibit synaptosomal uptake of dopamine and 5-HT. Pure CBG has low potential for abuse at doses up to 10 mg/kg and has minimal effects on behaviour up to 100 mg/kg.

THE PHYTOCANNABINOID, Δ^9-TETRAHYDROCANNABIVARIN, CAN ACT THROUGH 5-HT_{1A} RECEPTORS TO PRODUCE ANTI-PSYCHOTIC EFFECTS

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We have reported previously that three phytocannabinoids, cannabigerol (CBG), cannabidiol (CBD) and cannabidiolic acid (CBDA) can interact with serotoninergic 5-HT_{1A} receptors both *in vitro* and *in vivo* [1, 2, 3, 4]. Here, for the first time, we present evidence that another phytocannabinoid, Δ^9-tetrahydrocannabivarin (THCV) shares the ability of CBD, CBDA and CBG to interact with 5-HT_{1A} receptors *in vitro* and also produces apparent anti-psychotic effects in rats that are, at least in part, 5-HT_{1A} receptor-mediated.

Our *in vitro* experiments consisted of (a) 8-[^{3}H]-OH-DPAT displacement and [^{35}S]GTPγS binding assays performed with MF1 mouse whole brain membranes or Sprague Dawley rat brainstem membranes, and (b) 8-[^{3}H]-OH-DPAT dissociation kinetic assays performed with MF1 mouse whole brain membranes. In our *in vivo* experiments, vehicle- and THCV-treated Sprague Dawley rats were subjected to a spontaneous locomotor activity assay as well as to novel object recognition, social interaction and forced swim tests. These rats were all first pre-treated once daily for 7 days with the non-competitive NMDA receptor antagonist, phencyclidine (PCP), and then subjected to a 7-day withdrawal period.

We found that in both mouse whole brain and rat brainstem membranes (a) like the well-established 5-HT_{1A} receptor agonist, 8-OH-DPAT, THCV potently displaced [^{3}H]-8-OH-DPAT from specific binding sites, (b) the displacement produced by THCV was partial in nature, and (c) THCV resembles CBD and CBDA by displaying both an ability, at 100 nM, to produce significant enhancement of 8-OH-DPAT-induced activation of 5-HT_{1A} receptors and a bell-shaped dose-response curve for the production of this effect. THCV (100 nM) did not significantly affect the rate of dissociation of [^{3}H]-8-OH-DPAT from specific binding sites in mouse brain membranes. In PCP-treated rats, THCV shared the ability of clozapine to (a) antagonize stereotyped behaviour, (b) reduce the amount of time spent immobile in the forced swim test and (c) normalize hyperlocomotor activity, social behaviour and cognitive performance. The 5-HT_{1A} receptor antagonist, WAY100635, abolished the ability of THCV to modify PCP-induced stereotyped and social behaviour and partially reduced the suppressant effect of THCV on PCP-induced cognitive deficiency in the novel object recognition test, suggesting that some of the above apparent anti-psychotic effects of THCV are 5-HT_{1A} receptor mediated. We conclude that THCV shows therapeutic potential for ameliorating some of the negative, cognitive and affective symptoms of schizophrenia.

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THE PHYTOCANNABINOID CANNABIDIVARIN DEMONSTRATES NOTABLE ANTIEPILEPTIC PROPERTIES AND IS A GENUINE CANDIDATE FOR THE TREATMENT OF TEMPORAL LOBE EPILEPSY

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Epilepsy is a chronic and debilitating neurological condition characterised by recurrent seizures; 50 million people worldwide are affected. Temporal lobe epilepsy (TLE) is the most common epilepsy and one of the most refractory to existing medicines; ≥30% of patients are unresponsive to antiepileptic drugs (AEDs) while 20% have limited seizure control. Currently available AEDs have significant side-effects, adversely affecting alertness, motor control and cognition, making more efficacious and better tolerated AEDs an urgent requirement. Growing evidence from pre-clinical research on cannabis-derived compounds, and self-medication by epilepsy patients with cannabis, support the study of phytocannabinoids as new AEDs.

Here, the antiepileptic and tolerability profiles of a non-psychoactive phytocannabinoid, cannabidivarin (CBDV) were assessed. The repeated low dose lithium pilocarpine model of TLE was used to induce spontaneous recurrent seizures in male Wistar rats. Animals were treated with 200 mg/kg/day CBDV for 12 weeks and effects on seizure frequency and severity were behaviourally assessed daily for 3 weeks. CBDV significantly reduced both severe seizure incidence and seizure index (seizure severity x seizure frequency). Tolerability was assessed using static beam, grip strength and inclined screen tasks, where CBDV produced no motor function deficits or neurotoxicity. CBDV also attenuated motor function deficits observed in epileptic animals, further supporting its antiepileptic effects. Finally, electrocorticography (ECoG) recordings were used to assess the effects of acute CBDV administration (200 mg/kg) on baseline (non-seizure) neuronal activity of epileptic and non-epileptic animals. CBDV exerted few effects upon the power spectra of signals recorded from hippocampus and parietal cortex, further supporting CBDV's tolerability.

In conclusion, CBDV has significant antiepileptic properties and is extremely well tolerated, supporting CBDV as a genuine candidate for the treatment of human TLE.

PHARMACOLOGICAL PROPERTIES, PHARMACOKINETICS AND BIOAVAILABILITY OF PURE CANNABIDIOLIC ACID AND CANNABIDIOLIC ACID BOTANICAL DRUG SUBSTANCE

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Cannabidiolic acid (CBDA) has weak activity at CB_1 and CB_2 receptors, and components of the endocannabinoid system. CBDA has also been reported to desensitise TRPA1, TRPM8 and TRPV4 channels.

Here, the ability of pure CBDA and CBDA botanical drug substance (BDS) to displace radioligands from a range of receptors, channels and enzymes was evaluated; these were non-cannabinoid and non-TRP targets. Only compounds that displaced a radioligand at 10 μM were further investigated in order to obtain IC_{50} and K_i. Pure CBDA was found to bind to 5-HT_{5a} (IC_{50} 15 μM, K_i 750 nM). CBDA BDS was unable to displace any of the standard ligands.

In a separate investigation, plasma and brain samples were obtained from male Wistar rats to provide information on the concentrations of pure CBDA and CBDA BDS, derive pharmacokinetic (PK) data and evaluate absolute/relative cannabinoid bioavailabilities where possible. Pure CBDA was dosed by the intravenous (IV), oral (PO) and intraperitoneal (IP) routes and CBDA BDS by the PO and IP routes only. Pure CBDA and CBDA BDS were administered in a 1:1:18 ratio of ethanol, cremophor EL and physiological saline (0.9 % NaCl) as either a single bolus dose or repeatedly over a five day period at intervals of approximately 24 hours. In this study, the systemic bioavailability of CBDA following a single administration, ranked highest to lowest was as follows: pure CBDA, IP, 10 mg/kg (80 %); CBDA BDS, IP, 10 mg/kg (74 %*); pure CBDA, IP, 1 mg/kg (62 %); pure CBDA, IP, 0.1 mg/kg (36 %); CBDA BDS, PO, 10 mg/kg (36 %*); pure CBDA, PO, 10 mg/kg (19 %). Whereas, following repeated daily administration, the systemic bioavailability of CBDA, ranked highest to lowest was: pure CBDA, IP, 10 mg/kg (145 %); CBDA BDS, IP, 10 mg/kg (127 %*); pure CBDA, IP, 1 mg/kg (70 %); CBDA BDS, PO, 10 mg/kg (60 %*); pure CBDA, PO, 10 mg/kg (39 %); pure CBDA, IP, 0.1 mg/kg (7 %). The relative (*) and absolute oral bioavailability values were calculated using the IV dosed pure CBDA area under the curve (AUC_{0-t}) data, demonstrating high systemic exposure via the IP and PO routes.

In conclusion, neither pure CBDA nor CBDA BDS (at 10 μM) bind to many other traditional receptor targets with any great affinity in this screen. Also, PK and bioavailability data demonstrate good bioavailability for repeated and single doses of pure CBDA and CBDA BDS at 10 mg/kg.

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THE NON-PSYCHOTROPIC PLANT CANNABINOIDS, CANNABIDIVARIN (CBDV) AND CANNABIDIOL (CBD), ACTIVATE AND DESENSITIZE TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1) CHANNELS *IN VITRO*: POTENTIAL FOR THE TREATMENT OF NEURONAL HYPEREXCITABILITY

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Epilepsy is the most common neurological disorder in humans, with over 50 million people worldwide affected. Recent evidence suggests that the transient receptor potential cation channel subfamily V member 1 (TRPV1) may contribute to the onset and progression of some forms of epilepsy. Since the two non-psychotropic cannabinoids cannabidivarin (CBDV) and cannabidiol (CBD) exert anticonvulsant activity *in vivo* and produce TRPV1-mediated intracellular calcium elevation *in vitro*, we evaluated the effects of these two natural products on TRPV1 channel activation and desensitization and in an *in vitro* model of epileptiform activity.

Patch clamp analysis in transfected HEK293 cells demonstrated that CBD and CBDV dose-dependently (1-30 μM) activate and rapidly desensitize rat TRPV1, as well as rat TRP channels of subfamily V type 2 (TRPV2) and subfamily A type 1 (TRPA1). TRPV1 and TRPV2 transcripts were shown to be expressed in rat hippocampal slices by quantitative PCR, whereas TRPA1 was at the limit of detection.

When tested on epileptiform neuronal spike activity in such slices exposed to a Mg^{2+}-free solution using multi electrode arrays (MEAs), CBDV (10 μM) reduced both epileptiform burst amplitude and duration. The prototypical TRPV1 agonist, capsaicin (10 μM), produced similar, although not identical effects. CBDV effects were not always sensitive to IRTX (1 μM), a selective TRPV1 antagonist. These data suggest that CBDV anti-epileptiform effects in the Mg^{2+}-free model, are not uniquely mediated via activation of TRPV1.

As assessed by means of western blot analyses using a polyclonal antibody against its phosphorylated form, TRPV1 was strongly phosphorylated, and hence likely sensitized by capsaicin (10 μM) and CBDV (10 μM), but not IRTX (1 μM), in control hippocampal slices. An increase of TRPV1 phosphorylation was observed in hippocampal slices exposed to Mg^{2+} free solution. Interestingly, in this experimental condition, both capsaicin (10 μM) and CBDV (10 μM) caused instead dephosphorylation of TRPV1, consistent with its possible desensitization.

We propose that the fast desensitization of tonically activated TRPV1 determined by agonists and CBD or CBDV, could open a new therapeutic opportunity to treat neurological disorders caused by an excess of neuronal activity such as epilepsy, and that CBDV effects on TRP channels should be next assessed also in the context of *in vivo* models of epilepsy. **Acknowledgment**: Funded by GW Pharmaceuticals.

INFLUENCE OF HEPATIC METABOLISM OF Δ^9-TETRAHYDROCANNABIVARIN (THCV) AND CANNABIDIOL (CBD) ON THEIR ANTI-STEATOTIC ACTIONS *IN VITRO*

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The liver plays key roles in regulating total body energy homeostasis and its ability to do so is greatly affected by the occurrence of pathological conditions such as hepatosteatosis or non-alcoholic fatty liver disease (NAFLD), which contributes to hepatic insulin resistance, metabolic syndrome and potentially end-stage liver disease-related mortality. Triglyceride (TG) accumulation in hepatocytes of steatotic livers results from the incorporation of adipose-derived plasma free fatty acids as well as de novo biosynthesis. Recent reports indicate that the cannabinoids THCV and CBD can protect the liver in a various models of hepatosteatosis [1] [2]. Given that THCV and CBD are metabolized within the liver, resulting in the formation of the metabolites 11-OH-THCV and 7-OH-CBD (among others), respectively, we tested the ability of purified THCV, CBD, 11-OH-THCV and 7-OH-CBD to modulate intracellular TG levels directly in an *in vitro* hepatosteatosis model in hepatocytes, as well as in mature adipocytes. Additionally, given that TCHV is also able to inhibit the development of insulin-insensitivity in HHL5 hepatocytes [1], we determined if 11-OH-THCV has the same effect.

TG accumulation was induced in the HHL5 cell line of human hepatocytes by exposure to 100 μM oleic acid for 24 hours. Mature adipoctyes were derived from mouse 3T3-L1 cells. TG levels were quantified in each through the use us a commercially available Nile Red preparation to determine the effects of exposure to the cannabinoids at various concentrations. Assessment of insulin sensitivity was performed through Western blot analysis of insulin-induced AKT activation (phosphorylation) in HHL5 cells exposed to 200 μM palmitic acid to induce insulin resistance.

As shown previously [3], our results indicate that both THCV (5-20 μM) and CBD (2.5-20 μM) are able to markedly decrease intracellular TG levels in HHL5 cells exposed to 100 μM oleic acid for 24 hours. We found that while 11-OH-THCV (up to 20 μM) had no lipid lowering capability, the reverse was true for 7-OH-CBD (2.5-20 μM), which in fact was more potent than CBD at the higher concentrations tested. In mature 3T3-L1 adipocytes, as in hepatocytes, THCV (10 μM), CBD (5, 10 μM) and 7-OH-CBD (2.5, 5, 10 μM) all decreased intracellular TG stores upon 48 hr exposure, while, again, 11-OH-THCV (up to 10 μM) had no effect. Finally, in insulin-resistant HHL5 cells, while THCV (3 μM) re-sensitized cells to insulin, low concentrations of 11-OH-THCV (0.3, 1 μM) tended to slightly enhance insulin resistance and no effect was observed at the highest concentration (3 μM) tested.

These data highlight the differences that metabolism of THCV and CBD has on their lipid-lowering capacity and provide in vitro evidence that THCV, CBD and 7-OH-CBD hold promise as potential therapies for fatty liver disease and diabetes. The potential molecular targets through which these compounds exert their actions in hepatocytes do not include cannabinoid receptors and are currently under investigation.

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ADDITIVE NEUROPROTECTIVE EFFECT OF CANNABIDIOL AND HYPOTHERMIA IN HYPOXIC-ISCHEMIC PIGLETS

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Background: The proportion of newborns beneficed from hypothermia (HT) after brain hypoxia-ischemia (HI) is limited. Thus, looking for synergistic therapies is warranted. Cannabidiol (CBD) has shown neuroprotective effects in animal models of newborn HI brain damage.

Objective: To test the possible additive neuroprotective effects of CBD and HT.

Design/Methods: Sedated and ventilated piglets (1-2 day-old) underwent HI brain damage (hypoxia -FiO2 10%- + bilateral carotid artery compression for 30 min). Then, normothermic (NT) piglets were maintained at 37-38 C using a warmed air blanket; HT piglets were cooled by a cool water mattress to 33-34 °C. Thirty min after HI piglets received i.v. vehicle (VEH) or CBD 1 mg/kg. Six hours after HI brains were obtained for histological studies quantifying the number of neurons (Nissl) and astrocytes (GFAP) in parietal cortex, for proton magnetic resonance spectroscopy (H-MRS) to quantify Lac/NAA (neuronal damage), Glu/NAA (excitotoxicity) and GSH/Cr (oxidative stress) ratios and for Westerblot studies quantifying protein nitrosylation (Oxyblot, oxidative stress) and caspase-3 (apoptosis) and TNFα (inflammation) expression. Similarly studied animals without HI insult served as controls (SHM).

Results: Neuroprotection was CBD+HT>VEH+HT=CBD+NT>VEH+NT in terms of reduction of neuronal and astroglial death, apoptosis and Lac/NAA ratio increase (Table). Either CBD or HT similarly modulated HI-induced excitotoxicity, inflammation and oxidative stress, with both therapies together showing additive effects.

Parameter	SHM+NT	VEH+NT	CBD+NT	SHM+HT	VEH+HT	CBD+HT
Histology						
Necrotic neurons (%)	4.7(1.3)	*25.5(4.8)*	***11.8(3.2)***	1(0.1)	*13.6(1.3)*	**3.7(0.9)**
Astrocytes (n)	33(3.2)	31.1(2.2)	***39.4(3.8)***	33.4(1.3)	*23.8(0.6)*	**34.3(1.5)**
H-MRS						
Lac/NAA	2.4(0.2)	*6.6(2.3)*	**2.9(0.3)**	1.4(0.1)	*2.3(0.1)*	**1.6(0.1)**
Glu/NAA	0.51(.02)	*0.61(0.04)*	**0.49(0.03)**	0.42(0.02)	*0.45(0.02)*	**0.39(0.01)**
GSH/Cr	0.17(0.01)	*0.11(0.01)*	**0.17(0.01)**	0.16(0.01)	*0.14(0.01)*	**0.16(0.01)**
Westernblot						
OxyBlot	1.9(0.2)	*2.9(0.3)*	**2.2(0.2)**	1.7(0.1)	*2.3(0.2)*	**1.9(0.1)**
Caspase 3	0.19(0.01)	*0.35(0.02)*	***0.25(0.02)***	0.09(0.01)	*0.15(0.01)*	**0.11(0.01)**
TNFα	0.07(0.02)	*0.17(0.01)*	***0.14(0.01)***	0.03(0.02)	*0.10(0.01)*	**0.08(0.01)**

Mean(SEM). Lac: lactate. NAA: n-acylaspartate. Glu: glutamate. GSH: reduced glutathione. (*Italic*) P<0.05 vs SHM; **(Bold)** p<0.05 vs VEH

Conclusions: CBD is at least as efficient as neuroprotectant as HT, both therapies showing additive neuroprotective effects.

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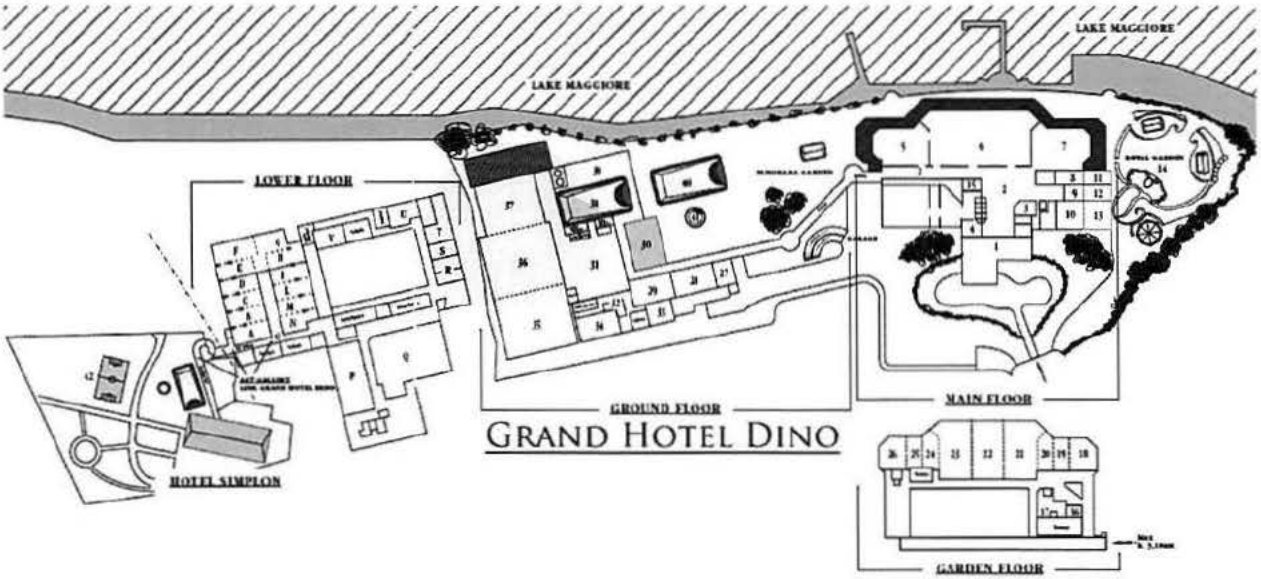
Map of Zacchera Hotels, Baveno, Italy



Baveno
ICRS2014

MAIN FLOOR

1 . Entrance
2 . Lobby
3 . Reception
4 . Internet corner
5 . Restaurant Panorama
6 . Restaurant 3 isole
7 . Main Bar
8 . Executive (TV Room)
9 . VIP
10 . President
11 . Royal A
12 . Royal B
13 . Royal C
14 . Royal Garden
15 . Luggage Room

GARDEN FLOOR

16 . Technical Room
17 . Cloakroom
18 . Titano
19 . Saturno
20 . Nettuno
21 . Venere
22 . Marte
23 . Giove
24 . Minerva
25 . Plutone
26 . Urano

GROUND FLOOR

27 . Camino
28 . Birreria
29 . Rustico
30 . Winter Garden
31 . Hall & Bar
32 . Business Office
33 . Rosa
34 . Galassia
35 . Carlo 1
36 . Carlo 2
37 . Carlo 3
38 . Swimming Pool
39 . Relax Area & Jacuzzi
40 . Outside swimming Pool
41 . Children swimming Pool
42 . Soccer

LOWER FLOOR

Planetario A
Planetario B
Planetario C
Planetario D
Planetario E
Planetario F
Planetario G
Planetario H
Planetario I
Planetario L
Planetario M
Planetario N
O . Gallery Hotel Simplon
P . Play room
Q . Fitness room
R . Massage room
S . Solarium
T . Turkish bath
U . Sauna Lady
V . Sauna Gentlemen

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ANNE LYNN MORGAN, M.D.

860 US HIGHWAY 1, SUITE 203 A

NORTH PALM BEACH, FL. 33408

954-592-0700

EDUCATION:

1985-1988	PGY-1, 2, & 3 in Family Practice Conemaugh Valley Memorial Hospital Johnstown, Pennsylvania
1983-1984	PGY-1 in Obstetrics and Gynecology Orlando Regional Medical Center Orlando, Florida
1981	Doctor in Medicine C.E.T.E.C. University Santo Domingo, Dominican Republic
1979-1981	Medical Clerkships: Medicine: Ravenswood Hospital Medical Center, Chicago, Illinois Surgery: MacNeal Memorial Hospital, Berwyn, Illinois Psychiatry: MacNeal Memorial Hospital, Berwyn, Illinois Obstetrics & Gynecology: MacNeal Memorial Hospital, Berwyn, Illinois Pediatrics: Children's Hospital of Michigan, Detroit, Michigan Ophthalmology: Kresge Eye Institute, Detroit, Michigan Pathology: Miami Heart Institute, Miami Beach, Florida
1978	Bachelor of Science University of Kentucky Lexington, Kentucky

BOARD CERTIFICATION:

July 1988	American Board of Family Practice

July 1994 Recertification

July 2000 Recertification

May 2014 Society of Cannabis Clinicians : Board Eligible

May 2014 American Academy of Cannabinoid Medicine: Board Eligible

June 2014 International Cannabis Research Society

MEDICAL SOCIETIES:
Past & Present*

American Medical Association
American Academy of Family Physicians
Florida Academy of Family Practice
Southern Medical Association
Broward County Medical Association
Palm Beach County Medical Association *
International Cannabis Research Society *

Hospital Privileges: (Former)
Miami Heart Institute, Miami Beach, Florida
Mount Sinai Medical Center, Miami Beach, Florida
Hollywood Medical Center, Hollywood, Florida
Memorial Hospital, Hollywood, Florida
Cleveland Clinic, Fort Lauderdale, Florida
North Ridge Medical Center, Fort Lauderdale, Florida
Holy Cross Hospital, Fort Lauderdale, Florida

Specialized Training: Value Health Sciences (1993)

LICENSURE:	FLORIDA	ME0049829	Active
	PENNSYLVANIA	MD 040410L	Active

PEER REVIEW: STATE OF FLORIDA, BOARD OF MEDICINE
VOLUNTEER EXPERT WITNESS REVIEWER

Examination of investigative files and provision of professional opinion.

PROFESSIONAL EXPERIENCE:

2007- Present Private Practice

Palm Beach County, Florida

2005-2007 METROPOLITAN HEALTHCARE, INC. West Palm Beach, Florida

MEDICAL DIRECTOR, South Florida Region

Establishment of Practices for Platinum Care Programs for Advanced Medical Homes: South Florida and Treasure Coast Medical Offices

Physician Advisor: Physicians in Wholly Owned Centers, Admitting Physicians and Consultants

MRA Assessment Tool: First Medical Risk Assessment Program for HMO reimbursements

Management, Training and Recruitment of Physicians and Medical Staff.

Peer Review and Medical Advisory

Establishment of Functional Triage System for Practices

Creation of Patient Satisfaction Surveys: real time and correlative data.

Resource Management: evaluation and financial analysis of Monthly and Quarterly Reports with recommendation to Chief Medical Officer,

President and Board of Directors

Coordinator, PCP Quarterly Meetings: Responsible for content and agenda.

Oversight of Primary Care Physicians in Network

Sales Force Focus Forum: Interdisciplinary member responsible for recruiting new HMO members with resultant increase in membership, South Florida.

1998-2005 Private Practice, Fort Lauderdale, Florida

Consultant, Managed Care Case Review

1997-1998 HUMANA, INC., Miramar, Florida

MEDICAL DIRECTOR

Medicare, H.M.O., Commercial P.P.O., P.O.S.

Utilization Review: pre-procedure, concurrent, and

prospective.
Determination and recommendation of Expedited Appeals.
Educational presentations to physicians and providers off-site.
Quarterly Meetings
Secondary review of H.M.O. Denial of Services
Creation and implementation of Quality Improvement Plans for Humana Medical Centers.

Review and analysis of Medical Facility Performance profiles with recommendation for appropriate action.
Medical resource for Patient Care Coordinators, Medical Case Managers and Pre-Certification Department.
Annual review and update of H.I.V. Case Coordinator and respective health care providers.
Revision, organization, and streamlining of the Medical Director - Patient Care Coordinator referral process.
Medical Director review of skilled nursing facility admissions, including appropriateness of admission, level of care, length of stay and coordination of discharge planning.
Creation and implementation of streamlined telephonic review program for University of Miami - Jackson Memorial Hospital admissions : training of admitting panel physicians.
Designated Humana spokesperson upon request by member's and member's family for contact with a Humana Medical Director.
Nursing Education Programs
Retrospective review of high-cost and out-of-area claims
Humana spokesperson to Hospital Administrators
Quality Management Case Referral Review
Financial performance and cost analysis of Providers and Humana Centers.
COMMITTEES:
Peer Review
Grievance and Appeals

Quality Management
Utilization Management
Credentialing

1995-1997 Private Practice

1993-1995 METLIFE HEALTH CARE NETWORK
UNITED HEALTHCARE, INC., Miami, Florida
MEDICAL DIRECTOR

Participated in negotiations and marketing presentations with prospective companies for employee benefit plans.
Participated in review of prospective vendors.
Directed and managed MetLife's Dade County Public Schools' Health Maintenance Plan totaling 40,000 enrollees.
Directed and Managed MetLife's point of Service Plans of South Florida totaling 75,000 enrollees.
Directed and managed MetLife's Managed Care Division comprised of thirty nurses, referral processors, and support staff.
Revision and streamlining of the Provider credentialing process.
Conducted telephonic follow-up to the Providers after on-site audits and medical record reviews.
Authored article and guideline for the MetLife Medical Reference Manual: Human Growth Hormone
Claims review, both initial and appeals for Dade County Public schools, point of service cases and Metlife's South Florida H.M.O.
Medical necessity determination of organ transplant cases.

Development and weekly presentation of nursing Continuing Education Programs.
COMMITTEES:
Claims Advisory
Technology Review
Medical Advisory- Chairperson
Peer Review- Chairperson

	Credentialing- Chairperson Specialty Advisory Board- Chairperson Utilization Management Quality Improvement- Chairperson Grievance and Appeals
1991-1993	MEDICAL FOUNDATION, Miami, Florida PHYSICIAN ADVISOR MEDICAL FOUNDATION : Elected Member BOARD OF DIRECTORS, 1993 Utilization Review: pre-procedure, concurrent, and prospective. Senior Advisor: Case Management Nursing Education Medical necessity review of Dade County Public Schools Hardship Leave Program M.R.S. for medical necessity, Value Health Sciences Management of Second Surgery Opinion program Senior Advisor: Ryan White Grant Program
1988- 1993	Private Practice Dade County, Florida Utilization Review Committee, Miami Heart Institute Guest Speaker, Nova Southeastern University, College of Osteopathic Medicine- Graduation Ceremony (1993, 1994), " Utilization Review & Q.I. In Your Future".
1982-1983	Private Duty R.N., Miami Heart Institute, Miami Beach, Florida
LANGUAGES:	English, Conversational Spanish

Bist, Kevin

From: ANNE <bestofalltherest@comcast.net>
Sent: Thursday, January 08, 2015 1:55 AM
To: Nelson, Patricia A
Subject: Good News

Follow Up Flag: Follow up
Flag Status: Flagged

Hi, Patti,
I am sending you good news. Dr. Sanchez Ramos has agreed to act as resource person for your office. He has the largest neuromuscular disorder group of patients in Florida and is a world renowned expert in his field.
I am sending this along in support to my request to be a member of the Rule Making Committee. As a former Medical Director, this os what I do...bring excellent physicians together in a collaborative effort to achieve our stated goals.
If I am not selected, Dr. Sanchez Ramos' offer, of course still is to be there for your Department.
See you in February!
Anne Lynn Morgan, M.D.

From: "Juan Sanchez-Ramos"
To: "ANNE"
Sent: Wednesday, January 7, 2015 9:25:43 PM
Subject: Re: Help

Anne,
I can serve as a resource on the medical use of cannabis, in particular its use in various formulations for neurologic diseases.
—Juan

Juan Sanchez-Ramos, PhD, MD
Ellis Professor of Neurology
University of South Florida

From: ANNE <bestofalltherest@comcast.net>
Date: Wednesday, January 7, 2015 at 11:30 AM
To: Juan <JSRAMOS@HEALTH.USF.EDU>
Subject: Help

Hi, Juan.
I am writing to ask if you would agree to be a "Resource" for the DOH, Office of Compassionate Care?
It would be great if you would agree so that if they have any questions in your field...that they could contact you.
Please let me know.

Thanks,
Anne Morgan, M.D.

Bist, Kevin

From: martin.dix@akerman.com
Sent: Thursday, January 08, 2015 9:10 AM
To: Nelson, Patricia A
Subject: RE: Low-THC Cannabis Rulemaking

Follow Up Flag: Follow up
Flag Status: Flagged

Hi Patricia:
Have you selected the attorney member yet or have someone in mind for the Committee? If not, I am interested in applying and would appreciate knowing the process.
Thank you.
Marty

Martin R. Dix
Partner
Board Certified in Health Law
Akerman LLP | Suite 1200 | 106 East College Avenue | Tallahassee, FL 32301
Main: 850.224.9634 | Fax: 850.222.0103
martin.dix@akerman.com

vCard | Bio | LinkedIn | Blog

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From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Monday, January 05, 2015 5:11 PM
To: DL 64-4 Interested Parties; Dunn, Nathan P
Subject: Low-THC Cannabis Rulemaking

Dear Interested Parties,

Please see the attached Notice of Negotiated Rulemaking (on page 1 of the attached document) scheduled for February 4 and 5, 2015, in Tallahassee.

Sincerely,
Patty

Patricia Nelson

Director
Office of Compassionate Use

Bist, Kevin

From: Nelson, Patricia A
Sent: Thursday, January 08, 2015 9:16 AM
To: Martin.dix@akerman.com
Subject: RE: Low-THC Cannabis Rulemaking

Follow Up Flag: Follow up
Flag Status: Flagged

The only process is to send me an email expressing your interest, including an explanation as to what you bring to the table, so to speak.

From: martin.dix@akerman.com [mailto:martin.dix@akerman.com]
Sent: Thursday, January 08, 2015 9:10 AM
To: Nelson, Patricia A
Subject: RE: Low-THC Cannabis Rulemaking

Hi Patricia:
Have you selected the attorney member yet or have someone in mind for the Committee? If not, I am interested in applying and would appreciate knowing the process.
Thank you.
Marty

Martin R. Dix
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Subject: Low-THC Cannabis Rulemaking

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Sincerely,
Patty

Patricia Nelson
Director
Office of Compassionate Use

Bist, Kevin

From: martin.dix@akerman.com
Sent: Thursday, January 08, 2015 10:22 AM
To: Nelson, Patricia A
Subject: RE: Low-THC Cannabis Rulemaking

Follow Up Flag: Follow up
Flag Status: Flagged

Thanks, Patricia.
I am interested in being considered for the attorney position on the Committee. I

- have practiced regulatory/administrative law since 1985;
- am board certified in health law;
- have a background in pharmacy law and am knowledgeable about DEA's regulatory processes as well as HIPAA and patient confidentiality issues;
- have participated in rule hearings before the Department of Health, Board of Pharmacy, AHCA, DBPR, DEP, DNR and other agencies and was the author of the DNR Myakka River Wild and Scenic River Rule (under contract with then DNR);
- have assisted clients with licensing applications before the Board of Pharmacy, Department of Health, AHCA, DBPR, Dept. of Agriculture, DEP, DNR, DEA, FDA, Army Corps, and other agencies;
- have challenged and defended agency decisions on numerous occasions including proposed and existing rule challenges;
- assisted in representing the Tallahassee-Leon County Planning Commission for 14 years so am knowledgeable on Sunshine Law, Public Records Act, Ethics in Government, etc.; and
- attended several of the prior rule hearings;

I can provide references if needed. My full bio is linked here: http://www.akerman.com/bios/bio.asp?id=704&name=Dix

Thank you for your consideration.
Marty Dix
Martin R. Dix
Partner
Board Certified in Health Law
Akerman LLP | Suite 1200 | 106 East College Avenue | Tallahassee, FL 32301
Main: 850.224.9634 | Fax: 850.222.0103
martin.dix@akerman.com

From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Thursday, January 08, 2015 9:16 AM
To: Dix, Marty (Ptnr-Tlh)
Subject: RE: Low-THC Cannabis Rulemaking

The only process is to send me an email expressing your interest, including an explanation as to what you bring to the table, so to speak.

From: martin.dix@akerman.com [mailto:martin.dix@akerman.com]
Sent: Thursday, January 08, 2015 9:10 AM

To: Nelson, Patricia A
Subject: RE: Low-THC Cannabis Rulemaking

Hi Patricia:
Have you selected the attorney member yet or have someone in mind for the Committee? If not, I am interested in applying and would appreciate knowing the process.
Thank you.
Marty

Martin R. Dix
Partner
Board Certified in Health Law
Akerman LLP | Suite 1200 | 106 East College Avenue | Tallahassee, FL 32301
Main: 850.224.9634 | Fax: 850.222.0103
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From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Monday, January 05, 2015 5:11 PM
To: DL 64-4 Interested Parties; Dunn, Nathan P
Subject: Low-THC Cannabis Rulemaking

Dear Interested Parties,

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Sincerely,
Patty

Patricia Nelson
Director
Office of Compassionate Use

Bist, Kevin

From:	zzzz Feedback, Compassionate Use
Sent:	Thursday, January 08, 2015 10:44 AM
To:	Nelson, Patricia A
Subject:	FW: Tree-King Comments on SB1030 Rule Rewrite
Attachments:	Dr. William Clark 12-19-14.pdf
Follow Up Flag:	Follow up
Flag Status:	Flagged

Patty,

This comes from our zzz feedback mailbox.

Kevin

From: Robert Buck [mailto:email@attorneybuck.com]
Sent: Monday, January 05, 2015 3:14 PM
To: Nelson, Patricia A; Patricia.nelson@eog.myflorida.com
Cc: zzzz Feedback, Compassionate Use
Subject: Re: Tree-King Comments on SB1030 Rule Rewrite

Ms. Nelson,

It appears that the statement from Dr. William Clark did not attach to my prior email. I apologize for the mistake. Please find attached a duplicate copy.

Please consider Dr. Clark's experience in your search for an expert panel. We consider his knowledge in chemistry and out-of-state rule making to be unparalleled.

Regards

--
Robert L. Buck, Esq., P.A.
PO Box 15146., Brooksville, FL 34604
352-584-2062 Phone
352-686-7455 Fax
email@attorneybuck.com
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Bist, Kevin

From: Bist, Kevin
Sent: Thursday, January 08, 2015 11:43 AM
To: ANNE; Nelson, Patricia A
Subject: RE: Hello from Anne Morgan, M.D

Dear Dr. Morgan,

Thank you for the information you provided in your attachments. I look forward to speaking with you soon.

Kind Regards,

Kevin

From: ANNE [mailto:bestofalltherest@comcast.net]
Sent: Wednesday, January 07, 2015 6:22 PM
To: Nelson, Patricia A
Cc: Bist, Kevin
Subject: Hello from Anne Morgan, M.D

Patty,

It was a pleasure to meet you at the Orlando workshop on Dec. 30, which I found very informative.

I am writing to ask your consideration to become a member of The Negotiated Rulemaking Committee.

I feel that I have a lot to bring to the table, both in medical management experience, my willingness to be open and honest about marijuana as a medicine, agree to negotiate in good faith and my goal of doing whatever I can as a physician to make sure that "We do it right in Florida."

At this early point in the history of cannabinoid medicine, very few physicians in Florida have the degree of interest and the background in cannabis education that I now have. I sincerely believe that my interests as a clinician and scientist is not represented on the Committee at present.

I am a Family Practitioner with more than 25 years of experience and no blemishes on my background. For quite a few years, I did pro bono work on the Peer Review Board for the Board Of Medicine.

A year ago, I became intrigued with the potential for cannabis as medicine, and have since spent a great deal of time getting up to speed.

I have applied for Board Certification with the American Academy Of Cannabinoid Medicine. In that effort, I have already passed the oral and written exam and will need to practice two years of cannabinoid medicine in order to become Board Certified in the field.

I attended the Patients Out of Time conference in Portland, Oregon last April, 2014.

The conference will be coming to the West Palm Beach Convention Center this Memorial Weekend and I will be playing a supporting role.

I expect to take the FMA CME course, completing that aspect by my application.

When at the Portland Conference, I met several scientists who invited me to become a member of the International Cannabinoid Research Society. I accepted their invitation and attended their 24th Annual Conference in Baveno, Italy last June/July. I learned that there is so much pertinent information that has not reached me or my colleagues in practice, especially in Florida.

I am taking this opportunity to send you the PDF file of the ICRS course syllabus. Several of the authors have agreed to act as "resource" persons when I asked them last June/July in advance of the upcoming Amendment 2, knowing that we would need help in Florida. I will do whatever I can to reach out to them in an effort to obtain as much medical/clinical information that we need to apply this law safely, efficiently, properly and fairly.

As a result, I was able to persuade (along with some others) the founders of Patients Out Of Time to bring their next conference to West Palm Beach this coming May.

I have asked Greg Gerdeman, Ph.D, if he would agree to be a resource person for the DOH and he has graciously agreed. He is a world renowned expert in his field and we are very fortunate to have him at Eckerd College.

I am taking this opportunity to send you a copy of my long-form C.V., since most Medical Management positions want to know exactly what departments and programs an applicant has created and developed.

I do state that I am a member of the Palm Beach County Medical Society "Medical Marijuana Task Force" which does not give endorsements of medical cannabis, but wishes to work to keep this properly, safely and efficiently managed in the State of Florida. A letter to that effect will be sent to you directly very soon.

Please feel free to call me if I can be of any assistance: 954-592-0700.

I wish you all the best in your endeavor,

I will see you at the meeting on February 4th.

Respectfully submitted,

Anne Lynn Morgan, M.D.

860 US Highway 1

Suite 203 A

North Palm Beach, Fl 33408

Bist, Kevin

From:	Erik Williams <erik.williams@beMINDFUL.today>
Sent:	Thursday, January 08, 2015 11:49 AM
To:	Nelson, Patricia A
Cc:	Jimeaton53@aol.com
Subject:	Request to serve - Proposed Rules Committee
Attachments:	FL_Request_ErikWilliams.pdf
Follow Up Flag:	Follow up
Flag Status:	Flagged

Dear Director Nelson,

Attached please find letter seeking appointment to the proposed rule making committee.

Thank you for your consideration and I look forward to hearing from you soon.

Best,

E

Erik Williams
Director of Government and Public Affairs
Mindful

860.805.3243
www.beMiNDFUL.today

MINDFUL

January 7, 2015

Patricia Nelson
Director
Office of Compassionate Use
2585 Merchants Row Boulevard , Bin # A
Tallahassee , FL 32399 -3265

and via email: patricia.nelson@flhealth.gov

Subject: Rulemaking Committee

Dear Director Nelson,

I am writing to formally request to be a Member of the Compassionate Use rule making committee. Thank you for your consideration of my qualifications outlined below. I look forward to working with you in the near future.

Specifically, I can be of the most help under the following:

7. An individual with demonstrated experience establishing or navigating regulatory structures for cannabis in other jurisdictions;

As Director of Government and Public Affairs for Denver-based Mindful and Partner with Marimind Consulting, I have in-depth working knowledge and demonstrated experience both establishing and working in different regulatory structures at various levels of government.

Most importantly, I am uniquely qualified in the United States in that my government and regulatory experience is coupled with actual successful cannabis businesses that operate in several states under several structures covering everything from cultivation and processing to ancillary products and retail. At the same time, I have impeccable bona fide relationships with patients and activists communities. Before entering the cannabis business, I spent years doing pro bono work through my political consulting firm, was Executive Director of Connecticut NORML (where I passed the first medical marijuana

program through a legislature), advised dozens of activist and patient groups, and was named the 2012 Outstanding Activist of the Year.

In addition to helping to create, knowing and understanding the political processes, laws, rules and regulations in the jurisdictions in which we do or intend to operate, I am one of the most knowledgeable individuals for all medical marijuana models throughout the country.

Law enforcement, local zoning and code enforcement, local elected officials, and fire safety officials in Florida may have limited experience dealing with medical marijuana. I've worked with these groups in multiple jurisdictions and can assist in bridging the gap between preconception and reality.

While I have worked on legislation, rules and regulations on municipal, county and state levels throughout the country, the most prominent have been in Illinois, New York, Connecticut and Colorado. See below for references.

Bryan Clenahan - Legal Counsel, NY State Senator Diane Savino - (518) 253-7057
I worked directly with Attorney Clenahan for almost three years on the New York legislation through its passage.

Jonathan Harris - Commissioner, CT Dept. of Consumer Protection - (860) 985-5299
Commissioner Harris is newly appointed, but he and I have worked together for 15 years through his elected tenure and his involvement at other levels of state government. He can speak to my involvement in Connecticut's medical marijuana program.

Bob Morgan - General Counsel, IL Dept. of Public Health - (312) 814-6033
Mr. Morgan developed the Rules and Regulations for the State of Illinois. I provided insight, testimony, and submitted written draft rules.

Kristen Thompson - Lobbyist, Heizer Paul, LLP - (303) 656-0054
Ms. Thompson is one of the most knowledgeable and reliable cannabis lobbyists in Colorado with detailed working knowledge of all laws, rules and regulations and their genesis since 2009. Ms. Thompson and I work closely together and can speak to my in-depth knowledge and understanding of Colorado's programs.

Paul Kuhn - Board, National NORML and Tennesseeans United - (615) 294-6187
Mr. Kuhn has been involved with cannabis reform issues nationally and in his home state of Tennessee for years and enjoys one of the most professional and intelligent reputations in the nation. I am working directly with he and his organization to craft and pass legislation in Tennessee. He can speak to my ability

to gain the confidence of and listen to all sides and help craft compromise language that benefits patients and communities.

More importantly for the Florida rule making process, I have been actively involved in the legislative efforts for SB 1030 debates and have participated continuously in the DOH rule development process from the beginning of the process and understand the legislative framework created, the issues brought up by JAPC and the DOAH judge as well as the intentions of the Department of Health and how the experience from other states can help inform your efforts.

In closing, I am certain that I can be a strong asset to your Committee and will work to propose rules that are beneficial for the patients and their families, the businesses that take on the risk of providing this medicine, the host communities and the state of Florida.

Thank you for your consideration,

Erik A. Williams
Director of Government and Public Affairs,
BeMindful

Bist, Kevin

From: Mary K.Van Kleunen <mary@changingperformance.com>
Sent: Thursday, January 08, 2015 12:41 PM
To: Nelson, Patricia A
Cc: kevin.bist@flhealth.org
Subject: Negotiated Rulemaking Board- Medical Marijuana
Attachments: CV_2015.docx

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Ms. Nelson,

I am writing to request consideration as a community member on the rulemaking panel for the new medical marijuana regulations. I've attached my CV, where I've highlighted some previous experience in serving in this type of capacity as well as past legislative experience as an aide.

Additionally, I am the mother of a child who had intractable seizures from the age of 6 months up to his death in 2011 at age 16. My son Andrew suffered many ill effects from the numerous drugs he needed to take in an attempt to control the seizures. At the time of his death, he was taking 6 very strong seizure medications and had a vagus nerve stimulator. How we would have loved to have had another option to try!

My interest in the panel is to reflect the face of all parents and children who are experiencing what my family went through. I believe that families want the rules promulgated quickly ("yesterday") and yet thoughtfully. I am also interested in ensuring that the outcomes of medical marijuana are studied and documented, to provide an evidence base to support appropriate dosing, effects of long-term use, etc, to potentially move it along from compassionate use to standard of care.

My only experience with the nursery business is some cousins in the tree farm business (who have no intention of becoming involved with marijuana) , but am willing to "do the homework," read, listen, and attempt to understand the various stakeholder's positions.

Please feel free to call me or email me with any questions you may have.

Thank you,
Mary Kathryn Van Kleunen
Winter Springs, FL

Mary Kathryn Van Kleunen
Healthcare Performance Consulting
407.718.1859
Healthcare Performance

Mary Kathryn Van Kleunen
1208 Cheetah Trail
Winter Springs, FL 32708
(407) 696-2554

Experience:

Healthcare Performance Consulting, Medical Education Process Outcomes Consultant and Project Manager, 2009 to present
Responsibilities:

- data collection and analysis
- create and conduct quantitative and qualitative research
- develop reports to national medical education providers and sponsors
- project management
- interface with shareholders

Owner, ProMed Research, Consultant in Clinical Research Coordination, 2009-present

- Contracted to University Pediatric Dentistry, Buffalo, NY, to assist in research coordination along with the creation of standards of care for children receiving dental care from UPD and to facilitate widespread adoption of standards.

- Contracted to Physician Associates of Florida for the Phase IV studies:
  - *A Randomized, Single-Blind Pilot Study to Compare the Efficiency and Cost-Effectiveness of Frovatriptan vs. Topiramate for the Prevention of Migraine*, published Journal Headache 2012;52(5):749-764
  - *An observational, multicenter, parallel group Study [TRX112617] to evaluate the Impact of TREXIMET versus oral Triptan alone on work-related productivity loss due to migraine when administered during the mild, moderate, or severe phase of a migraine occurring prior to, or during, a scheduled work day.*
  - *Lipigesic™M for Acute Treatment of Migraine in Children and Adolescents*

Responsibilities: Advising on or performing:

- recruiting and enrolling human subjects
- data collection and monitoring
- case management of protocol participants
- protection of participants and participants' rights through IRB relations
- preparation of adverse event experience reports; construction and monitoring of case report forms
- maintenance of drug accountability records
- report preparation
- education of patients and families regarding clinical trials

Interim Institutional Review Board (IRB) Coordinator, Nemours-Delaware IRB, January to May, 2009

Responsibilities:

- research administration
- assured effective functioning of the IRB including pre-meeting and post-meeting research review activities as required by law, regulation, and Nemours policy and procedure
- worked in concert with the IRB Chairperson to assure that all research and related items submitted to the IRB were reviewed and acted upon in a timely manner in accordance with NOHSP policy and established Nemours IRB performance standards
- assured, by monitoring and interaction with research team members, that IRB submissions were complete and accurate prior to scheduling them for IRB review
- provided customer service to IRB stakeholders

Project Coordinator, Nemours Clinical Management Program, 2004-2009

Responsibilities:

- coordinated outcomes research and medical quality improvement initiatives
- collected and audited data
- liaison to IRB for protection of human subjects and subjects' rights
- developed Informed Consents, Parental Permission Forms, and Child Assent forms under the supervision of various Principal Investigators
- assisted in project budget development
- prepared and delivered reports via Power Point presentations; live web meetings; written and oral presentations
- along with clinical thought-leaders, developed critical data elements to imbed best practices into electronic medical record

Surgery Scheduler, Nemours Children's Clinic, Division of Urology, 2000-2004

Responsibilities:

Surgery Precertification; Patient Insurance Verification

Senior Interviewer, Nemours Children's Clinic, Center for Health Services Research, 1998-2000

Responsibilities:

- provided research administration support for a complex, multi-year, multi-site research protocol, *Managed Care and the Access to Health Care for Children with Cystic Fibrosis*
- site Liaison: Maintained all required regulatory documents from participating Institutional Review Boards and coordinated correspondence with study institutions.
- participant recruitment
- subject Interviews
- data entry
- coding
- insurance verification

Realtor, 1984-1997

The Prudential Florida Realty, Orlando, FL 1988-1997

Coldwell Banker,M.M.Parrish and Associates, Gainesville, FL 1987-1988

South Island Real Estate, Melbourne Beach, FL 1984-1987

- Board of Realtors Honor Society; 1986,1987,1989,1990,1991,1992

- Florida Association of Realtors Honor Society; 1986,1987,1990,1992
- Achieved Graduate, Realtor Institute (GRI) designation 1989
- Received Certified Residential Specialist designation, 1993

1977-1984: Legislative Aide, Florida House of Representatives

Responsibilities: Assistant to State Representative

- Solved constituent problems. Acted as liaison to state bureaucracy
- Public relations; written and verbal
- Made presentations to legislative committees to advance particular legislation to ultimate passage by the House and Senate
- Legal research
- Assisted in passage of Florida Housing Finance Authority Act, as well as other laws currently on books

1976-1977: Bill Drafting, Florida Senate

Responsibilities: Prepared bills for floor consideration

Performance Skills:

- Communication skills
- Computer skills (Word, Excel, Power Point, MS Project, QuestionPro, Visio, and more)
- Ability to work as a team member across sites with a high level of cooperation and supportiveness
- Ability to teach and delegate specific tasks to other staff
- Ability to read, interpret, and communicate regulation related to Human Subject Protection
- Understanding of research ethics principles
- Customer service skills

Education: Florida State University, B.A. English

Community

- Patient Representative Member, Collaborative Quality Advisory Committee, Orlando Health 2012-current
- Chairman/Executive Director, Drew's Care Connection, a foundation providing medical and therapeutic supplies and equipment to children with special medical needs
- AREA 7 Agency for Persons with Disabilities Quality Improvement Steering Committee, 2005 and 2006
- Early Steps (Birth to Three Early Intervention) Curriculum Development project, State of Florida Department of Health in conjunction with the University of Central Florida
- Gubernatorial appointee, Family Care Council, Agency for Persons with Disabilities, 1998-2006
- Chairman, Legislative Committee, Family Care Council, 1998-2000
- Director, Florida Association of Realtors (396 Directors out of 59,000 members), 1986, 1987, 1992.

- Chairman, Candidate Screening Subcommittee, Florida Association of Realtors, 1988.
- Chairman, Political Affairs Committee, Orlando Area Association of Realtors, 1991;
- Melbourne Area Board of Realtors, 1986, 1987.
- Chairman, Issues Mobilization Committee, Gainesville Board of Realtors, 1988.
- Service on two committees setting the standards for real estate sales professionalism and ethics, 1990, 1991.
- Outstanding Award, Realtor Active in Politics, 1990.
- Director, American Cancer Society, Brevard County Unit, 1986.
- Junior Leagues of Lakeland, Melbourne, Gainesville, Orlando
- Homeowners Association Director, 1990-91.

Other areas of interest/expertise:

- Neurology: Neuromuscular disorders, Seizure disorders and treatments/medications
- Pulmonology: Lung disorders and treatments/medications. Hands-on use of Bi-Pap, ventilator, pulse oximeter, respiratory therapy and equipment, ABI Vest. Tracheostomy care, suctioning, and equipment
- Orthopedics: Scoliosis and treatment
- Endocrinology: Thyroid disorders and treatments.
- Gastroenterology: Enteral and parenteral feeding, Disorders of the digestive tract and treatment/medications
- Special Education: Familiarity with IDEA, Part B, Part C. Provide classroom support and assist in identifying and obtaining resources to meet classroom needs
- Advocacy and Resource Counseling: Identifying and advocating for resources for equipment, services, medications, and medical treatments and assisting other families in similar endeavors

References: Available upon request.

Bist, Kevin

From: Michael Sjuggerud <Mike@cfglawoffice.com>
Sent: Thursday, January 08, 2015 1:15 PM
To: Nelson, Patricia A
Subject: Application to serve on negotiated rulemaking committee to address the regulatory structure for dispensing organizations of low-THC cannabis
Attachments: Michael Sjuggerud bio.pdf; Notice of Development of Rulemaking.doc

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Ms. Nelson,

Please consider this as my application to serve on the negotiated rulemaking committee to address the regulatory structure for dispensing organizations of low-THC cannabis pursuant to the attached Notice of Development of Rulemaking.

I understand that the negotiated rulemaking committee will be selected from a variety of representative groups. I am eligible to serve on the negotiated rulemaking committee because I satisfy the requirements of two of the representative groups:

- I am member of the Florida Bar with experience in administrative law, and
- I am an individual with experience navigating regulatory structures for cannabis in other jurisdictions inasmuch as my legal practice includes assisting Florida clients with cannabis-related laws in Washington state, California, Oregon, and Colorado.

Regarding my credentials, attached please find a copy of my resume (also available at the following link: Bio for Michael Sjuggerud, Esq.)

Please let me know if you have any questions or further information about the negotiated rulemaking committee. I look forward to hearing from you.

Regards,

Michael Sjuggerud, Esq.
Cantwell & Goldman, P.A.
96 Willard Street, Suite 302
Cocoa, Florida 32922

321-639-1320 x 108
321-639-9950 (fax)
mike@cfglawoffice.com

Michael Sjuggerud

Download VCard
Email: mike@cfglawoffice.com

Phone: 321-639-1320 ext. 108
Fax: 321-639-9950

96 Willard St, #302
Cocoa, Florida 32922

PRACTICE AREAS

- » **Business Law**
- » **Real Estate Law**
- » **Commercial Loans & Debt Restructuring**
- » **Commercial Sales & Leases**
- » **Private International Law (International Transactions)**
- » International Business and Trade Law

Michael Sjuggerud was raised in Central Florida. He has a global education and a unique background in international business and law. Michael holds an undergraduate degree in finance, an MBA in international business, and a law degree. He has studied at universities in the U.S., Mexico, Italy, and Japan and worked for major law firms in the United States and Japan. His practice areas include the following:

BUSINESS LAW

- **Establishment of businesses.** Michael advises on the selection of business entities and drafts and files business formation documents to establish corporations, partnerships, limited partnerships, limited liability companies (LLC's) and other business entities. He advises on structuring a new business, drafting founding documents and completing required state and federal filings.
- **Purchases and sales of businesses.** Michael advises on the purchase and sale of businesses, business equipment and other assets. He advises privately held businesses on loan transactions, mergers, acquisitions and raising capital.
- **Contract drafting for businesses.** Michael drafts and reviews non-compete agreements, separation agreements, buyout agreements, shareholder agreements, purchase agreements, sales agreements and other business contracts.
- **Commercial contract negotiations.** Michael advises clients on business contracts and commercial contract negotiations in the U.S., Asia, Latin America, and Europe.

COMMERCIAL REAL ESTATE

- **Purchase and sales of commercial real estate.** Michael drafts and reviews agreements related to the purchase and sale of commercial real estate. He procures title commitments, resolves title issues, conducts commercial real estate closings throughout Florida, and procures owner's title insurance policies.
- **Financing (and refinancing) of commercial real estate.** Michael drafts and reviews commercial mortgages, assignments of rents, and UCC financing statements. He advises banks, credit unions,

and financial institutions on the financing of loans, loan modifications and extensions, and loan refinancing. He procures title commitments, resolves title issues, and procures lender's title insurance policies.

- **Commercial leases.** Michael drafts and reviews leases for commercial real estate. He advises commercial landlords and tenants on the terms of gross leases and net leases. Michael advises commercial clients on assignments and assumptions of leases.

INTERNATIONAL LAW

- **U.S. companies engaging in international business.** Michael advises U.S. companies on laws and treaties governing international commercial transactions. International commercial transactions include joint ventures with overseas partners, licensing technology and intellectual property to overseas companies, sales of goods to or by overseas companies, and international finance. He drafts and reviews legal documents necessary to carry out international transactions and works with local counsel in foreign countries to ensure that transactions comply with local law. Through years of experience working at the law firm of Anderson Mori & Tomotsune in Tokyo, Japan and in the Latin American region on behalf of Siemens, Michael has developed excellent working relationships with counsel across the globe.
- **Foreign companies doing business with U.S. companies.** Many foreign companies consider the U.S.'s legal system to have a high risk of litigation. Michael advises foreign companies on how to do business with U.S. companies, the types of contracts (and contract provisions) which are customary in the U.S., and what steps should be taken do business in the U.S.

LITIGATION

- **Pre-suit dispute resolution.** Michael spent the initial part of his career in the litigation department of Holland & Knight LLP. Michael consults with clients and develops legal strategies to successfully resolve business disputes.
- **Litigation.** If the parties are unable to amicably resolve their dispute and one of the parties desires to file a lawsuit or a lawsuit has been already been filed, Michael can serve as counsel, co-counsel, or local counsel in commercial disputes.

LAW SCHOOL ACTIVITIES & AWARDS

- Florida State University Law Review:
    - Notes & Comments Editor
    - Meritorious Service Award
    - Best Student Law Review Article
- American Bankruptcy Institute: Medal of Excellence
- Florida Lawyers Legal Insurance Company Florida Lawyers Legal Insurance Company/Dan Bradley Memorial Award
- Book Awards

PUBLICATIONS

- "New International Jurisdiction Rules: Foreign Companies Must Brace For Change," International Law Office (2011)
- "Usury claims: Challenging Grey-Zone Interest," International Law Office (2010)
- "Student Loans in Bankruptcy: Policy Analysis and Portfolio Review," Banking Law Journal (2006)
- "The Basics of Bankruptcy Risk Management when Making Consumer Loans," Banking Law Journal (2004)
- "Defeating the Self-Settled Spendthrift Trust in Bankruptcy," Florida State University Law Review (2001)

SPEAKING ENGAGEMENTS

- Municipal Law: Fundamentals of Bankruptcy (2014)
- Cocoa Beach Regional Chamber of Commerce: Roadmap for Business Owners & Management (2014)

Education

J.D., Florida State University College of Law
Cum Laude
M.B.A., Clemson University & CIMBA Italy
B.S.B.A. (Finance), University of Florida
with High Honors

Jurisdictions Admitted to Practice

Florida
District of Columbia
New York
Washington

Languages

Spanish (second language)

---

96 Willard St, #302
Cocoa, FL 32922
Phone: 321-639-1320
Fax: 321-639-9950

We serve the following localities: Brevard County, Cape Canaveral, Cocoa, Cocoa Beach, Indialantic, Melbourne, Merritt Island, Mims, Orlando, Palm Bay, Rockledge, Satellite Beach, Sebastian, Titusville, and Miami.

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NOTICE OF DEVELOPMENT OF RULEMAKING

DEPARTMENT OF HEALTH
Office of Compassionate Use

RULE NOS.:	RULE TITLES:
64-4.001	Regulatory Structure Rule 1
64-4.002	Regulatory Structure Rule 2
64-4.003	Regulatory Structure Rule 3
64-4.004	Regulatory Structure Rule 4
64-4.005	Regulatory Structure Rule 5
64-4.006	Regulatory Structure Rule 6
64-4.007	Regulatory Structure Rule 7
64-4.008	Regulatory Structure Rule 8
64-4.009	Compassionate Use Registry

PURPOSE AND EFFECT: The Department of Health announces the convening of a negotiated rulemaking proceeding to address the regulatory structure for dispensing organizations of low-THC cannabis. The purpose of the negotiated rulemaking is to draft mutually acceptable proposed rules.

SUBJECT TO BE ADDRESSED: The subject and scope of the rules to be developed through negotiated rulemaking will be the regulatory structure for dispensing organizations of low-THC cannabis.

RULEMAKING AUTHORITY: 381.986 FS.

LAW IMPLEMENTED: 381.986 FS.

NEGOTIATED RULEMAKING COMMITTEE: The negotiated rulemaking committee members will be selected from the following representative groups:

1. A nursery that meets the criteria in Section 381.986(5)(b)1., Florida Statutes;
2. A qualified patient or patient representative;
3. A testing laboratory;
4. A member of the Florida Bar experienced in administrative law;
5. An individual with demonstrated experience in sound agricultural practices and necessary regulation;
6. A physician authorized to order low-THC Cannabis products for qualified patients;
7. An individual with demonstrated experience establishing or navigating regulatory structures for cannabis in other jurisdictions; and
8. Representatives of the Department of Health.

If you believe that your interests are not adequately represented by the committee members listed above, you may apply to participate within 30 days of the date of publication of this notice. Your application must contain the following information: your name, business address, and telephone number; the name of any organization you are representing; a description of the organization or the members of the organization; a description of how the proposed rulemaking proceedings will affect you or the parties that you represent; a statement identifying the reasons why you believe the representative groups listed above will not adequately represent your interests; and a statement that you are willing to negotiate in good faith and can attend the scheduled meeting. Please submit your application to Patricia Nelson, Department of Health, 4052 Bald Cypress Way, Bin A-02, Tallahassee, Florida 32399, email address: Patricia.Nelson@flhealth.gov.

NEGOTIATED RULEMAKING COMMITTEE MEETING:

The committee will meet at the following date, time, and place to discuss rule development: February 4, 2015, 8:00 a.m. - 10:00 p.m. and February 5, 2015, 8:00 a.m. until concluded, Department of Health, Room 301, 4052 Bald Cypress Way, Tallahassee, FL 32399.

Pursuant to provisions of the Americans with Disabilities Act, any person requiring special accommodations to participate in this meeting is asked to advise the agency at least 72 hours before the meeting by contacting Sophia Flowers, Department of Health, (850)245-4005, Sophia.Flowers@flhealth.gov. If you are hearing or speech impaired, please contact the agency using the Florida Relay Service, 1 (800)955-8771 (TDD) or 1 (800)955-9770 (Voice).

THE PERSON TO BE CONTACTED REGARDING THE PROPOSED RULE DEVELOPMENT AND A COPY OF THE PRELIMINARY DRAFT, IF AVAILABLE IS: Patricia Nelson, Department of Health, 4052 Bald Cypress Way, Bin A-02, Tallahassee, Florida 32399, Email address: Patricia.Nelson@flhealth.gov
THE PRELIMINARY TEXT OF THE PROPOSED RULE DEVELOPMENT IS NOT AVAILABLE.

Bist, Kevin

From: Ryantlaw@aol.com
Sent: Thursday, January 08, 2015 1:50 PM
To: Nelson, Patricia A
Subject: (no subject)

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Ms Nelson:
I received your e-mail about the rules making committee. I do not know if all of the members for the committee have been chosen. The number 7 position is of interest to me. I am a Florida attorney who has been practicing in Florida since 1980. In 2012 I began teaching Medical Marijuana Law in Colorado at Greenway University, a Colorado department of educational certified marijuana university. I wrote a comparative study of the marijuana laws of all 50 states and a study of the conflicts with Federal law. I also taught people how to license, open and run dispensaries and grows legally and how to become a budtender. I also gave legal seminars in Arizona to teach people how to comply with Arizona law. In 2013 I moved to California and became a qualified grower for licensed dispensaries. I developed and built out grows for others and ran multiple grows. I returned to Florida in March of 2014 and wrote two Florida CLE medical marijuana courses and have given seminars through my company Department of Medical Marijuana Education of Florida, LLC . If you have an opening I would like to discuss the opportunity with you.
The other question I had was wether the public will be allowed to attend the rules making sessions.
If you have any questions please do not hesitate contacting me.
Thomas F. Ryan
(561) 723-5725
Ryantlaw@aol.com
Fl Bar 293180

Bist, Kevin

From: Jeremy Lawton Susac <JSusac@bergersingerman.com>
Sent: Thursday, January 08, 2015 3:54 PM
To: Nelson, Patricia A
Subject: Negotiated Rulemaking Committee Question

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Patricia, I trust all is well. Would sitting on the Negotiated Rulemaking Committee disqualify that appointed person/entity from being a future applicant? My reading is that it would not, but I wanted to double-check. Thanks for any guidance.

BERGER SINGERMAN
Jeremy L. Susac
125 South Gadsden Street | Suite 300 | Tallahassee FL 32301
office: (850) 561-3010 | *direct:* (850) 521-6736 | *fax:* (850) 561-3013
email: JSusac@bergersingerman.com
website: www.bergersingerman.com
doing business in Florida resource: www.flabusinesslaw.com

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****
****

Bist, Kevin

From:	Desiree Mufson <desireemufson@gmail.com>
Sent:	Thursday, January 08, 2015 7:03 PM
To:	Nelson, Patricia A
Subject:	Re: Low-THC Cannabis Rulemaking
Attachments:	Coastal Cannalabs Jan 2015.docx
Follow Up Flag:	Follow up
Flag Status:	Flagged

Hello Ms. Nelson;

I wish to be considered as one of the committee members to represent the laboratory portion for the Medical Marijuana Rule Revision to be held on February 4-5 in Tallahassee.

Please see enclosed on why I feel I can make a difference.

Best,

Desiree Ardito Mufson
Coastal Cannalabs
772-260-8636
Desireemufson@gmail.com

On Mon, Jan 5, 2015 at 5:11 PM, Nelson, Patricia A <Patricia.Nelson@flhealth.gov> wrote:

Dear Interested Parties,

Please see the attached Notice of Negotiated Rulemaking (on page 1 of the attached document) scheduled for February 4 and 5, 2015, in Tallahassee.

Sincerely,

Patty

Patricia Nelson

Director

Office of Compassionate Use

--
Desiree Ardito Mufson
New Vision Productions, Inc.

Stuart, Fl
772-219-0140

January 7, 2015

Dear Ms. Nelson;

I am writing to you to be considered as a participant on the committee to draft mutually acceptable proposed rules for Medical Marijuana.

My name is Desiree Ardito Mufson and I am the owner of Coastal Cannalabs here in Florida. Although the bill was crushed in November,
I still recognize the need for labs to analyze low THC and would like to be counted among those that are interested in pursuit of this business. Since the last session in September, I have affiliated with people that could strengthen my knowledge in the MMJ Laboratory Business resulting with

safe products patients can trust. Besides experts in the business, I am also associated with two major investing firms that place money into new and upcoming medial marijuana businesses. The software company, Biotech THC is one that I just recently introduced to my investors to allow them to go public.

Who is Desiree Ardito Mufson, MT (ASCP), MBA

My background has been honed to basically fit into any area of the new Cannabis Industry.
As a young teenager, I started out by working on my family's 2 acre farm and then I graduated with honors in 1985 from Thomas Jefferson University only to move on to study marketing at Wharton's School of Business.

I worked in hospital laboratories for 20 years holding various positions from Blood Bank Supervisor to Head of the Laboratory here at Palm Beach Gardens Medical Center. In 1987 I was one one of the pioneers in autologous donation procedure whereas a surgical patient self donates their blood prior to the surgery in case of need for transfusion. I wrote the standards and procedure for that hospital and gained their accreditation through the College of American Pathologists.
I am licensed by American Society of Clinical Pathologists and is an inspector for CAP, AABB, and OSHA.
My work in quality control have been published in multiple journals.
I started my own clinical laboratory consulting firm: DA Laboratory Consultants, and was a consultants to laboratories that wished to become accredited or to meet compliance standards.

Seminars I attended:

In June, I attended the Medical Cannabis Symposium and toured various facilities.
This past October, I attended the Infused Product and Extraction Symposium in Denver this to gain knowledge in new product development and regulation with regards to edibles. At this symposium I had the pleasure and good fortune to meet some of the industry's leaders in MMJ regulation and gained valuable information and alliances with them.

Slow and Low Program

One of the principal speakers that I met was, Meg Collins, Executive Director of the Cannabis Business Alliance. She is instrumental in the development of the legislative and regulatory framework implementing Colorado's retail marijuana market and is one of the creators of the new "Slow and Low" campaign to urge newcomers to edibles to consume a small quantity at lower doses. The new low dose is 5 mg which is being marketed throughout the State. The awareness of this campaign has a direct correlation in lowering the number of individuals entering the Emergency Room due to overdose. They want a first timers experience to be healthy and pleasant and not toxic requiring an Emergency Room Visit. Although I realize that it is still a bit early for us to discuss different campaigns for Medical Marijuana in Florida, I wanted you to know that Ms. Collins offered to help us to work on a similar project for our state. If Coastal Cannalabs is chosen as a laboratory for the state, I wish to chair or be part of the committee for the same type of campaign for the State of Florida. Their marketing campaign is an excellent way of creating awareness and educating the patient population to start with a nibble to stay safe.

Contacts

During my time in Denver, I was introduced to Christian Sederberg from the Attorneys of Vincente Sederberg LLC, one of the leading firms whose primary focus is on the regulatory compliance and government affairs relating to the legalization of marijuana. Christian and I discussed MMJ in Florida and he offered to assist me in any way possible. Other speakers that I was introduced to were: Danica Lee, Food Safety Section Manager of the Denver Department of Environmental Health, the agency that regulates food businesses in the City and County of Denver; Tripp Keber, Chief Executive Officer, Dixie Brands, Inc.; Joshua Kappel, Partner of Vincente Sederberg LLC; Chris Hudalla, Chief Scientific Officer ProVerde Laboratories, Ata Gonzalez, Founder and CEO of G FarmaLabs; Mike Elliott, Executive Director, Marijuana Industry group (MIG) besides many more.

In addition to attending the seminar, I have been contacted by Jeannine M Machon, the Business Director of CMT Laboratories in Denver Colorado to help set up Coastal Cannalabs to meet and exceed compliance regulations. She wishes to be part of my board to help see Coastal succeed here in Florida. I am fortunate to affiliate with Jennine as not only she offers expertise in working with MMJ but she is coming aboard with full respect that this is a Florida based company and will offer consultation to streamline the start-up
process.

Coastal Cannalabs

Mission Statement: Coastal Cannalabs objective is to provide accurate marijuana testing utilizing cutting edge technology to growers, extractors, and concentrate makers to analyze marijuana's quality, purity, potency and cannabinoid effects

in order to ensure the safety and quality to their patient base.

The law in Colorado, and in all states, describes that Cannabis must be tested for purity and concentration. Patient safety is of paramount concern and Coastal Cannalabs will be offering purity as well as concentration levels in order to ensure the safety and quality of medicinal plant materials. The laboratory will provide data to patients so they can better predict how marijuana will help them and so they know their marijuana is free of harmful pathogens, residue, pesticides and debris. Growers will use the lab data to monitor and upgrade their cultivation techniques. Our clients will consist of growers, extractors, dispensaries, edibles and concentrate makers.

Patients and doctors must have an accurate cannabinoid profile of each flower, edible or concentrate to select the proper strain and dosage for treating any number of symptoms. Additionally, growing cannabis creates conditions that allow for the germination of harmful bacteria. Coastal Cannalabs will provide technology to screen for the many contaminates that could affect a user's health and well-being. Concentrates, like hash oil, are made using a solvent-based extraction process to strip the cannabinoids and terpenoids from the plant material. Common solvents are butane, propane, carbon dioxide, isopropyl alcohol, acetone and ethanol. Coastal Cannalabs will provide the technology for residual solvent testing to guarantee the highest quality concentrates are being created and free of impurities and odorants.

I have created a website: Coastal Cannalabs to offer patients and clients more information about our

company. My web person is also a web designer for Wells Fargo and is in the process of creating proprietary software for the laboratory that offers real time information to patients and physicians.

In addition, Coastal would like to be part of the Biotek world and is investigating start up on this project.

I feel in order for the State of Florida to perform due diligence in creating protocol for safe and effective methods of handling and distribution of medical marijuana the laboratory testing is the MOST Important step in the process. The laboratory must be operated by knowledgeable personnel with experience in analytical and microbiology type testing. The laboratory MUST be a stand alone and independent laboratory with no ties to a grow. We at Coastal Cannalabs are prepared to take on the challenge set by the state to offer safe and reliable testing of product. Accuracy that Patients and Physicians Can Depend Upon to help with their particular disease. We are the only laboratory offering a Pharmaceutical Analytical Chemist and Microbiologist as well as a Medical Director (Dr. Anne Morgan) who is committed to researching the best way of delivering safe products. Best of all we are Florida Based Company utilizing medical and pharmaceutical experience coupled with gained knowledge from known Cannabis laboratory experts to create a quality laboratory. We have the knowledge and the financial backing to make it happen in multiple areas of the state. We at Coastal Cannalabs are committed to offer Testing You Can Count On.

I feel blessed to have investors and associations with the top people who care about patient care and have the expertise to guide and fund me through this venture.

Please know that I am always willing to share the information and contacts that I have collected throughout this educational process in order for Florida to be on the

cutting edge of patient care when the time is right.

Best,

Desiree Ardito Mufson
Founder/Director
Coastal Cannalabs LLC
Desireemufson@gmail.com
Stuart, FL
772-260-8636

Bist, Kevin

From: Manny Johnson <manny@mannyjohnson.net>
Sent: Friday, January 09, 2015 4:02 AM
To: Nelson, Patricia A
Subject: Low-THC Cannabis Rulemaking

Importance: High

Follow Up Flag: Follow up
Flag Status: Flagged

January 9, 2015
Dear Ms. Patty Nelson,
I hope the new year brings you and your family the best life has to offer! You add a wonderful, compassionate, professional persona to these difficult proceedings. Kudos to you and your staff.
I would like to apply to attend the Development of Proposed Rules and Negotiated Rulemaking on Feb 4-5 for the SB 1030 hearings.
My name is E. Manny Johnson, 127 West Fairbanks Avenue, #507, Winter Park, FL32789. (813) 789-1300.
My organization is the Compassionate Cannabis Community, LLC- we provide books, research/scientific information, interviews of noted individuals in the Medicinal Cannabis industry, and videos free of charge. We represent Veterans, Children, and patients who might benefit from this low-THC Cannabis oil. I have attended all of the hearings thus far and have always found that the interests of our members and of myself are not properly represented because the vast majority of individuals at the hearings have an ulterior motif; generally money of some kind. I will and always have negotiated through the hearings in good faith and shown everyone the utmost respect.
I have continued to provide your organization with a great deal of free knowledge that is not always in agreement with others. I am a 30 year Nurseryman and could apply for a license if I desired, but I do not desire to apply for a number of reasons. Four days out of high school I joined the U.S. Army to attend the USMA at West Point and Honorably served my Country. I started a nursery with my wife's and my hands and built it into one of the most respected family-owned specialty nurseries in the world. We had nurseries in the U.S., Germany and China. I am also a qualified patient suffering from colon cancer but hope to be off that list before the hearings. I have served as a consultant on the application process without any monetary gain for 3 dispensaries in Colorado, 1 in Oregon, 2 Washington, 1 Illinois, and ZERO in Florida. I am a writer and documentary film maker with an MFA in Film and Screenwriting from UCLA. I have visited Israel, Canada, California, Colorado, Oregon, Washington, Illinois to observe the various aspects of medicinal cannabis. I attended the first Continuing Medical Education Course on Medicinal Cannabis in Denver. I am a member of the Society of Cannabis Clinicians and passed their accreditation course with a 97%. I have vast experience in managing a real, working nursery in the state of Florida. I have a Pesticide license. As you might see I have a plethora of knowledge in a vast area that intersects essentially every area of the Negotiated Rulemaking Committee. I can serve as a true buffer in a wide variety of areas because I know the truth based on experience and I have no money to gain or lose no matter who becomes the Dispensing Organizations. I do believe the best applicant is the one who can prove he/she has managed their nursery through a myriad of difficult economic climates and is partnered with citizens of good moral standing.
I offer my skill-set in this matter because I am concerned that "smoke and mirrors and money" lurk around every corner. I receive calls at least once a week from investors who want to back me in this application endeavor. They offer more money than I have ever had. They inform me they do not mind losing money for a few years until the recreational cannabis is the norm. I will have no part of this. I am all about the VETS, KIDS and Patients-in-need!
Also, please let me know if I am accepted to attend as I keep a very busy schedule!
Respectfully Submitted,
E. Manson Johnson
E. Manson Johnson
127 W. Fairbanks Avenue, # 507

Winter Park, FL 32789
(813) 789-1300 cell
Manny@mannyjohnson.net

From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Monday, January 05, 2015 5:11 PM
To: DL 64-4 Interested Parties; Dunn, Nathan P
Subject: Low-THC Cannabis Rulemaking

Dear Interested Parties,

Please see the attached Notice of Negotiated Rulemaking (on page 1 of the attached document) scheduled for February 4 and 5, 2015, in Tallahassee.

Sincerely,
Patty
Patricia Nelson
Director
Office of Compassionate Use

Bist, Kevin

From:	Meg Sanders <meg.sanders@bemindful.today>
Sent:	Friday, January 09, 2015 8:13 AM
To:	Nelson, Patricia A
Subject:	Erik Williams- Rules Committee
Attachments:	letter of rec EW.docx; ATT00001.htm; PastedGraphic-2.tiff; ATT00002.htm
Follow Up Flag:	Follow up
Flag Status:	Flagged

Dear Director Nelson,
Please see attached letter for recommendation for the rules committee.
Thank you,
Megl

MiNDFUL

January 7. 2015

Mrs. Smith, CEO
Weed Inc.,
420 Pot St.
Mary Jane, CO 42000

Dear _____

It is with great pleasure that I write this letter of recommendation for Erik Williams. As CEO of MiNDFUL, I've worked with Erik for the past three years on policy and rule making for medical marijuana on both a local and state level. Erik has guided me through several rule making processes, including my involvement on the Amendment 64 Governor's Task Force. I was the only industry representative.

Erik Williams is hands down the best resource available for marijuana policy. His deep understanding of rules and regulations in multiple states is an enormous asset. His insight and experience of promulgation and implementation of policy is invaluable. Erik has guided multiple states and stakeholders through this difficult process. He is keenly aware of unintended consequences of strict regulation applied to a living plant as well as understanding the correct balance between too little and too much regulation. There are many sides to this issue- law enforcement, patients, running a successful business- for example. Managing the concerns of each stakeholder and finding acceptable compromise of conflicting issues is one of Erik's specialties.

Including Erik Williams on the rule committee for the Florida Compassionate Use program will ensure its success.

Sincerley,

Meg Sanders
303-981-2453

Bist, Kevin

From:	Joel Ewusiak <joel@ewusiaklaw.com>
Sent:	Friday, January 09, 2015 10:49 AM
To:	Nelson, Patricia A
Subject:	RE: Application for Rulemaking Committee for Florida's Low THC Cannabis Law - Joel Ewusiak
Attachments:	Application for Committee - Joel Ewusiak - Signed.pdf
Follow Up Flag:	Follow up
Flag Status:	Flagged

Patty:
As a follow-up to my email below, attached please find my formal application to serve as a committee member. At your convenience, please confirm your receipt of this email.
Thanks for your consideration,
-Joel
Joel Ewusiak
Ewusiak Law, P.A.
100 Main St., Suite 205
Safety Harbor, FL 34695
P: 727.286.3559 | F: 727.286.3219 | www.ewusiaklaw.com
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From: Joel Ewusiak [mailto:joel@ewusiaklaw.com]
Sent: Tuesday, January 6, 2015 2:31 PM
To: 'Nelson, Patricia A'
Subject: RE: Rulemaking Committee for Low THC Cannabis Law
Thanks, Patty. I'll send you a request via email tomorrow. I think I would offer a fair and balanced approach to the rule-making process that is consistent with the law. I represent one of the few doctors currently authorized in Florida to order low-THC Cannabis (she has taken and passed the CLE course), as well as several Florida businesses and individuals wishing to apply for dispensary and cultivation licenses in the future. During last year's legislative session, I also extensively analyzed all of Florida's proposed medical marijuana laws, including the low THC Cannabis law that passed, so I am more than familiar with the issues at hand. By way of example only, please see:
http://www.flamedicalmarijuanalawyer.com/news/2014/2/13/several-part-series-on-floridas-new-proposed-medical-marijuana-bill.
Anyway, I will be in touch and thank you for your consideration.
-Joel
Joel Ewusiak
Ewusiak Law, P.A.
100 Main St., Suite 205
Safety Harbor, FL 34695
P: 727.286.3559 | F: 727.286.3219 | www.ewusiaklaw.com
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strictly prohibited. If you have received this transmission in error, please immediately reply to the sender that you have received this communication in error and then delete it. Thank you.

From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Tuesday, January 6, 2015 1:07 PM
To: joel@ewusiaklaw.com
Subject: RE: Rulemaking Committee for Low THC Cannabis Law

Joel,

The negotiating committee has not been selected. Any person interested in being on the committee should send a request to me by email. The request should include an explanation of why they are interested in being on the committee as well as what qualifications they believe would make them a good choice for the committee.

Patty

From: Joel Ewusiak [mailto:joel@ewusiaklaw.com]
Sent: Monday, January 05, 2015 1:32 PM
To: Nelson, Patricia A
Subject: Rulemaking Committee for Low THC Cannabis Law

Hi Patricia:

I've reviewed the attached Notice of Development of Rulemaking for the low THC cannabis law. How are the committee members being selected and if they are already selected, who are the committee members? The notice outlines a procedure that must be followed in order to apply to participate in the February 2015 meeting, but the procedure seems to apply to non-committee members only.

Thanks,

-Joel

Joel Ewusiak
Ewusiak Law, P.A.
100 Main St., Suite 205
Safety Harbor, FL 34695
P: 727.286.3559 | F: 727.286.3219 | www.ewusiaklaw.com

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EWUSIAK LAW

Reply to: joel@ewusiaklaw.com

100 MAIN STREET, SUITE 205
SAFETY HARBOR, FL 34695
P: 727.286.3559 | F: 727.286.3219

January 9, 2015

Via Email Only (Patricia.Nelson@flhealth.gov)
Patricia Nelson
Department of Health
4052 Bald Cypress Way, Bin A-02
Tallahassee, Florida 32399

Re: Application for Membership on Negotiated Rulemaking Committee for Low THC Cannabis Law (Fla. Stat. Section 381.986. "Compassionate use of low-THC cannabis.")
Section 1: Notice of Development of Proposed Rules and Negotiated Rulemaking, Florida Administrative Register Volume 41, Number 2, January 5, 2015

Dear Ms. Nelson:

Please accept this email as my application to serve as a committee member for the negotiated rulemaking process.

For the past thirteen and a half years, I have continuously practiced law in the state of Florida and represented clients in a wide range of complex legal matters. My resume may be found on my website here: http://www.ewusiaklaw.com/joel-ewusiak/. Given the nature of my practice, I have frequently interpreted and actively litigated the meaning, application, and constitutionality of statutes for a wide range of clients, including individuals, professionals, corporations and small businesses. Moreover, my practice consists of representing plaintiffs, defendants, and third parties, so I have an appreciation for the viewpoint of all parties to a dispute. In recent years, I have also been involved in handling transactions matters for clients. As for medical marijuana laws, rules, regulations and attendant issues, over the past couple years, I have become extensively versed in all proposed and adopted medical marijuana laws in Florida. I have also paid particularly close attention to medical marijuana statutes and regulations adopted in other states with strictly controlled medical marijuana schemes, such as Illinois, as well as the practical impact of those laws.

My analysis and commentary on Florida's Low-THC Cannabis law (and the rule making process to date), Florida's broader proposed medical marijuana law during prior legislative sessions, and proposed and adopted ordinances in Florida cities, may be found on my blog. Examples of my analysis and commentary over the past year may be found at the following links:

1. http://www.flamedicalmarijuanalawyer.com/news/impact-low-thc-bill-florida-medical-marijuana-application-requirements
2. http://www.flamedicalmarijuanalawyer.com/news/quick-take-away-and-key-provisions-of-final-version-of-floridas-low-thc-medical-marijuana-law
3. http://www.flamedicalmarijuanalawyer.com/news/2014/2/13/several-part-series-on-floridas-new-proposed-medical-marijuana-bill
4. http://www.flamedicalmarijuanalawyer.com/news/department-releases-final-version-of-floridas-low-thc-medical-marijuana-rules
5. http://www.flamedicalmarijuanalawyer.com/news/ruling-by-florida-judge-on-floridas-low-thc-cannabis-rules-and-regulations

I represent one of the few doctors currently authorized in Florida to order low-THC cannabis (she has taken and passed the applicable CLE course, and I represented her both before and after she took the course), and I have provided advice to at least two other Florida doctors who plan to seek authorization to order low-THC cannabis in the future. Thus, I am more than familiar with medical marijuana laws and regulations impacting physicians on a federal and state level. From time to time, I have also provided advice to qualified patients seeking to use low-THC cannabis for medical conditions. Finally, I also currently represent and counsel several Florida businesses and individuals wishing to apply for dispensary and cultivation licenses in the future, if and when a broader medical marijuana law is implemented in Florida.

Given the legal representation and advice that I provide to several clients who wish to participate in different aspects of Florida's proposed and adopted medical marijuana laws, as well as my knowledge of the proposed and adopted medical marijuana laws in Florida and other states, I believe that I can provide a balanced and thoughtful approach to all interested parties as part of the rule making process, as well as provide commentary on whether the draft rules and regulations comply with the spirit and intent of the Low THC Cannabis Law and the administrative judge's ruling. Given my background in litigation, I believe that I can also assist in drafting objective criteria/rules governing the application and selection process for applicants/nurseries, thereby minimizing or eliminating legal challenges from those who are not selected as one of the five licensed businesses under the Low THC Cannabis Law. Finally, I believe that my input and commentary on the low-THC cannabis rules and regulations will also prove valuable for purposes of any future rules and regulations that will be necessary if and when Florida adopts a broader medical marijuana law. I would be happy to attend the scheduled meeting in Tallahassee in early February and will participate in good faith.

Should you have any questions or concerns about my experience and interest in the rule-making process for the Low-THC Cannabis Law, please feel free to contact me. My business address and telephone number is outlined in the letterhead above. As reflected in this application, I can offer input on a wide range of issues and would be happy to offer input on all

such issues or as a representative for one particular stakeholder, such as Florida physicians. If requested by you, and after obtaining client consent (subject to any confidentiality issues), I am happy to disclose the name of my physician client referenced above, as well as the names of some of the other Florida business and individual clients whom I represent and wish to apply for dispensary and cultivation licenses in the future, if and when a broader medical marijuana law is adopted in Florida. For purposes of clarification, none of my existing clients have any interest in applying for one of the five licenses available under the Low-THC Cannabis Law, and I have no intentions of representing any future clients who may wish to apply for such licenses.

Thank you for your attention to this matter.

Sincerely,

Joel Ewusiak

JE/je

Bist, Kevin

From:	Arianna Cabrera <arianna@costafarms.com>
Sent:	Friday, January 09, 2015 11:23 AM
To:	Nelson, Patricia A
Cc:	Arianna Cabrera; Katharyn Field; Peter Freyre
Subject:	Nomination for Negotiated Rulemaking Committee
Attachments:	Costa Farms Committee Nomination 1-9-15.pdf; Costa Farms Executive Bios.pdf
Follow Up Flag:	Follow up
Flag Status:	Flagged

Hi, Patricia. In connection with the Notice of Development of Proposed Rules and Negotiated Rulemaking published on January 5, 2015, Costa Farms would like to be considered to serve on the negotiated rulemaking committee as, "a nursery that meets the criteria in Section 381.986(5)(b)1., Florida Statutes." I have attached a brief narrative describing Costa Farms, together with the biographies of our Executive Team.

If you have any questions, please feel free to reach out to me. My contact information is below.

Thank you!

Arianna Cabrera, Esq.
General Counsel

Costa Farms

o: 786-866-5227 | f: 786-272-6137 | c: 305-608-6572
21800 SW 162nd Avenue | Miami, FL 33170

Costa Farms

Costa Farms is the largest producer of indoor house plants in the world. Founded in 1961 by Jose Costa, Costa Farms is a third generation, family owned business that globally stretches over 4,200 acres and employs approximately 3,600 people, 75% of which are Floridians. Along with foliage, annual, and perennial plant divisions, Costa Farms operates merchandising and transportation companies as part of its infrastructure, with operations domestically in Florida, North Carolina, and South Carolina, and abroad in the Dominican Republic and Far East.

Over five decades, Costa Farms has grown into a thriving business. During 2014 we sold $340 million in plants to big box retailers such as Lowe's, Walmart, Home Depot, Costco, and Sam's Club, and in 2015 we project to sell $385 million. We currently grow 500+ genera and thousands of species, a large majority of which are nonnative to Florida. In 2014 we sold over 34 million plants through our foliage division alone. We ship to all 50 states, Puerto Rico, and Canada. Although our focus is on tropical foliage, Costa Farms also produces plants intended for human consumption such as tomatoes, strawberries, peppers, and herbs.

Our ability to grow a multitude of nonnative plants in different environments, efficiently bring plants to the mass market, and offer our customers a turn-key solution is unmatched by other nurseries. We grow over one thousand types of nonnative plants in the demanding Florida climate using a variety of environments: greenhouse, shade, and sun. Our research and development team features an array of botanical experts. One such expert, Mike Rimland, served on the Plants for Clean Air Counsel and launched O2 for You with Dr. Bill Wolverton of NASA, spearheaded Dummen Mandevilla to make us the fifth largest grower of Mandevilla in the world with 1 to 2 million plants produced annually, and established several proprietary programs including Glowee, Velvet Touch, and WaterWick, among others.

These qualities should be represented when developing a rule to implement the Compassionate Use Act. The statute allows only five producers to supply a market the size of Florida with a product derived from a plant nonnative to the state. Success is critical to ensure that vulnerable patients receive the medication as quickly as possible. We have proven the ability to do so, and more, with our differentiated, market leading approach.

The success of Costa Farms has resulted from years of hard work by a team that is best in class. Our team crosses all main functions: sourcing, growing, production, logistics, IT, sales and marketing, category management, and finance, and features vice presidents, directors, and managers with diverse and exceptional credentials. For example, prior to joining Costa Farms, Pedro Freyre, Vice President of the Foliage Division, consulted with Bain & Company for Fortune 500 companies and graduated from Tuck Business School at Dartmouth. Arianna Cabrera, our general counsel, worked at Akerman Senterfitt, a national law firm, after earning a Juris Doctor from Harvard Law School. The family members that run Costa Farms have nearly 70 years of combined farming experience. With such expertise and business

21800 SW 162nd Avenue | Miami, Florida 33170 | T 305-247-3248 | F 786-272-6137

acumen, we will effectively and efficiently represent the nursery industry and will facilitate an informed discussion among all of the parties present at the negotiation.

Also consider that since Governor Scott signed Senate Bill 1030 into law, Costa Farms has actively participated in DOH's rulemaking efforts. We believe this topic is important and complex and thus have dedicated personnel from our research and development team as well as senior executives to the task of fully understanding scientific and business requirements of cultivating, processing and dispensing low-THC cannabis.

Indeed, we have used this knowledge to responsibly participate at each juncture; we attended every workshop and hearing and provided constructive comments on the rule drafts. Through our participation we have developed a deep understanding of the process and the various interests at stake. Above all is the patient interest: to obtain a safe and high quality medication in as short a time frame as possible. We will facilitate a continuity of successful ideas from the past, bring new and thoughtful concepts to bear on the future rule, and help guide the negotiated rulemaking committee around previously experienced pitfalls.

Finally, Costa Farms will use expertise to shed light on operational issues such as market size and revenues and costs to start up and operate, each of which affect profitability. Understanding the business model will help us advise DOH's economic analysis and the implications for pricing. We aim to ensure that the most cost effective production model is used so that patients may obtain the medication at a reasonable price point. Costa Farms aspires to develop and usher in a rule that allows for the production of the most effective medicine in the most time-sensitive and responsible manner possible.

Costa Farms, LLC – Executive Team Bios

Jose "Joche" Smith
President and Chief Executive Officer

Joche has been part of the Costa Farms senior management team since 1992, having served as President during most of that time. During his first full year at Costa Farms, annual revenues were $13M. Since then, the company's annual revenues have exceeded $300M. Joche's primary roles with Costa Farms center around sales, customer relationships and corporate finance. Joche has led the company's transformation from a production and sales-oriented organization to one with a significant focus on information systems, research and development, and innovation. Under his guidance, Costa Farms has transformed itself into a partner for its customers – providing IT solutions, logistics solutions, product development, and marketing support. Joche has also been instrumental in expanding the portfolio of products offered by Costa Farms to include annuals and perennials, and expansion of the company's geographic scope from South Florida to Central Florida, North Carolina, and South Carolina. Prior to joining Costa Farms, Jose Smith worked as a financial analyst at Ryder System, Inc.

In 2012, Mr. Smith was selected to serve on the Lowe's Vendor Advisory Council, where, together with 10 other vendors, he spent two years providing feedback to the senior management of Lowe's. He is currently serving on the Miami-Dade County Mayor's Roundtable, having been selected to provide advice on local civic matters. He earned both his Bachelor of Science in Industrial Engineering and his M.B.A. from the University of Florida.

Jose Costa III
Executive Vice President- Foliage Division

Jose joined Costa Farms full-time in 1995 and currently serves as Executive Vice President of the Foliage division. Jose oversees production as well as numerous enterprise support areas, including logistics, purchasing and the company's banking relationships. Jose was instrumental in the establishment of a buying office in the Far East, professionalization of the purchasing function, implementation of innovative logistics solutions, expansion of local business into the Dominican Republic markets, and the absorption of multiple acquisitions into Costa Farms including: First Foliage, Fancy Flora, Premier Nursery, Geerlings Nursery, and Hermann Engelmann Greenhouses. Jose also manages the acquisition and management of landholding assets in South Florida, Central Florida, North Carolina, South Carolina and the Dominican Republic. During his career at Costa Farms, the company's annual revenues grew over 10 times- from $30M to over $300M. He was instrumental in the leadership during this period of rapid growth and oversaw infrastructure developments to support the company's expansion.

Mr. Costa earned his Bachelor of Science from the College of Agriculture at the University of Florida and his M.B.A. from the University of Miami. He currently serves on the Board of Log Cabin Enterprises, Inc., a part of the Sunrise Community of organizations dedicated to helping people with physical and intellectual challenges achieve greater independence, dignity, and personal freedom.

Maria Costa Smith
Executive Vice President- Color Division

Maria has worked at Costa Farms since 1991 and currently serves as the Executive Vice President of the Color divisions located in Miami, Florida; North Carolina; and South Carolina, encompassing both annual and perennial operations. Her responsibilities include production, sales, operations, and systems development. Maria oversees replenishment, inventory, category management, and all other main operating functions for the Color division. During her tenure, the young plant unit, Total Growth solutions, has expanded significantly. She also leads IT and R&D for the entire company of Costa Farms. Maria has served in several positions throughout the organization, notably heading the Foliage division from 1991 to 1997.

Mrs. Smith earned her Master's degree in agriculture engineering from the University of Florida as well as a Bachelor's degree in ornamental horticulture from the University of Florida. She was named a Woman of Distinction in Agriculture (2010) sponsored by the Farm Bureau of Dade County among others. She is a member of the Board of Directors for Homestead Hospital which is part of Baptist Health Systems. Mrs. Smith has been instrumental in mission trips for Medical Students in Action, providing medical care to villages throughout the Dominican Republic.

Arianna Cabrera
General Counsel and Director of Government Affairs

Arianna has served as General Counsel of Costa Farms since February 2006. She has led the legal department for the last 9 years managing litigation, employment practices, intellectual property, transportation, real estate, and general corporate legal matters for all of the company's divisions. As the Director of Government Affairs, she serves as a liaison between the Company and a myriad of government and regulatory compliance agencies, including the Department of Agriculture, the Fish and Wildlife Commission, the USDA and several water management districts. She was instrumental in guiding the company in its expansion through 10+ major acquisitions. Prior to joining Costa Farms, she was an attorney at the Miami office of Akerman Senterfitt, a national law firm. While at Akerman Senterfitt, Arianna worked in the Corporate Tax Group, specializing in mergers and acquisitions, private equity funding, SEC compliance, employment and compensation matters, and international tax planning. Between 1999 and 2000 she worked for Gerald Stevens Inc., a Huizenga group start-up company, and accomplished over 50 acquisitions in the floral retail market.

Ms. Cabrera earned her Juris Doctor from Harvard Law School and graduated with honors from the University of Miami as Valedictorian with a Bachelor's degree in business administration with a concentration in accounting.

Pedro Freyre
Vice President- Foliage Division

Pedro has been with Costa Farms since January 2011. During Pedro's time with the company, he implemented an integrated forecasting production planning system, incorporated lean manufacturing practices throughout farm operations, and created a category management function to optimize sales and increase profitability. Pedro also oversees operations of the Foliage division and enterprise-wide functions such as: warehouse management, process engineering, the Far East sourcing office, and the Dominican Republic farms and operations. Prior to joining Costa Farms, he spent seven years at Bain & Co., a global management consulting firm, in their Boston and Atlanta offices. During his time at Bain, Pedro served corporate and private equity clients leading a range of projects, including Corporate Strategy, M&A, Performance Improvement, and Organizational Development. Prior to Bain, Pedro spent five years at Visa International, where he served in a number of roles, including leading the FP&A and Business Intelligence organizations for the Latin American and Caribbean Region. While at Visa he also led the marketing, finance, and human resources efforts for a startup joint venture merchant processor in the Dominican Republic. Previous to Visa, Pedro spent 3 years at Price Waterhouse LLP focused on delivering macro-economic and political risk analyses to clients with financial exposure in Latin America.

Mr. Freyre earned his B.A. in International Affairs from George Washington University and his M.B.A. from the Tuck School of Business at Dartmouth College.

Jose Alvarez
Chief Financial Officer

Jose joined Costa Farms in February 2012. In his role as Chief Financial Officer, he oversees the Company's financial, accounting, and human resources departments. His responsibilities also include oversight of the company's treasury and risk management functions. While at Costa Farms, Jose has implemented strict financial reporting protocols, rolled out integrated manufacturing costing standards, and launched an enterprise-wide Human Resource Information System. Prior to joining Costa Farms, Jose served in various executive management roles at private equity owned Navarro Discount Pharmacies, the largest Hispanic owned pharmacy chain in the country. In his most recent role there he served as Vice President of Retail Merchandising and was responsible for developing and executing the Company's front end sales and margin growth strategy. Prior to that role, Jose served as Navarro's Vice President of Finance, overseeing and upgrading all aspects of the financial operations of the chain, including the financial reporting, FP&A, treasury, inventory valuation, and accounting functions. Prior to joining Navarro, Jose served as the Manager of Financial Reporting at PBS&J, an SEC registered company. He began his career at Ernst and Young LLP in Miami where he held the position of senior auditor. Jose's professional credentials include over 14 years of broad-based financial management and accounting experience, having worked in various roles within publicly traded organizations.

Mr. Alvarez is a graduate of the University of Miami where he earned a Bachelor's degree with honors, in business administration with a concentration in accounting. As an active member of the community, Mr. Alvarez has served in several advisory roles for local non-profit organizations.

Charlie Acevedo
Vice President, Sales and Marketing

Charlie has been with Costa Farms since 2006 and joined the company with over 20 years of experience in sales and marketing. On the sales side, Charlie manages sales directors for all Costa Farms' accounts as well as the sales replenishment and analysis team. He is an active participant in the strategic planning and business development process to grow revenues across the company. Charlie takes a customer-centric approach to identify and develop these opportunities with key accounts. He has led initiatives to focus on a new store environment for customers as well as increasing penetration. On the marketing side, he sets strategic vision and direction, and oversees product development and innovation. Charlie spearheaded a net promoter initiative by spending time with Zappos and integrating best practices at Costa Farms. He helped transform the culture to make the company a fun yet professional and productive place to work. Prior to Costa Farms, he owned a market-leading Anheuser Busch distributorship that serviced South Miami Dade and Monroe counties. During that time, he sat on the Anheuser Busch wholesaler national advisory board, representing over 700 distributors. Charlie worked for Anheuser Busch in the US and in Latin America. He focused on sales and brand management, managing the Bud Light brand for 2 years. He was Vice President of Latin America for 5 years, and he oversaw joint ventures and distributor partnerships in Central and South America. Charlie also sat on the board of Budweiser Brasil.

Mr. Acevedo earned his Bachelor of Science in business administration from the University of Florida. He is on the advisory board for the University of Florida's Tropical Research and Education Center.

LJ Contillo
Vice President- Color Division

LJ has worked with Costa Farms since inception of the Color division in Miami, Florida in 1997. Acting in the capacity of Operations Manager, LJ's leadership role was instrumental in building the business from the ground floor up including design and build of the infrastructure, recruitment and development of team members, forecasting/production planning, and other critical enterprise functions. He is now Vice President as a result of his experience and success in initially launching this business unit. LJ's current responsibilities include oversight of the Color division supply chain management, live goods purchasing and procurement, cost reduction, and product development. He has also made a significant contribution to the TGS young plant business and R&D. LJ has developed a keen knowledge of the customers- the retailers- and continues to nurture these relationships thus reducing customer churn.

Prior to joining Costa Farms, LJ spent seven years at Caple Farms, one of the largest producers of annuals in the eastern US. During his time at Caple Farms, LJ's responsibilities included sales, merchandising management, inventory management, distribution, order writing, sales budgeting/forecasting, and production planning. He attended College at Walter State in Morristown, TN.

Bist, Kevin

From:	Jill Lamoureux <jill.lamoureux@cannlabs.com>
Sent:	Friday, January 09, 2015 2:35 PM
To:	Nelson, Patricia A
Cc:	Bist, Kevin
Subject:	NEGOTIATED RULEMAKING COMMITTEE MEETING
Follow Up Flag:	Follow up
Flag Status:	Flagged

Hello Patricia -

CannLabs (www.cannlabs.com), a medical cannabis testing laboratory with over four year's experience in Colorado and Connecticut would like to participate in the Negotiated Rulemaking Committee process in February. I would serve as the representative for the organization. Information on the company and my bio is below. Thank you in advance for your consideration. Please confirm you have received this and if you could let me know when you will make selections, I would very much appreciate it. Sincerely, Jill

Jill Lamoureux
Carbon Bond Holdings (gov't relations for CannLabs, Inc. in CO & CT)
3888 E. Mexico Ave. #202
Denver, CO 80210
Main: 303-309-0105
Direct: 720-335-1386

CannLabs, Inc. Description

CannLabs, Inc., is a
testing and science
solutions provider with intellectual property, proprietary cloud-based analytics and scientific methods to serve the cannabis industry. Through Carbon Bond Holdings Inc., CannLabs provides laboratories with the necessary business intelligence technology and other solutions required to serve the cannabis industry, and these include the first state-licensed laboratory in Denver, Colorado.

CannLabs enables growers, dispensaries and edible makers to implement best practices while furthering product advancements. In addition, the CannLabs website allows consumers to easily locate certified tested products to meet their specific needs.

CannLabs has been at the forefront of educating the public, in addition to state and local governments as more states legalize marijuana for various uses. The Company provides client education around safe cultivation, identifying contamination problems, consulting on legal cases and the reformulation of cannabis products, throughout the U.S. and Canada.

Jill Lamoureux Background

Jill Lamoureux is the Director of Government Relations and Compliance for Carbon Bond Holdings. She is a nationally recognized former cannabis business operator in Colorado with extensive experience in cultivation, manufacturing and distribution. Jill was the only industry representative to serve on both the Colorado Department of Public Health and Environment and the Colorado Department of Revenue

medical marijuana advisory committees. She also serves as a subject matter expert to BOTEC Analysis Corporation and provides consulting services to operators in multiple jurisdictions.

Jill has personally experienced the positive quality-of-life impact medical cannabis has on individuals and their families and has been an ardent patient advocate. Due to her first-hand experience with developing a newly regulated system in Colorado, Jill understands the importance of addressing patient, industry, and regulator needs in an effective system.

Jill has presented on cannabis operations, financing, and regulation in multiple venues including finance conferences, continuing legal education courses, law review panels, and most recently at RAND's public policy workshop. She earned her MBA from the University of Denver and has worked in small business start-ups and quasi-governmental entities. Jill served as a municipal bond trustee, finance director, and treasurer for eight years before entering the medical cannabis industry.

She served as the second Chair of the National Cannabis Industry Association. Jill currently serves as the Chair of the Patient Focus Certification Review Board and as a member of the American Herbal Products Association Cannabis Committee and Women Grow.

Experience Helping Regulations in Other States

Jill was a member of the first rulemaking group for the Colorado Department of Revenue's Marijuana Enforcement Division which crafted the first set of regulations around medical marijuana in 2010. She was also the first business owner representative to also serve on the Colorado Department of Health and Environment's first (and only) workgroup to develop regulations for patients, caregivers and doctors related to the Medical Marijuana Act. Since then Jill served on multiple subgroups in Colorado including working as an independent contractor to all three trade associations on drafting rules revisions. Jill also served as a subject matter expert to BOTEC Analysis Corp. which was the consultant to the Washington State Liquor Control Board developing rules for retail marijuana. Through client groups, Jill provided feedback on multiple regulatory models including CT, MA, IL and NV. Most recently Jill has worked directly with legislators and regulators in GA and MD commenting on rule drafts.

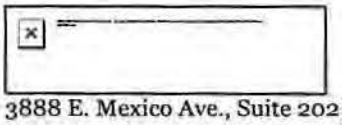
How Rulemaking Process will Affect Parties Represented

At this date, Jill does not have any clients seeking a license. However, CannLabs, Inc. has not made a determination regarding an independent testing lab in FL. A decision will be made based on final rules which will impact CannLabs estimate of the market (quantity of tests mandated, etc.) and its ability to survive as an independent lab. For instance, Jill has assisted with drafting in GA, but based on the restrictive nature of the program, CannLabs, Inc. will not seek to open a lab in GA at this time.

Statement of Good Faith

Jill Lamoureux is able to attend all necessary meetings and will negotiate in good faith. Jill's priorities in rulemaking are a workable system for operators and regulators which also protects the safety and interest of patients.

Jill Lamoureux
Director of Government Affairs
720 | 335-1386
jill.lamoureux@cannlabs.com



3888 E. Mexico Ave., Suite 202| Denver, CO 80210

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Bist, Kevin

From: Pence, Heather <HeatherPence@fdle.state.fl.us>
Sent: Friday, January 09, 2015 3:18 PM
To: Nelson, Patricia A
Subject: FW: Low-THC Cannabis Rulemaking
Attachments: FAR.1.5.15.pdf

Follow Up Flag: Follow up
Flag Status: Flagged

Patty,

Have you heard from other entities trying to seek a law enforcement representative on the committee? Also, do you know when the committee representatives will be selected?

Thanks,

Heather

Heather Pence
Office of External Affairs | Florida Department of Law Enforcement
Office: (850) 410 - 7147

From: Nelson, Patricia A [mailto:Patricia.Nelson@flhealth.gov]
Sent: Monday, January 05, 2015 5:11 PM
To: DL 64-4 Interested Parties; Dunn, Nathan P
Subject: Low-THC Cannabis Rulemaking

Dear Interested Parties,

Please see the attached Notice of Negotiated Rulemaking (on page 1 of the attached document) scheduled for February 4 and 5, 2015, in Tallahassee.

Sincerely,
Patty

Patricia Nelson
Director
Office of Compassionate Use

Section I
Notice of Development of Proposed Rules and Negotiated Rulemaking

DEPARTMENT OF HEALTH
Office of Compassionate Use

RULE NOS.:	RULE TITLES:
64-4.001	Regulatory Structure Rule 1
64-4.002	Regulatory Structure Rule 2
64-4.003	Regulatory Structure Rule 3
64-4.004	Regulatory Structure Rule 4
64-4.005	Regulatory Structure Rule 5
64-4.006	Regulatory Structure Rule 6
64-4.007	Regulatory Structure Rule 7
64-4.008	Regulatory Structure Rule 8
64-4.009	Compassionate Use Registry

PURPOSE AND EFFECT: The Department of Health announces the convening of a negotiated rulemaking proceeding to address the regulatory structure for dispensing organizations of low-THC cannabis. The purpose of the negotiated rulemaking is to draft mutually acceptable proposed rules.

SUBJECT TO BE ADDRESSED: The subject and scope of the rules to be developed through negotiated rulemaking will be the regulatory structure for dispensing organizations of low-THC cannabis.

RULEMAKING AUTHORITY: 381.986 FS.

LAW IMPLEMENTED: 381.986 FS.

NEGOTIATED RULEMAKING COMMITTEE: The negotiated rulemaking committee members will be selected from the following representative groups:

1. A nursery that meets the criteria in Section 381.986(5)(b)1., Florida Statutes;
2. A qualified patient or patient representative;
3. A testing laboratory;
4. A member of the Florida Bar experienced in administrative law;
5. An individual with demonstrated experience in sound agricultural practices and necessary regulation;
6. A physician authorized to order low-THC Cannabis products for qualified patients;
7. An individual with demonstrated experience establishing or navigating regulatory structures for cannabis in other jurisdictions; and
8. Representatives of the Department of Health.

If you believe that your interests are not adequately represented by the committee members listed above, you may apply to participate within 30 days of the date of publication of this notice. Your application must contain the following information: your name, business address, and telephone number; the name of any organization you are representing; a description of the organization or the members of the organization; a description of how the proposed rulemaking proceedings will affect you or the parties that you represent; a statement identifying the reasons why you believe the representative groups listed above will not adequately represent your interests; and a statement that you are willing to negotiate in good faith and can attend the scheduled meeting. Please submit your application to Patricia Nelson, Department of Health, 4052 Bald Cypress Way, Bin A-02, Tallahassee, Florida 32399, email address: Patricia.Nelson@flhealth.gov.

NEGOTIATED RULEMAKING COMMITTEE MEETING: The committee will meet at the following date, time, and place to discuss rule development: February 4, 2015, 8:00 a.m. - 10:00 p.m. and February 5, 2015, 8:00 a.m. until concluded, Department of Health, Room 301, 4052 Bald Cypress Way, Tallahassee, FL 32399.

Pursuant to provisions of the Americans with Disabilities Act, any person requiring special accommodations to participate in this meeting is asked to advise the agency at least 72 hours before the meeting by contacting Sophia Flowers, Department of Health, (850)245-4005, Sophia.Flowers@flhealth.gov. If you are hearing or speech impaired, please contact the agency using the Florida Relay Service, 1 (800)955-8771 (TDD) or 1 (800)955-9770 (Voice).

THE PERSON TO BE CONTACTED REGARDING THE PROPOSED RULE DEVELOPMENT AND A COPY OF THE PRELIMINARY DRAFT, IF AVAILABLE IS: Patricia Nelson, Department of Health, 4052 Bald Cypress Way, Bin A-02, Tallahassee, Florida 32399, Email address: Patricia.Nelson@flhealth.gov

THE PRELIMINARY TEXT OF THE PROPOSED RULE DEVELOPMENT IS NOT AVAILABLE.

Section II
Proposed Rules

AGENCY FOR HEALTH CARE ADMINISTRATION
Certificate of Need

RULE NOS.:	RULE TITLES:
59C-1.040	Hospital Inpatient General Psychiatric Services
59C-1.041	Hospital Inpatient Substance Abuse Services

PURPOSE AND EFFECT: The Agency is proposing to amend Rules 59C-1.040 and 59C-1.041, F.A.C., to remove, update and condense language regarding new hospital inpatient psychiatric services and hospital inpatient substance abuse services. The amendments will also incorporate material regarding population estimates.

SUMMARY: The proposed amendments to this rule include: updates to definitions, updates to language on excluded hospitals, adding incorporated material, updating statutory language, improving/condensing the language for the needs assessment methodology, removing references to State and Local Health Council District health plans, removing language citing Rule 59C-1.038, F.A.C. (repealed), removing language requiring accreditation pursuant to Section 408.043(4), F.S., removing obsolete language and items from the utilization reporting requirement, removing language regarding amended licenses and removing language regarding the applicability of the amended rule.

SUMMARY OF STATEMENT OF ESTIMATED REGULATORY COSTS AND LEGISLATIVE RATIFICATION:

The Agency has determined that this will not have an adverse impact on small business or likely increase directly or indirectly regulatory costs in excess of $200,000 in the aggregate within one year after the implementation of the rule. A SERC has not been prepared by the Agency

The Agency has determined that the proposed rule is not expected to require legislative ratification based on the statement of estimated regulatory costs or if no SERC is required, the information expressly relied upon and described herein: A checklist was prepared by the Agency to determine the need for a SERC. As there will be no impact on economic growth, job creation or employment, private-sector investment, or business competitiveness and no increase in regulatory costs – no adverse impact is likely.

Any person who wishes to provide information regarding a statement of estimated regulatory costs, or provide a proposal for a lower cost regulatory alternative must do so in writing within 21 days of this notice.

RULEMAKING AUTHORITY: 408.034(3), (8), 408.15(8) FS.

LAW IMPLEMENTED: 408.034(3), 408.035, 408.036(1)(b), (c), 408.039(4)(a) FS.

IF REQUESTED WITHIN 21 DAYS OF THE DATE OF THIS NOTICE, A HEARING WILL BE HELD AT THE DATE, TIME AND PLACE SHOWN BELOW (IF NOT REQUESTED, THIS HEARING WILL NOT BE HELD):

DATE AND TIME: January 27, 2015, 8:00 a.m. – 10:00 a.m.

PLACE: The Agency for Health Care Administration, 2727 Mahan Drive, Building 3, Conference Room C, Tallahassee, Florida 32308

Pursuant to the provisions of the Americans with Disabilities Act, any person requiring special accommodations to participate in this workshop/meeting is asked to advise the agency at least 1 days before the workshop/meeting by contacting: Marisol Fitch, Certificate of Need, 2727 Mahan Drive, Mail Stop 28, Building 1, Tallahassee, Florida or call (850)412-4346 or email at Marisol.fitch@ahca.myflorida.com. If you are hearing or speech impaired, please contact the agency using the Florida Relay Service, 1(800)955-8771 (TDD) or 1(800)955-8770 (Voice).

THE PERSON TO BE CONTACTED REGARDING THE PROPOSED RULE IS: Marisol Fitch, Certificate of Need, 2727 Mahan Drive, Mail Stop 28, Building 1, Tallahassee, Florida or call (850)412-4346 or email at Marisol.fitch@ahca.myflorida.com

THE FULL TEXT OF THE PROPOSED RULE IS:

59C-1.040 New Hospital Inpatient ~~General~~ Psychiatric Services.

(1) Agency Intent. This rule implements the provisions of Sections 408.032(9), 408.034(3), 408.034(6), 408.036(1)(b) and (c), 395.002(15), and 395.003(4), F.S. It is the intent of the agency to ensure the availability of hospital inpatient general psychiatric services as defined in this rule for children, adolescents and adults in need of these services regardless of their ability to pay. This rule regulates the establishment of new ~~hospital~~ inpatient ~~general~~ psychiatric hospitals and new inpatient intensive residential treatment facilities for children and adolescents ~~services, the construction or addition of new hospital inpatient general psychiatric beds, the conversion of licensed hospital beds to hospital inpatient general psychiatric beds,~~ and specifies which services can be provided by licensed or approved providers of hospital inpatient general psychiatric services.

(2) Definitions.

(a) "Adolescent." A person age 14 through 17.

(b) "Adult." A person age 18 and over.

(c) "Agency." The Agency for Health Care Administration. ~~"Approved Hospital Inpatient General Psychiatric Bed." A proposed hospital inpatient general psychiatric bed for which a certificate of need, a letter of intent to grant a certificate of need, a signed stipulated agreement, or a final order granting a certificate of need was issued, consistent with the provisions of paragraph 59C-1.008(2)(b), F.A.C., as of the most recent published deadline for agency initial decisions prior to publication of the fixed need pool, as specified in paragraph 59C-1.008(1)(g), F.A.C.~~

(d) "Approved Hospital Inpatient General Psychiatric Bed." A proposed hospital inpatient general psychiatric bed for which an exemption pursuant to paragraph 59C-1.005(6)(i), F.A.C., or notification pursuant to subsection 59C-1.0085(2), F.A.C., a letter of intent to grant a certificate of need, a signed stipulated agreement, or a final order granting a certificate of need was issued, consistent with the provisions of paragraph 59C-1.008(2)(b), F.A.C., as of the most recent published deadline for agency initial decisions prior to publication of the fixed need pool, as specified in paragraph 59C-1.008(1)(g),

F.A.C. ~~"Charity Care." The portion of hospital charges for which there is no compensation, for care provided to patients whose family income as applicable for the 12 months preceding the determination does not exceed 150 percent of the current Federal Poverty Guidelines, or for care provided to patients for whom the hospital charges are greater than 25 percent of an annual family income which does not exceed four times the current Federal Poverty Level for a family of four, as defined in the Health Care Board's Florida Hospital Uniform Reporting System Manual, Chapter III, Section 3223.~~

(e) "Charity Care." As defined in Section 409.911(1), F.S., charity care is the portion of hospital charges reported to the Agency for Health Care Administration for which there is no compensation, other than restricted or unrestricted revenues provided to a hospital by local governments or tax districts regardless of the method of payment, for care provided to a patients whose family income for the 12 months preceding the determination is less than or equal to 200 percent of the federal poverty level, unless the amount of hospital charges due from the patient exceeds 25 percent of the annual family income. However, in no case shall the hospital charges for a patient whose family income exceeds four times the federal poverty level for a family of four be considered charity. ~~"Child." A person under the age of 14 years.~~

(f) "Child." A person under the age of 14 years. ~~"Department." The Agency for Health Care Administration.~~

(g) "District." A district of the agency defined in Section 408.032(5), F.S.

(h) "Fixed Bed Need Pool." The numerical hospital inpatient general psychiatric bed need for adults, or for children and adolescents, for the applicable planning horizon, as established by the agency in accordance with this rule and subsection 59C-1.008(2), F.A.C.

(i) "General Hospital." Means any facility which meets the provisions of Section 395.002(12), F.S., and which makes its facilities and ~~A hospital which provides~~ services available to the general population ~~and does not restrict its services to any specified medical or psychiatric illness or to any specified age or gender group of the population, as defined in Section 395.002(10), F.S.~~

(j) "Gross Bed Need." The number of hospital inpatient ~~general~~ psychiatric beds projected for a district for the applicable planning horizon under paragraphs (4)(c) or (4)(f) of this rule, except that the number of licensed beds and approved beds is not subtracted from the projected total.

(k) "Hospital Inpatient ~~General~~ Psychiatric Bed." A bed designated for the exclusive use of patients receiving hospital inpatient ~~general~~ psychiatric services as defined by this rule.

(l) "Hospital Inpatient ~~General~~ Psychiatric Services." Services provided under the direction of a psychiatrist or clinical psychologist to persons whose sole diagnosis, or in the event of more than one diagnosis, the principal diagnosis ~~as defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R)~~ is a psychiatric disorder defined in paragraph (2)(p) of this rule.

(m) "Intensive Residential Treatment Program for Children and Adolescents." An inpatient program which provides intensive residential treatment services for children and adolescents, as described in Section 395.002(15), F.S., including 24-hour care and diagnosis and treatment of patients under the age of 18 having psychiatric disorders. A facility may seek certificate of need approval for an intensive residential treatment program for children and adolescents in order to have the program beds licensed as specialty hospital beds for hospital inpatient ~~general~~ psychiatric services, in accordance with Section 395.003(2)(c)~~(f)~~, F.S., and Chapter 59A-3, F.A.C. The net need for intensive residential treatment program beds for children and adolescents in facilities seeking to have the beds licensed as specialty hospital beds is included in the net need for hospital inpatient ~~general~~ psychiatric beds for children and adolescents calculated under paragraph (4)(f) of this rule.

(n) "Local Health Council." The council referenced in Section 408.033, F.S.

(o) "Planning Horizon." The projected date by which a proposed hospital inpatient ~~general~~ psychiatric service would be initiated. For purposes of this rule, the planning horizon for applications submitted between January 1 and June 30 is July of the year 5 years subsequent to the year the application is submitted; the planning horizon for applications submitted between July 1 and December 31 is January of the year 5 years subsequent to the year which follows the year the application is submitted.

(p) "Psychiatric Disorder." For purposes of this rule, a psychiatric disorder is a mental illness as defined in Section 394.455(18), F.S., which requires inpatient hospitalization. ~~disorder coded in any sub-classification of category 290 or coded in any sub-classification of categories 293 through 302 or coded in any sub-classification of categories 306 through 316, in Axis I or Axis II, consistent with the diagnostic categories defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R), incorporated herein by reference; or equivalent codes in the following sub-classifications in the International Classification of Disease (ICD-9), incorporated herein by reference: category 290, 293 through 302, or 306 through 316.~~

(q) "Separately Organized Unit." A specific section, ward, wing, or floor with a separate nursing station designated exclusively for the care of hospital inpatient ~~general~~ psychiatric services patients.

(r) "Specialty Beds." Specialty beds include ~~comprehensive medical rehabilitation beds,~~ hospital inpatient substance abuse beds, ~~and the~~ hospital inpatient ~~general~~ psychiatric beds and beds in intensive residential treatment programs for children and adolescents licensed as specialty hospital beds ~~which are regulated under this rule~~.

(s) "Specialty Hospital." A specialty hospital is as defined by subsection 395.002(28), F.S. ~~which restricts its services to a specified category of medical or psychiatric illness or to a specified age or gender group of the population, as defined in Section 395.002(28), F.S., including a facility with an intensive residential treatment program for children and adolescents which is licensed as a specialty hospital~~.

(t) "Substance Abuse." The misuse or abuse of, or dependence on alcohol, illicit drugs, or prescription medications which requires inpatient hospitalization. ~~A disorder coded in any sub-classification of categories 291, 292, 303, 304 or 305 in Axis I or Axis II consistent with the diagnostic categories defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R), incorporated herein by reference; or~~ equivalent ~~codes in any sub-classification of categories 291, 292, 303, 304 or 305 consistent with the diagnostic categories defined in the International Classification of Diseases (ICD-9), incorporated herein by reference~~.

(3) General Provisions.

(a) Admissions to Hospital Inpatient ~~General~~ Psychiatric Services. Admission to facilities with a certificate of need for hospital inpatient ~~general~~ psychiatric services is limited to persons whose sole diagnosis, or in the event of more than one diagnosis, the principal diagnosis, is a psychiatric disorder as defined in paragraph (2)(p) ~~in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R) is a disorder coded in any sub-classification of category 290 or coded in any sub-classification of categories 293 through 302 or coded in any sub-classification of categories 306 through 316, in Axis I or Axis II, consistent with the diagnostic categories defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R), incorporated herein by reference; or equivalent codes in the following sub-classifications in the International Classification of Disease (ICD-9), incorporated herein by reference: category 290, 293 through 302, or 306 through 316~~. Psychiatric patients in need of medical/surgical care may be treated in acute care medical/ surgical beds for their medical/surgical care needs or in a psychiatric services unit if the unit is properly staffed and equipped to care for the medical/surgical problem.

(b) Service Location. This rule applies to the establishment of new psychiatric inpatient ~~The hospital inpatient general psychiatric services regulated under this rule may be provided in a hospital licensed as a general hospital or licensed as a~~ specialty hospitals, including a facility with an intensive residential treatment program for children and adolescents which is licensed as a specialty hospital.

(c) Separate Regulation of Age Categories. The agency regulates two types of hospital inpatient general psychiatric services, those services which are used for ~~general~~ psychiatric treatment of adults, and those services used for ~~general~~ psychiatric treatment of children and adolescents. Certificate of need applications for the establishment of hospital inpatient ~~general~~ psychiatric services for adults shall be reviewed separately from certificate of need applications for hospital inpatient ~~general~~ psychiatric services for children and adolescents. A separate certificate of need shall be issued for each service.

(d) Separately Organized Units. Hospital inpatient ~~general~~ psychiatric services for adults shall be provided in one or more separately organized units within a ~~general hospital or~~ specialty hospital. Hospital inpatient ~~general~~ psychiatric services for children and adolescents shall be provided in one or more separately organized units within ~~a general hospital or~~ specialty hospital.

(e) Minimum Size of Specialty Hospitals. A specialty hospital providing hospital inpatient ~~general~~ psychiatric services, or an intensive residential treatment program for children and adolescents licensed as a specialty hospital, shall have a minimum total capacity of 40 beds. The minimum capacity of a specialty hospital providing hospital inpatient ~~general~~ psychiatric services may include beds used for hospital inpatient substance abuse services regulated under Rule 59C-1.041, F.A.C. The separately organized units for hospital inpatient ~~general~~ psychiatric services for adults or for children and adolescents in specialty hospitals shall meet the minimum size requirements stated in subsection (5) of this rule. ~~Hospitals inventoried consistent with the provisions of subsection (11) of this rule are exempt from meeting the minimum capacity and minimum unit size requirements of this paragraph unless or until they submit a proposal which would modify the number of beds listed in the inventory.~~

(f) Conformance with the Criteria for Approval. A certificate of need for the establishment of new ~~hospital~~ inpatient ~~general~~ psychiatric specialty hospital ~~services, or the expansion of existing services by addition of beds,~~ shall not normally be approved unless the applicant meets the applicable review criteria in Section 408.035, F.S., and the standards and need determination criteria set forth in this rule.

(g) Required Services. Hospital inpatient psychiatric services whether provided directly by the hospital or under contract shall include, at a minimum, emergency screening services, pharmacology, individual therapy, family therapy, activities therapy, discharge planning, and referral services. ~~Accreditation of Intensive Residential Treatment Programs for Children and Adolescents. A licensed intensive residential treatment program for children and adolescents applying for a certificate of need in order to have the program beds licensed as specialty hospital beds shall be accredited by the Joint Commission on Accreditation for Healthcare Organizations prior to submitting a certificate of need application.~~

(h) Excluded Hospitals. Hospitals operated by the State of Florida are not regulated under this rule pursuant to Section 408.036(d), (r) and (s), F.S. ~~Required Services. Hospital inpatient general psychiatric services whether provided directly by the hospital or under contract shall include, at a minimum, emergency screening services, pharmacology, individual therapy, family therapy, activities therapy, discharge planning, and referral services.~~

~~(i) Excluded Hospitals. Hospitals operated by the State of Florida or the federal government are not regulated under this rule.~~

(4) Criteria for Determination of Need.

(a) Bed Need. A favorable need determination for proposed new inpatient psychiatric ~~or expanded~~ hospitals ~~inpatient general psychiatric services~~ shall not normally be made unless a bed need exists according to the numeric need methodology specified in paragraph (4)(c) or (4)(f) of this rule.

(b) Fixed Bed Need Pool. The future need for hospital inpatient ~~general~~ psychiatric services for adults shall be determined twice a year and published by the agency as a fixed bed need pool for the applicable planning horizon. The future need for hospital inpatient ~~general~~ psychiatric services for children and adolescents shall be determined twice a year and published by the agency as a fixed bed need pool for the applicable planning horizon.

(c) Need Formula for Hospital Inpatient ~~General~~ Psychiatric Beds for Adults. The net bed need for hospital inpatient ~~general~~ psychiatric beds for adults in each district shall be calculated in accordance with the following formula:

NNA = ((PDA/PA × PPA) / (365 × .75)) - LBA - ABA

where:

1. NNA equals the net need for hospital inpatient ~~general~~ psychiatric beds for adults in a district.

2. PDA equals the number of inpatient days in hospital inpatient ~~general~~ psychiatric beds for adults in a district for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.

3. PA equals the estimated population age 18 and over in the district. For applications submitted between January 1 and June 30, PA is the population estimate for January of the preceding year; for applications submitted between July 1 and December 31, PA is the population estimate for July of the preceding year. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool. The following material is incorporated by reference within this rule: the Florida Population Estimates and Projections by AHCA District 2010 to 2030, released September 2013. This publication is available on the Agency website at http://ahca.myflorida.com/MCHQ/CON_FA/Publications/index.shtml and http://www.flrules.org/Gateway/reference.asp?No=Ref-01677.

4. PPA equals the estimated population age 18 and over in the district for the applicable planning horizon. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool.

5. .75 equals the desired average annual occupancy rate for hospital inpatient ~~general~~ psychiatric beds for adults in the district.

6. LBA equals the district's number of licensed hospital inpatient ~~general~~ psychiatric beds for adults as of the most recent published deadline for agency initial decisions prior to publication of the fixed bed need pool.

7. ABA equals the district's number of approved hospital inpatient ~~general~~ psychiatric beds for adults, as determined consistent with the provisions of paragraph (2)(c) of this rule.

(d) Need Formula for Hospital Inpatient Psychiatric Beds for Children and Adolescents. The net bed need for hospital inpatient psychiatric beds for children and adolescents in each district, including beds in intensive residential treatment programs for children and adolescents seeking to have the program beds licensed as specialty hospital beds, shall be calculated in accordance with the following formula: ~~Special Circumstances for Approval of Expanded Capacity at Hospitals with Licensed Hospital Inpatient General Psychiatric Services for Adults. Need for additional hospital inpatient general psychiatric beds for adults is demonstrated at a hospital with licensed hospital inpatient general psychiatric services for adults in the absence of need shown under the formula in paragraph (4)(c), or the provision specified in subparagraph (4)(e)3., and regardless of the average annual district occupancy rate determined under subparagraph (4)(e)4., if the occupancy rate or the hospital's inpatient general psychiatric beds for adults equalled or exceeded 85 percent for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

NNCA = ((PDCA/PCA × PPCA) / (365 × .75)) - LBCA - ABCA

where:

1. NNCA equals the net need for hospital inpatient psychiatric beds for children and adolescents in a district, including beds in intensive residential treatment programs for children and adolescents seeking to have the program beds licensed as specialty hospital beds.

2. PDCA equals the number of inpatient days in hospital inpatient psychiatric beds for children and adolescents in a district, including days in intensive residential treatment program beds for children and adolescents which are licensed as specialty hospital beds, for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.

3. PCA equals the estimated population under age 18 in the district. For applications submitted between January 1 and June 30 of each year, PCA is the population estimate for January of the preceding year; for applications submitted between July 1 and December 31 of each year, PCA is the population estimate for July of the preceding year. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool.

4. PPCA equals the estimated population under age 18 in the district for the applicable planning horizon. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool.

5. .75 equals the desired average annual occupancy rate for hospital inpatient general psychiatric beds for children and adolescents in the district, including beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds.

6. LBCA equals the district's number of licensed hospital inpatient psychiatric beds for children and adolescents as of the most recent published deadline for agency initial decisions prior to publication of the fixed bed need pool, including beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds.

7. ABCA equals the district's number of approved hospital inpatient psychiatric beds for children and adolescents, as determined consistent with the provisions of paragraph (2)(c) of this rule, including beds in intensive residential treatment programs for children and adolescents which are seeking to have the program beds licensed as specialty hospital beds.

(e) Other Factors to be Considered in the Review of Certificate of Need Applications for Hospital Inpatient Psychiatric Services for Children and Adolescents. ~~Other Factors to be Considered in the Review of Certificate of Need Applications for Hospital Inpatient General Psychiatric Services for Adults.~~

The applicant for an intensive residential treatment program for children and adolescents seeking to have the program beds licensed as specialty hospital beds shall provide documentation that the district's licensed non-hospital residential treatment programs for children and adolescents with psychiatric disorders do not meet the need for the proposed service. This documentation shall include the number of residential facilities and beds in the district, the type of clients served, and the type of program provided. In addition, the applicant shall provide letters of support from other mental health professionals in the district for the proposed facility.

~~1. Applicants shall provide evidence in their applications that their proposal is consistent with the needs of the community and other criteria contained in Local Health Council Plans, the district Alcohol, Drug Abuse and Mental Health Plan, and the State Health Plan.~~

~~2. Applications from general hospitals for new or expanded hospital inpatient general psychiatric beds for adults shall normally be approved only if the applicant converts a number of acute care beds, as defined in Rule 59C-1.038, F.A.C., excluding specialty beds, which is equal to the number of hospital inpatient general psychiatric beds for adults proposed, unless the applicant can reasonably project an annual occupancy rate of 75 percent for the applicable planning horizon, based on historical utilization patterns, for all acute care beds, excluding specialty beds. If conversion of the number of acute care beds which equals the number of proposed hospital inpatient general psychiatric beds for adults would result in an annual acute care occupancy exceeding 75 percent for the applicable planning horizon, the applicant shall only be required to convert the number of beds necessary to achieve a projected annual 75 percent acute care occupancy for the applicable planning horizon, excluding specialty beds.~~

~~3. In order to ensure access to hospital inpatient general psychiatric services for Medicaid eligible and charity care adults, 40 percent of the gross bed need allocated to each district for hospital inpatient general psychiatric services for adults should be allocated to general hospitals.~~

~~4. Regardless of whether bed need is shown under the need formula in paragraph (4)(c), no additional hospital inpatient general psychiatric beds for adults shall normally be approved in a district unless the average annual occupancy rate of the licensed hospital inpatient general psychiatric beds for adults in the district equals or exceeds 75 percent for the 12 month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

(f) Preferences Among Competing Applicants for Hospital Inpatient Psychiatric Services. In weighing and balancing statutory and rule review criteria, preference will be given to applicants who: ~~Need Formula for Hospital Inpatient General Psychiatric Beds for Children and Adolescents. The net bed need for hospital inpatient general psychiatric beds for children and adolescents in each district, including beds in intensive residential treatment programs for children and adolescents seeking to have the program beds licensed as specialty hospital beds, shall be calculated in accordance with the following formula:~~

~~NNCA = ((PDCA/PCA × PPCA) / (365 × .75)) – LBCA – ABCA~~

~~where:~~

1. Provide Medicaid and charity care days as a percentage of their total patient days equal to or greater than the average percentage of Medicaid and charity care patient days of total patient days provided by other hospitals in the district, as determined by the Florida Center for Health Information and Policy Analysis hospital discharge data for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool. ~~NNCA equals the net need for hospital inpatient general psychiatric beds for children and adolescents in a district, including beds in intensive residential treatment programs for children and adolescents seeking to have the program beds licensed as specialty hospital beds.~~

2. Propose to serve the most seriously mentally ill patients (e.g. suicidal patients; patients with acute schizophrenia; patients with severe depression) to the extent that these patients can benefit from a hospital-based organized inpatient treatment program. ~~PDCA equals the number of inpatient days in hospital inpatient general psychiatric beds for children and adolescents in a district, including days in intensive residential treatment program beds for children and adolescents which are licensed as specialty hospital beds, for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

3. Propose to serve Medicaid-eligible persons. ~~PCA equals the estimated population under age 18 in the district. For applications submitted between January 1 and June 30 of each year, PCA is the population estimate for January of the preceding year; for applications submitted between July 1 and December 31 of each year, PCA is the population estimate for July of the preceding year. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool.~~

4. Propose to serve individuals without regard to their ability to pay. ~~PPCA equals the estimated population under age 18 in the district for the applicable planning horizon. The population estimate shall be the most recent estimate published by the Office of the Governor and available to the agency at least 6 weeks prior to publication of the fixed bed need pool.~~

5. Agree to be a designated public or private receiving facility. ~~.75 equals the desired average annual occupancy rate for hospital inpatient general psychiatric beds for children and adolescents in the district, including beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds.~~

6. Provide a continuum of psychiatric services for children and adolescents, including services following discharge. ~~LBCA equals the district's number of licensed hospital inpatient general psychiatric beds for children and adolescents as of the most recent published deadline for agency initial decisions prior to publication of the fixed bed need pool, including beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds.~~

~~7. ABCA equals the district's number of approved hospital inpatient general psychiatric beds for children and adolescents, as determined consistent with the provisions of paragraph (2)(c) of this rule, including beds in intensive residential treatment programs for children and adolescents which are seeking to have the program beds licensed as specialty hospital beds.~~

(g) Non-Competitive Applicants. The factors contained in paragraph (i) shall also be considered in the review of a single non-competitive certificate of need application within the district. ~~Special Circumstances for Approval of Expanded Capacity at Hospitals with Licensed Hospital Inpatient General Psychiatric Services for Children and Adolescents. Need for additional hospital inpatient general psychiatric beds for children and adolescents is demonstrated at a hospital with licensed hospital inpatient general psychiatric services for children and adolescents, or in a facility with an intensive residential treatment program for children and adolescents having beds licensed as specialty hospital beds, in the absence of need shown under the formula in paragraph (4)(f) and regardless of the average annual district occupancy rate determined under subparagraph (4)(h)4., if the occupancy rate of the hospital's inpatient general psychiatric beds for children and adolescents or occupancy rate of the facility with intensive residential treatment program beds for children and adolescents equalled or exceeded 85 percent for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~(h) Other Factors to be Considered in the Review of Certificate of Need Applications for Hospital Inpatient General Psychiatric Services for Children and Adolescents.~~

~~1. Applicants shall provide evidence in their applications that their proposal is consistent with the need of the community and other criteria contained in Local Health Council Plans, the district Alcohol, Drug Abuse and Mental Health Plan, and the State Health Plan.~~

~~2. Applications from general hospitals for new or expanded hospital inpatient general psychiatric beds for children and adolescents shall normally be approved only if the applicant converts a number of acute care beds, as defined in Rule 59C-1.038, F.A.C., excluding specialty beds, which is equal to the number of hospital inpatient general psychiatric beds for children and adolescents proposed, unless the applicant can reasonably project an annual occupancy rate of 75 percent for the applicable planning horizon, based on historical utilization patterns, for all acute care beds, excluding specialty beds. If conversion of the number of acute care beds which equals the number of proposed hospital inpatient general psychiatric beds for children and adolescents would result in an annual acute care occupancy exceeding 75 percent for the applicable planning horizon, the applicant shall only be required to convert the number of beds necessary to achieve a projected annual 75 percent acute care occupancy for the applicable planning horizon, excluding specialty beds.~~

~~3. In order to ensure access to hospital inpatient general psychiatric services for Medicaid eligible and charity care children and adolescents, 40 percent of the gross bed need allocated to each district for hospital inpatient general psychiatric services for children and adolescents should be allocated to general hospitals.~~

~~4. Regardless of whether bed need is shown under the need formula in paragraph (4)(f), no additional hospital inpatient general psychiatric beds for children and adolescents or additional beds in an intensive residential treatment program for children and adolescents seeking to have the program beds licensed as specialty hospital beds shall normally be approved in a district unless the average annual occupancy rate of the licensed hospital inpatient general psychiatric beds for children and adolescents in the district, including beds in intensive residential treatment programs for children and adolescents licensed as specialty hospital beds, equals or exceeds 75 percent for the 12 month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~5. The applicant for an intensive residential treatment program for children and adolescents seeking to have the program beds licensed as specialty hospital beds shall provide documentation that the district's licensed non-hospital residential treatment programs for children and adolescents with psychiatric disorders do not meet the need for the proposed service. This documentation shall include the number of residential facilities and beds in the district, the type of clients served, and the type of program provided. In addition, the applicant shall provide letters of support from other mental health professionals in the district for the proposed facility.~~

~~(i) Preferences Among Competing Applicants for Hospital Inpatient General Psychiatric Services. In weighing and balancing statutory and rule review criteria, preference will be given to applicants who:~~

~~1. Provide Medicaid and charity care days as a percentage of their total patient days equal to or greater than the average percentage of Medicaid and charity care patient days of total patient days provided by other hospitals in the district, as determined for the most recent calendar year prior to the year of the application for which data are available from the Health Care Board.~~

~~2. Propose to serve the most seriously mentally ill patients (e.g. suicidal patients; patients with acute schizophrenia; patients with severe depression) to the extent that these patients can benefit from a hospital based organized inpatient treatment program.~~

~~3. Propose to serve Medicaid eligible persons.~~

~~4. Propose to serve individuals without regard to their ability to pay.~~

~~5. Agree to be a designated public or private receiving facility.~~

~~6. Provide a continuum of psychiatric services for children and adolescents, including services following discharge.~~

~~(j) Non-Competitive Applicants. The factors contained in paragraph (i) shall also be considered in the review of a single non-competitive certificate of need application within the district.~~

(5) Unit Size. A separately organized unit for hospital inpatient general psychiatric services for adults shall have a minimum of 15 beds. A separately organized unit for hospital inpatient general psychiatric services for children and adolescents shall have a minimum of 10 beds. ~~Facilities inventoried consistent with the provisions of subsection (11) of this rule are exempt from meeting the minimum unit size requirements of this subsection unless or until they submit a proposal which would modify the number of beds listed in the inventory.~~

(6) Access Standard. Hospital inpatient ~~general~~ psychiatric services should be available within a maximum ground travel time of 45 minutes under average travel conditions for at least 90 percent of the district's total population.

(7) Quality of Care.

(a) Compliance with Agency Standards. Hospital inpatient ~~general~~ psychiatric services for adults or for children and adolescents shall comply with the agency standards for program licensure described in Chapter 59A-3, F.A.C. Intensive

residential treatment programs for children and adolescents with beds licensed as specialty hospital beds shall comply with the agency standards for program licensure described in Chapter 59A-3, F.A.C. Applicants who include a statement in their certificate of need application that they will meet applicable agency licensure standards are deemed to be in compliance with this provision.

(b) Hospital Inpatient ~~General~~ Psychiatric Services for Children. As required by Section 394.4875(2), F.S., facilities providing hospital inpatient ~~general~~ psychiatric services to children must have beds and common areas designated for children which cannot be used by adults. Adolescents may be treated in the units designated for children. Adolescents may only be treated in units designated for adult hospital inpatient ~~general~~ psychiatric services if the admitting physician indicates that such placement is medically indicated, or for reasons of safety.

(c) ~~Accreditation. Applicants proposing to add beds to a licensed hospital inpatient general psychiatric service shall be accredited by the Joint Commission on Accreditation of Healthcare Organizations consistent with the standards applicable to psychiatric services provided in inpatient settings for adults or for children and adolescents.~~ Applicants proposing a new ~~hospital~~ inpatient ~~general~~ psychiatric service shall state how they will comply with the provisions of hospital licensure as defined in Rule 59A-3.300, F.A.C. ~~meet the accreditation standards of the Joint Commission on Accreditation of Healthcare Organizations~~ applicable to the establishment of a new inpatient psychiatric hospital ~~psychiatric services provided in inpatient settings for adults or for children and adolescents~~.

(d) Continuity. Providers of hospital inpatient ~~general~~ psychiatric services shall also provide outpatient services, either directly or through written agreements with community outpatient mental health programs, such as local psychiatrists, local psychologists, community mental health programs, or other local mental health outpatient programs.

(e) Screening Program. All facilities providing hospital inpatient ~~general~~ psychiatric services shall have a screening program to assess the most appropriate treatment for the patient. Patients with a dual diagnosis of a psychiatric disorder, as defined in paragraph (2)(p) and substance abuse, as defined in paragraph (2)(t), shall be evaluated to determine the types of treatment needed, the appropriate treatment setting, and, if necessary, the appropriate sequence of treatment for the psychiatric and substance abuse disorders.

(8) Services Description. An applicant for a new ~~hospital~~ inpatient ~~general~~ psychiatric hospital ~~services~~ shall provide a detailed program description in its certificate of need application including:

(a) Age groups to be served.

(b) Specialty programs to be provided (e.g., programs for eating disorders, stress management programs).

(c) Proposed staffing, including the qualifications of the clinical director and a description of staffing appropriate for any specialty program.

(d) Patient groups ~~by primary diagnosis ICD-9 code~~ that will be excluded from treatment.

(e) Therapeutic approaches to be used.

(f) Expected sources of patient referrals.

(g) Expected average length of stay for the hospital inpatient ~~general~~ psychiatric services discharges by age group.

(h) Projected number of hospital inpatient ~~general~~ psychiatric services patient days by payer type, including Medicare, Medicaid, Baker Act, private insurance, self-pay and charity care patient days for the first 2 years of operation after completion of the proposed project.

(i) Admission policies of the facility with regard to charity care patients.

(9) Quarterly Reports. Facilities providing licensed hospital inpatient psychiatric services, including facilities with intensive residential treatment program beds for children and adolescents licensed as specialty hospital beds, shall report to the agency or its designee, within 45 days after the end of each calendar quarter, the number of hospital inpatient psychiatric services admissions and patient days by age groups:patients under age 18 years and adults. ~~Applications from Licensed Providers of Hospital Inpatient Psychiatric Services or Intensive Residential Treatment Programs for Children and Adolescents. A facility providing licensed hospital inpatient general psychiatric services seeking certificate of need approval for additional hospital inpatient general psychiatric beds, or a licensed intensive residential treatment program for children and adolescents seeking certificate of need approval in order to have the program beds licensed as specialty hospital beds, shall provide the following information in its certificate of need application in addition to the information required by subsection (8):~~

~~(a) Number of hospital inpatient general psychiatric services admissions and patient days or number of intensive residential treatment program admissions and patient days by age group and primary diagnosis ICD-9 code for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~(b) Number of hospital inpatient general psychiatric services patient days or intensive residential treatment program patient days by payer type, including Medicare, Medicaid, Baker Act, private insurance, self-pay and charity care patient days, for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~(c) Gross revenues by payer source for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~(d) Patient days by primary diagnosis ICD-9 code for the 12-month period ending 6 months prior to the beginning date of the quarter of the publication of the fixed bed need pool.~~

~~(e) Current staffing.~~

~~(f) Current specialized treatment programs (e.g. eating disorders, stress management, autism).~~

(10) Intensive Residential Treatment Programs for Children and Adolescents. An intensive residential treatment program for children and adolescents which is licensed as a specialty hospital consistent with the requirements of Chapter 59A-3, F.A.C., or a provider who has received Certificate of Need approval for an intensive residential treatment program for children and adolescents which is not yet licensed, may apply to the agency for licensure of the program as a psychiatric hospital with services restricted to children and adolescents if it complies with the requirements specified in Chapter 59A-3, F.A.C., which are applicable to licensure of a psychiatric hospital. ~~Quarterly Reports. Facilities providing licensed hospital inpatient general psychiatric services, including facilities with intensive residential treatment program beds for children and adolescents licensed as specialty hospital beds, shall report to the agency or its designee, within 45 days after the end of each calendar quarter, the number of hospital inpatient general psychiatric services admissions and patient days by age and primary diagnosis ICD-9 code.~~

~~(11) Establishment of An Inventory of Hospital Inpatient General Psychiatric Beds for Adults and Hospital Inpatient General Psychiatric Beds for Children and Adolescents.~~

~~(a) Inventory Process. The agency shall inventory the number of hospital inpatient general psychiatric beds for adults and the number of hospital inpatient general psychiatric beds for children and adolescents, including the number of beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds. The inventory shall be based on information in certificates of need and an agency survey of facilities with short-term hospital inpatient psychiatric beds or long-term psychiatric beds that were licensed prior to the effective date of this rule. Facilities with intensive residential treatment program beds for children and adolescents which were licensed as specialty hospital beds prior to the effective date of this rule will also be surveyed. All information reported in the agency survey shall be subject to verification by the agency.~~

~~(b) Separate Identification of Beds for Children or Adolescents Required. The determination of the number of hospital inpatient general psychiatric beds for adults and for children and adolescents shall be based on the certificate of need issued or the provider's survey response. Providers who designate hospital inpatient general psychiatric beds for children or adolescents shall document that these beds are located in units which are organized separately from units with hospital inpatient general psychiatric beds for adults. All hospital inpatient general psychiatric beds which are not located in documented separately organized units for children or adolescents will be listed in the inventory as hospital inpatient general psychiatric beds for adults. All beds in intensive residential treatment programs for children and adolescents which are licensed as specialty hospital beds will be deemed to meet the requirement for separate identification of beds for children and adolescents.~~

~~(c) Current Restrictions on Age Groups Treated. Any restrictions imposed by a certificate of need issued prior to the effective date of this rule regarding age groups treated in a facility's short-term hospital inpatient psychiatric services or long-term psychiatric services will be reflected in the inventory.~~

~~(d) Approved Hospital Inpatient General Psychiatric Beds. Short-term hospital inpatient psychiatric beds and long-term psychiatric beds which are approved but not licensed as of the effective date of this rule will be listed in the inventory as hospital inpatient general psychiatric beds for adults unless the application for the certificate of need which was approved identified a specific number of beds to be used for children or adolescents. Beds in approved intensive residential treatment programs for children and adolescents seeking to have the program beds licensed as specialty hospital beds will be listed in the inventory as approved hospital inpatient general psychiatric beds for children and adolescents.~~

~~(e) Publication. The agency shall publish a preliminary inventory of the licensed or approved hospital inpatient general psychiatric services for adults and for children and adolescents in the Florida Administrative Weekly. Providers shall have 21 days after the initial publication of the inventory to contest the inventory. Subsequent to the resolution of any issues pertaining to the preliminary inventory, the agency shall publish a final inventory.~~

~~(12) Facilities with Short-Term or Long-Term Psychiatric Services. Facilities currently licensed for the provision of short-term or long-term psychiatric services shall be issued an amended license authorizing the provision of hospital inpatient general psychiatric services to adults or hospital inpatient general psychiatric services to children and adolescents, regardless of the length of stay, based on the inventory established by this rule.~~

~~(13) Intensive Residential Treatment Programs for Children and Adolescents. An intensive residential treatment program for children and adolescents which is licensed as a specialty hospital consistent with the requirements of Chapter 59A-3, F.A.C., or a provider who has received Certificate of Need approval for an intensive residential treatment program~~

~~for children and adolescents which is not yet licensed, may apply to the agency for licensure of the program as a psychiatric hospital with services restricted to children and adolescents if it complies with the requirements specified in Chapter 59A-3, F.A.C., which are applicable to licensure of a psychiatric hospital.~~

~~(14) Applicability of this amended rule. This amended rule shall not be applied to applications that are pending final agency action as of the effective date of the rule.~~

Rulemaking Authority 408.034(3), (~~8~~6), 408.15(8) FS. Law Implemented 408.034(3), 408.035, 408.036(1)(b), (c), 408.039(4)(a) FS. History–New 1-1-77, Amended 11-1-77, 6-5-79, 4-24-80, 2-1-81, 4-1-82, 11-9-82, 2-14-83, 4-7-83, 6-9-83, 6-10-83, 12-12-83, 3-5-84, 5-14-84, 7-16-84, 8-30-84, 10-15-84, 12-25-84, 4-9-85, Formerly 10-5.11, Amended 6-19-86, 11-24-86, 1-25-87, 3-2-87, 3-12-87, 8-11-87, 8-7-88, 8-28-88, 9-12-88, 4-19-89, 10-19-89, 5-30-90, 7-11-90, 8-6-90, 10-10-90, 12-23-90, Formerly 10-5.011(1)(o), 10-5.040, Amended 8-24-93, 2-22-95,_____.

59C-1.041 New Hospital Inpatient Substance Abuse Services.

(1) Agency Intent. This rule implements the provisions of Sections 408.032(9), 408.034(3), 408.034(6), 408.036(1)(b) and (c), and Section 395.003(4), F.S. It is the intent of the agency to ensure the availability of hospital inpatient substance abuse services for children, adolescents and adults in need of these services regardless of their ability to pay. This rule regulates the establishment of new ~~hospital~~ inpatient substance abuse hospitals ~~services, the construction or addition of new hospital inpatient substance abuse beds, the conversion of licensed hospital beds to hospital inpatient substance abuse beds,~~ and specifies which services can be provided by licensed or approved providers of hospital inpatient substance abuse services.

(2) Definitions.

(a) "Adolescent." A person age 14 through 17. ~~"Acute Detoxification Services." Hospital inpatient services provided under the direction of a physician intended to treat the physiological effects of acute alcohol or drug intoxication during or immediately after the acute intoxication.~~

(b) "Adult." A person age 18 and over. ~~"Acute Severe Psychiatric Disorder." A psychiatric disorder not currently in full remission coded in any sub-classification of categories 293 through 295, except for 293.00, or coded as 296.24, 296.34, 296.44, 296.54 or 296.64, or coded in any sub-classification of categories 297 through 299, or coded as 310.10, in Axis I or Axis II, consistent with the diagnostic categories defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R), incorporated herein by reference; or equivalent codes in the following sub-classifications in the International Classification of Disease (ICD-9), incorporated herein by reference: category 293 through 295, except for 293.00, or coded as 296.24, 296.34, 296.44, 296.54 or 296.64, 297 through 299, or 310.10.~~

(c) "Agency." The Agency for Health Care Administration. ~~"Adolescent." A person age 14 through 17.~~

(d) "Approved Hospital Inpatient Substance Abuse Bed." A proposed hospital inpatient substance abuse bed for which an exemption, notification, a certificate of need, a letter of intent to grant a certificate of need, a signed stipulated agreement, or a final order granting a certificate of need was issued, consistent with the provisions of paragraph 59C-1.008(2)(b), F.A.C., as of the most recent published deadline for agency initial decisions prior to publication of the fixed need pool, as specified in paragraph 59C-1.008(1)(g), F.A.C. ~~"Adult." A person age 18 and over.~~

(e) "Charity Care." As defined in Section 409.91 (1), F.S., charity care is the portion of hospital charges reported to the Agency for Health Care Administration for which there is no compensation, other than restricted or unrestricted revenues provided to a hospital by local governments or tax districts regardless of the method of payment, for care provided to a patients whose family income for the 12 months preceding the determination is less than or equal to 200 percent of the federal poverty level, unless the amount of hospital charges due from the patient exceeds 25 percent of the annual family income. However, in no case shall the hospital charges for a patient whose family income exceeds four times the federal poverty level for a family of four be considered charity. ~~"Approved Hospital Inpatient Substance Abuse Bed." A proposed hospital inpatient substance abuse bed for which a certificate of need, a letter of intent to grant a certificate of need, a signed stipulated agreement, or a final order granting a certificate of need was issued, consistent with the provisions of paragraph 59C-1.008(2)(b), F.A.C., as of the most recent published deadline for agency initial decisions prior to publication of the fixed need pool, as specified in paragraph 59C-1.008(1)(g), F.A.C.~~

(f) "Child." A person under the age of 14 years. ~~"Charity Care." The portion of hospital charges for which there is no compensation, for care provided to patients whose family income as applicable for the 12 months preceding the determination does not exceed 150 percent of the current Federal Poverty Guidelines, or for care provided to patients for whom the hospital charges are greater than 25 percent of an annual family income which does not exceed four times the current Federal Poverty Level for a family of four, as defined in the Health Care Board's Florida Hospital Uniform Reporting System Manual, Chapter III, Section 3223.~~

(g) "District." A district of the agency defined in Section 408.032(5), F.S. ~~"Child." A person under the age of 14 years.~~