Project 1: Research on the therapeutic effects of cannabinoids in models of Alzheimer's disease.

Dr Caroline Herron

This project is suitable for students who have a background in Neuroscience, Physiology, Biochemistry or Biomedical Sciences.

Background

This project will use two models of Alzheimer's disease (AD) to investigate the therapeutic effects of analogues of cannabidiol (CBD). CBD is a naturally occurring phyto cannabinoid which is the non psycho active component of cannabis. It is non toxic and is formulated with THC in "Sativex" for the treatment of multiple sclerosis. It has also recently been shown to be beneficial in the form of "Epidiolex" form GW pharmaceuticals in a phase three clinical trial to treat juvenile epilepsy (Dravet syndrome). In the laboratory, we have shown that CBD can reverse the acute effects of application of the neurotoxic peptide beta amyloid peptide (AB1-42) (Hughes and Herron 2013) which is found in high concentration in post mortem AD brains. In addition, we have shown recently that in the APPswe/PS1dE9 mouse model of Alzheimer's disease that hippocampal slices from these mice have a deficit in long term potentiation at 8 months. (Metais et al., J Alzheimers Dis.;39:315-29). This is likely due to increased levels of Abeta 1-42 and alterations in biochemical signaling associated with increased levels of this protein. Chronic treatment of APPswe/PS1dE9 mice for 28 days with CBD via I/P injection between months 7-8 can reverse the deficit in LTP in these animals. (Hughes 2016) This demonstrates that the reduction in synaptic plasticity associated with models of AD can be reversed by CBD. LTP is used as a cellular model of learning and memory and agents that are known to cause a reduction in LTP reduce cognition.

Methods: Hippocampal Long term potentiation is a use dependent form of synaptic plasticity that is a cellular model of learning and can last for hours in vitro and days in vivo. Beta amyloid peptide (Abeta), that are produced in Alzheimers disease has previously been shown to attenuate hippocampal LTP. As such, any agent that can attenuate the neurotoxic effects of Abeta has therapeutic potential.

Acute Electrophysiological experiments to investigate the effects of Amyloid derived diffusible ligands and the protective effects of Cannabidiol analogues on hippocampal Long term potentiation

Experimental procedures will be similar to those previously used in the laboratory (Metais et al., 2014; 2015). Recording electrodes will be made from borosilicate capillary glass (GC150 F-10, Havard apparatus), using a horizontal puller (DMZ universal puller, Germany). Electrodes (2-5MO) will be filled with artificial cerebrospinal fluid (aCSF); (NaCl 119 mM; D-glucose 11 mM; NaHCO3 26 mM; KCl 2.5 mM; MgSO4 1 mM; CaCl2 2.5mM; Na2HPO4 1 mM). The voltage signal will be filtered at 5 kHz and stored for off-line analysis using a personal computer interfaced with a CED/ National Instruments A/D board and WinCP software (J. Dempster, Strathclyde University), alternatively a digidata 1440 A/D board coupled to Pclamp 10 and Clampex will be used/ The Shaffer-collateral pathway will be stimulated using a monopolar electrode (FHC, Bowdoin, USA) at 0.033Hz (duration: 100µs), the return electrode will be a silver/silver chloride wire placed in the recording bath. Extracellular field recordings will be made from the stratum radiatum of the CA1 region at 29-30 °C. Signals will be amplified by a HS2A headstage (Molecular Devices, USA) connected to an Axoclamp 2B system (Molecular Devices, USA) and a Brownlee 410 Precision preamplifier. A Master 8 (AMPI) timer or pClamp 10 will be used to deliver and time the stimulus trigger. Stable field excitatory postsynaptic potentials (EPSPs) will be recorded for 20 minutes, at 40-50% maximum response prior to the application of high frequency stimulation (HFS) to induce Long Term Potentiation (LTP). LTP will be induced using two trains of stimuli at 100 Hz for 1s, with an inter-train interval of 30 seconds. Following the application of HFS, the synaptic response will be recorded for a further period of 60 minutes. Statistical analysis will be performed using ANOVA. and t-tests. All results will be presented as mean ± SEM. To test the effects of Beta amyloid on LTP a final bath concentration of 500 nM ADDLs (Beta amyloid peptide)(see below) will be used. ADDLs will be added to the perfusing solution 30 min prior to induction of LTP. To test if the cannabinoid agent is neuroprotective it will be bath applied 30 min prior to ADDLS to determine if the agent alone has any effect on baseline synaptic transmission and/ or can attenuate the neurotoxic effects of Abeta on LTP.
**Amyloid Derived Difusible ligands (ADDLs)**

Beta amyloid peptide that is found in high concentrations in post mortem Alzheimer’s brain tissue is thought to be highly neurotoxic in the soluble oligomeric form. We will therefore use Beta amyloid derived difusible ligands (ADDLs) that are prepared to provide synthetic Abeta 1-42 oligomers at a final concentration of 500nM for our hippocampal slice experiments. ADDLs will be prepared using synthetic Aβ1-42 peptide. Briefly, the Aβ1-42 peptide will be dissolved in 1,1,3,3,3-Hexafluoro-2-propanol to 1 mM, then the solvent will be evaporated and the peptide will be stored on a dried film at –80 °C. When needed, this film will be resuspended in DMSO to a final concentration of 5 mM, vortexed thoroughly, and sonicated for 10 min. The resulting solution will be diluted with ice-cooled, phenol red-free Ham’s F12 medium to 200 μM and stored at 4 °C overnight to allow Aβ oligomers to form. The oligomer solution will be subsequently centrifuged briefly, and the supernatant containing soluble Aβ oligomers (e.g., ADDLs) will be collected. Alloquots will then be stored at –80 °C.

**Transgenic experiments**

Double transgenic APPswe/PS1dE9 mice and age matched control littermates bred on a C57BL/6 background will be used in the second part of this study. All procedures will be approved by the University Research Ethics Committee, and licensed by the Health Products Regulatory Authority (HPRA) in accordance with European Communities Council Directive of 24 November 1986 (86/609/ECC). Experiments will be conducted on mice aged 8 months. Mice will be housed in the Biomedical Facility at University College Dublin with a dark/ light cycle of 12h, fed with chow and water ad libitum. Founder wild type C57BL/6 females and heterozygous APPswe/PS1dE9 males will be obtained from Charles River and Jackson Laboratories respectively. APPswe/PS1dE9 mice have two transgenes (humanised mouse mutant APP and PS1) inserted at a single locus under the control of a prion promoter (Borchelt et al., 1996; 1997). These mice express a Mo/HuAPP695swe, transgene allowing the mice to secrete human A-beta peptide. The APP Swedish mutation increases the total amount of Aβ produced and the PS1 sequence lacks Exon 9 (dE9) which increases the relative amount of Aβ42 compared to Aβ 40 (Borchelt et al., 1996, 1997). The effects of chronic treatment with cannabinoid compounds will be performed. Mice will receive a daily I.P injection of compound in vehicle (tween and alcohol) at a dose of 1, 10 and 100mg/kg for 28 days. Following a 3 day rest period the mice will be used to prepare hippocampal slices for electrophysiology. APPswe/PS1dE9 heterozygote mice and age matched control littermates (male and female balanced groups) will be used. In addition to making hippocampal slices, blood will be harvested and serum samples will be collected as well as brain tissue samples and liver samples. (these will be snap frozen for later collection/ analysis). Electrophysiological experiments to investigate the level of Long Term Potentiation (LTP) in the CA1 region will be performed on hippocampal slices (similar methods to acute experiments a above). Baseline synaptic transmission will be examined and input/ output curves and paired pulse facilitation (50ms inter pulse interval) to determine any differences between control and cannabinoid treated treated groups of mice.

**Genotyping**

All mice bred in the transgenic colony will need to be genotyped to determined control and transgenic groups. (TG mice are heterozygous for APPswe/PS1dE9). Punched ear tissue samples will obtained from each mouse and will be incubated with proteinase K (Qiagen) at 56 °C (180 minutes or overnight) and a DNeasy kit (Qiagen) will be used for DNA extraction. Samples will be stored at -20°C before PCR assay experiments. Polymerase chain reaction (PCR) will be performed using a thermal cycler program (master cycler gradient Eppendorf) for 35 cycles (each cycle: 95°C for 2 min, 95°C for 30 sec, 59.6°C for 1 min, 72°C for 3 min, 72°C for 10 min) and held at 4°C prior to electrophoresis. PCR will be performed using two primer sets, one designed to amplify mouse Prn-p gene control DNA fragments (MPID1: ATATCTAGGGAACAGCCCA and MPID2-2: GCAAAGAGGAAGTCCTGCTAGTGA purchased from Operon) the other set designed to amplify the human APP695/PSN1dE9 cDNA (PrP-SJ=CCAAAGCTAGAACCAGAATGCG and S-36=CGGAGATCTCTGAAAGATGGATG purchased from MWG). Two separate sets of 25 μl PCR samples will be prepared (first 12.5 μl of GoTaq Green master mix (Promega), 10 pmol of MPID1 and MPID2 and 8.5 μl of DNA; second 12.5 μl of TaqMix, 10 pmol of S-36 and PrP-SJ primers and 8.5μl of DNA extract) and run to avoid any cross reactions between the PrP control and AbPP primer sets. Electrophoresis will be performed using a 1.5% w/v agarose gel in 0.5 x Tris Acetate-EDTA buffer, (Sigma) using a Helixx technologies iMy Run apparatus in 0.5x TAE buffer solution (Sigma) at 100 V for 45 minutes. Ethidium bromide fluorescent bands will be examined using UV (λ= 260 nm) illumination using a Gene Genius Boimaging system.

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Western Blots and Chemical Analysis
Using standard procedures, Western Blot analysis will be performed on tissue samples that have been treated acutely with ADDIs +/- cannabinoid and also on brain tissue from APPswe/PS1dE9 mice and control littermates. Markers of inflammation and apoptosis will be investigated along with levels of proteins associated with AD e.g. JNK, GSK3β, DKK1, pCREB. Beta amyloid levels will be assessed by ELISA in drug treated and vehicle treated groups as well levels of Caspase 3.


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Project 2: Investigating the pharmacology and therapeutic potential of cannabis-derived terpenes

Dr Andy Irving

Compelling evidence has shown that phytocannabinoid-terpenoid interactions can produce synergy with respect to treatment of pain and inflammation [1]. The main objective of this research will focus on investigating the mechanism of action and signalling of terpenes and to investigate their role within the so-called “entourage effect”, where they may modify the biological actions of classical cannabinoids. Terpenes act through a variety of targets, including ion channels and GPCRs. It is plausible that certain terpenes, which possess anti-inflammatory and analgesic activity, may act through immunoregulatory GPCRs. For example, geranyl pyrophosphate (GPP) is formed as a precursor in cannabis [2], and is a parent compound to both phytocannabinoids and terpenes. Both GPP and related compound farnesyl pyrophosphate (FPP) are isoprenoids within the mevalonate-cholesterol biosynthetic pathway, and a precursor to the biosynthesis of sesquiterpenes [3]. Interestingly FPP is known to be an endogenous agonist for GPR92, a lipid-sensing GPCR and our pilot data suggest that geranyl-geranyl pyrophosphate (GGPP), which is formed from FPP, is also highly active at this receptor.

Due to the chemical similarity between FPP, GGPP and terpenes, it is possible that GPR92 may also be regulated by these compounds, either directly or via an allosteric mechanism. GPR92 is thought to play a role in nociception being co-localised with TRPV1 in spinal cord and Dorsal Root Ganglia. In animal studies, GPR92 knockouts
demonstrate an insensitivity to neuropathic pain [3,4]. In addition, our pilot data suggests that GPR92 is present in immune cell populations, where its expression levels are influenced by the activation state. GPR92 is known to couple to a variety of G proteins, where it can promote calcium release and CREB phosphorylation. Terpenes may also modulate a range of target-sites involved in nociception and inflammation signaling pathways (prostaglandin, TRPs, PPARs) [1,5]. The relationship between these actions and cannabinoid receptor function will be an important factor in understanding the complex actions of cannabis at the cellular level. This is highlighted by the finding that β-caryophyllene, a terpene present in cannabis, can modulate CB2 receptor function and has anti-inflammatory actions [6]. Thus a better understanding of the complementary pharmacological activities of terpenes and classical cannabinoids might unravel novel targets for a safe and effective treatment of pain and inflammatory conditions. Experimental tools such as calcium imaging, fluorescence microscopy and signalling assays will be used in order to effectively characterize agonism, partial agonism and antagonism of compounds at receptors and co-operative or allosteric effectors, depict accurate rank order potencies of terpenes, thereby proving their specificity and mode of action at the receptor. Effects on recombinant expression systems for CB1, CB2, GPR92 and related GPCRs will be established. In particular, it would be interesting to evaluate terpene actions at GPR18 as this receptor may be an important modulator of immune function [7] and exhibits considerable pharmacological crossover with classical cannabinoid receptors. These studies will then be supported by studies on immune cells (eg macrophages and microglia) and experimental models of inflammation (including MS and arthritis). Ideally, further work in humans would evaluate changes in disease progression and inflammatory markers in blood samples from patients treated with selected terpenes.

References:

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Project 3: Investigation of the Potential of Master Regulators of Proliferation as Prognostic Indicators in Early Stage Breast Cancer

Prof. William Gallagher

A majority of women with early stage breast cancer have been traditionally over-treated with chemotherapy. There is an urgent need for more refined tools to better discriminate patients which have an inherent low versus
high risk of distant metastasis following primary surgery and hormone therapy. Our lab has identified a promising multi-marker signature, OncoMasTR, which offers superior prognostic utility compared to existing assays in this arena. The OncoMasTR signature is comprised of a discrete set of master regulators of proliferation, originally identified via reverse engineering of transcriptomic datasets from patients with breast cancer.

The project is part of a recently funded SFI Investigator programme award led by Prof. Gallagher, called OPTi-PREDICT, which is focused on validating this novel signature in a selection of patient cohorts. The PhD student would work closely with a highly experienced Research Fellow to create tissue microarrays of breast tumour specimens, assist in antibody validation, and perform immunohistochemical staining of human tissues. Extensive training in respect to manual and automated assessment of immunohistochemical staining would be provided, along with training in biostatistics. In parallel, this student will also develop a new line of research on a promising biomarker called Pali1, which is an ER-interacting protein with potential clinical relevance in breast cancer. This latter aspect of the work will take place in close collaboration with Dr. Adrian Bracken (TCD). Training will also be provided by our industry collaborator, OncoMark, as well as pathology colleagues in Lund University, Sweden.

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Project 4: The prion as a regulator of X11alpha.

Dr Hilary McMahon

Brief description:
X11alpha is an adaptor protein with numerous partners that it regulates, amongst these partners is the amyloid precursor protein (APP) that is associated with Alzheimer's disease. We have recently identified that the normal and prion disease associated protein regulate X11alpha levels and function. This relationship and its significance in Alzheimer's disease will be investigated in this project.

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Project 5: Targeting the Polycomb Repressive Complex chromatin remodeling machinery for therapeutic benefit in Diabetic Retinopathy.

Dr John Crean

Background
The International Diabetes Federation (IDF) sets out its global strategy 2011-2021, recognising the priority need to fulfill the WHO objectives of ‘turning the tide of diabetes”. Explicitly, this plan sets out three key objectives which require urgent action to support an effective global response to the diabetes epidemic and illustrate this strategy with a powerful statement of intent “Prevention and control of diabetes are not alternative or phased options; they are equally important. Investing in both simultaneously is feasible and brings tangible economic returns across a range of areas and health conditions.” In Ireland, it is estimated that 190,000 (5.6%) of the Irish population have diabetes (Institute of Public Health in Ireland, 2007), while a significant proportion of these patients (20-30%) remain undiagnosed. It is projected that the number of people with Type 2 diabetes will increase by 60% over the
next 10-15 years. Against this backdrop of an increasing prevalence of predominantly Type 2 diabetes in the community, the Diabetes Programme (http://www.hse.ie/eng/about/Who/clinical/natclinprog/diabetesprogramme/) established a National Diabetes Working Group with the joint involvement of healthcare providers in primary, secondary and tertiary care sectors to devise methodologies for dealing with these patients, specifically focusing on saving the “lives, eyes and limbs” of these patients; a tacit acknowledgement of the insidious nature of the microvascular complications.

Summary
Developmental cell fate decisions are controlled by the interplay of transcription factors and epigenetic modifiers, which together determine cellular identity. During the initiation and progression of diabetic complications, cells within affected tissues undergo a process of reprogramming, evoking gene expression profiles reminiscent of ontogenesis. In vitro and in vivo studies identified members of the Transforming Growth Factor-β superfamily as central to these processes and established this signaling network as a prime candidate for therapeutic manipulation. We have identified a switch enhancing complex comprising Smad3 and PRC2 that regulates cell fate during embryonic differentiation and is reactivated during fibrotic processes, with potential for therapeutic manipulation during the progression of retinal disease. Molecular, genetic, genomic, and biochemical methods will be used to study the role of a newly identified interaction between the EZH2 subunit of PRC2 and Smad3 to facilitate cellular reprogramming in vivo and in vitro. Direct association of Smad3, Oct4 and EZH2 suggests cooperative recruitment of PRC2 to regulate gene expression during fate determination. The recapitulation of this process during the initiation and progression of diabetic retinopathy identifies a new therapeutic paradigm—epigenetic reprogramming in vivo to promote the resolution of retinal injury and the regression of fibrosis.

Aims: The goal of this proposal is to define and exploit for therapeutic benefit, chromatin mechanisms that regulate pathogenic gene expression in retinal disease, principally diabetic retinopathy. Objectives: The principal objectives of this programme are:

i) To determine mechanisms of Smad3-PRC2 promoter targeting and its molecular role in gene silencing in vitro and in vivo and

ii) Exploitation of the Smad3-PRC2 interaction for therapeutic gain in the treatment of diabetic retinopathy.

The focus of this proposal is on the Polycomb Repressive Complex 2 (PRC2), which is a chromatin-modifying enzyme complex that methylates histone H3 on lysine 27 (K27), a hallmark of transcriptionally silenced chromatin in genomes of higher eukaryotes (Khan, 2015 #46). Molecular, genetic, genomic, and biochemical methods will be used to study the role of a newly identified interaction between the EZH2 subunit of PRC2 and Smad3 to facilitate cellular reprogramming in vivo and in vitro.

- We will define the in vivo and in vitro mechanisms and consequences of histone methylation at normal sites of Polycomb Group silencing in retinal disease.
- Manipulation of the interaction between EZH2 and Smad3 will be exploited via perturbation of the miR-302/Let7 regulatory circuit to facilitate the specification of retinal epithelial cells from embryonic stem cells and induced pluripotent stem cells, for potential therapeutic purposes.
- Pharmacological targeting of the histone methyl transferase activity of EZH2 in models of retinal injury will establish efficacy of therapeutic intervention.

Hypothesis: We propose that the replacement or normalisation of cells that underlie the fibrotic response represents a rational therapeutic approach. The successful fulfillment of these aims will advance knowledge of mechanisms in gene silencing and facilitate the manipulation of epigenetic processes that control human cell fates underlying the pathogenesis of diabetic retinopathy.

For more information and to discuss this project please contact Dr John Crean. John.crean@ucd.ie
Figure 1. Understanding the role of Smad3-EZH2 circuitry and its manipulation for renal epithelial specification. (A) Clustering of the PRC2 components in ES cells. (B) Proteomic analysis of the PRC2 interactome during neuronal cell specification identified a number of novel binding partners for the histone methyltransferase EZH2 including members of the Smad family of transcription factors, notably Smad2 and Smad3. (C) This interaction was validated by coimmunoprecipitation experiments that showed that EZH2 and phosphorylated Smad3 interact with each other and are found only in chromatin associated fractions.

Figure 2. Metabolic memory and the diabetic condition regulation of gene expression in D1R mediated by histone lysine modifications. (A) In the context of retinal epithelial differentiation in response to TGFβ, the interaction between Smad3 and EZH2 was validated by both proteomic analysis (data not shown) and coimmunoprecipitation. Increased levels of EZH2 were coincident with the appearance of the repressive H3K27me3 mark and repression of E-cadherin expression. Pharmacological inhibition of EZH2 during TGFβ mediated EMT resulted in rescue of epithelial phenotype as evidenced by restoration of ZO-1 (white arrows) (E) containing tight junction and sustained expression of E-cadherin (B). Original magnification 200x. Similarly, perturbation of the interaction between EZH2 and Smad3 by use of a TGFβ receptor silencing miR (miR-382) protected against epithelial dedifferentiation (C) and facilitated in vivo expression of E-cadherin in mesenchymal cells (D).

Figure 3. Epigenetic therapy: Establishing the therapeutic efficacy of targeting the Smad3-EZH2 interaction in diabetic retinopathy. Purification of Histone H3 demonstrates specific association of EZH2, while inhibition of the methyl transfer activity with both DZNep and GSK343 results in decreased H3K27me3.

Hypothesis: Direct association of Smad3 and EZH2 suggests cooperative recruitment of PRC2 to regulate gene expression during fetal determination and the recapitulation of this process during the initiation and progression of diabetic retinopathy identifies a new therapeutic paradigm: epigenetic reprogramming in vivo to promote the resolution of retinal injury and the regression of fibrosis.