QMB Signal Transduction
G protein-coupled receptors: form, function and therapeutics
Abstracts

S1: Structural insights into class B GPCR activation

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Class B GPCRs are important therapeutic targets for the treatment of major disease including migraine, irritable bowel syndrome, diabetes, obesity and neurodegeneration. These are important therapeutic target class but have been difficult to study at a structural level due to high conformational flexibility and poor stability in solution. In particular, fully-active GPCRs in complex with their key signalling partners (G proteins) was refractory to traditional x-ray crystallography approaches. We have applied single particle cryo-EM to determination of active GPCR structures. Using this approach, we have determined novel structures of class B GPCR bound to agonist and G protein.

These structures reveal the peptide ligand engages with both the ECD and TM bundle of receptors. Structures of different class B receptors reveal distinctions in how different ligands engage within their respective receptors, including conformational differences induced within their ECDs and the tops of TMs 6 and 7. However, these differences converge to common macromolecular changes at the intracellular face of the receptor associated with G protein activation that allow these receptors to couple to a common transducer. This includes a sharp kink induced within the centre of helix 6, resulting an outward movement of intracellular ends of helices 6 and 7 to accommodate G protein. Collectively, these structures advance our understanding of how class B GPCRs are activated by agonists that allows them to couple to their canonical G protein.

S2: A molecular ruler for ligand efficacy: Using NMR to probe ligand induced changes in α_{1A}-adrenoceptor conformational equilibria and characterize fragment hits

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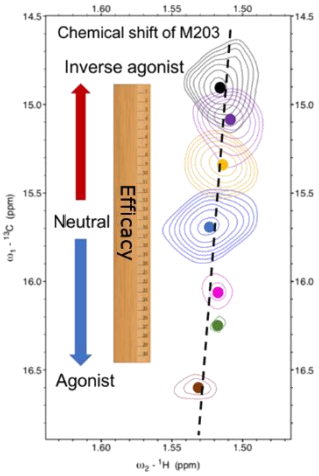

α_1-adrenoceptors (α_1-ARs) comprise three G protein-coupled receptor (GPCRs) subtypes that stimulate smooth muscle contraction in response to binding adrenaline and noradrenaline. α_{1A}-AR and α_{1B}-AR are clinically targeted for treating hypertension and benign prostatic hyperplasia but are putative drug targets for neurodegenerative diseases. New subtype-selective tool compounds are required to probe the role of these receptors in the brain and to validate them as drug targets for neurodegenerative diseases. GPCRs are allosteric machines that sample multiple conformations existing in equilibrium. Agonist binding shifts the equilibrium to active states to promote G protein activation. Recent crystal structures give us snapshots of inactive and active states, but not the dynamics that underlie GPCR activation. Here, we isotopically labeled six methionines in α_{1A}-AR to probe how different ligands modulate the conformational equilibrium of this GPCR using NMR. Met292 sits in the orthosteric ligand binding pocket and its chemical shift was unique upon binding different ligands. Met203 on-the-other-hand, is located on the intracellular side of the receptor where G proteins interact. We found the resonance of Met203 shifts upfield in the presence of inverse agonists, downfield upon agonist binding and that the chemical shift changes correlated well with ligand efficacy. The linear dependence of the chemical shifts is consistent with a conformational selection mechanism, while the resonance broadening in the presence of agonist suggests increased microsecond motion. We subsequently used this molecular efficacy ruler to validate the pharmacology of two novel hits from a trial fragment screen and the peptide toxin, τ-Tia. In conclusion, this study validates the current conformational equilibrium-based hypothesis of GPCR function and establishes NMR for screening and characterizing novel GPCR ligands.

S3: A single domain i-body against CXCR4 as a therapy for fibrosis

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i-bodies are small, stable, human scaffolds engineered from information gained from the shark single domain antibodies. The presence of a long CDR3 enables better access to complex proteins such as GPCRs and ion channels. AD-214 is a single domain i-body with affinity for CXCR4, a GPCR which is known to be upregulated in a number of cancers and recently has been implicated in fibrosis. When AD-214 was injected intraperitoneally it was found to completely block SDF-1-induced leukocyte recruitment in an air pouch model of inflammation in mice. Importantly and unlike most other CXCR4 antagonists, it did not mobilize stem cells from the bone marrow. We have shown that AD-214 can block the recruitment of fibrocytes into the lungs of mice with bleomycin induced pulmonary fibrosis and that the anti CXCR4 i-bodies have anti-inflammatory and anti-fibrotic effects in several different animal models of fibrosis.

S4: Formyl peptide receptors: novel targets for heart disease

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Introduction: Myocardial infarction remain the leading cause of mortality and morbidity, thus **developing innovative approaches against this condition is highly desirable**. Formyl peptide receptor (FPR) are GPCRs integral to regulation and resolution of inflammation, but it can elicit opposing downstream cellular response. This study tested the hypothesis that FPR biased ligand is superior than balanced ligand providing cardio-protection against MI.

Method: Signalling fingerprints of the small-molecular agonist Compound 17b and Compounds 43 at FPR1 and FPR2 were systemically assessed in CHO cells stably-expressing human FPR1 and FPR2. This was contrasted to their impact on cardiac injury response, both in cardiomyocytes, cardiofibroblasts and across 4 different time points post ischaemic insult in adult male mice.

Results: Compound 17b exhibited a distinct signaling fingerprint to Compound 43 at both hFPR subtypes. Compound 17b post-receptor signaling was biased away from potentially detrimental intracellular calcium mobilisation by 30 fold, while maintaining the beneficial reperfusion risk salvage pathway. The biased agonist agonist Cmpd17b (50 mg/kg/day, i.p.) elicited significant cardioprotection when administered at reperfusion, reducing cardiac necrosis (44$\pm$4% to 29$\pm$5%), cardiac neutrophil content at 48h from 3.5$\pm$0.4 to 1.9$\pm$0.2 AU, early cardiac remodeling and more importantly preserving cardiac function (25$\pm$4 to 35$\pm$5%) when compared to vehicle treated mice subjected to ischaemic insult (n=6-14, $p<0.05$, one way ANOVA with Tukey's post-hoc).

Conclusion: We demonstrated that FPR1/2-biased agonism with the prototype molecule Compound17b is superior than the balanced ligand Compound 43 in vitro and in vivo. These finding supports the development of small molecule FPR agonist to treat myocardial infarction.

S5: Structural insights into biased allosteric modulation of metabotropic glutamate receptor subtype 5

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Metabotropic glutamate receptor subtype 5 (mGlu5) modulates neurotransmission in response to the major excitatory neurotransmitter glutamate and is a promising target for multiple neurological and psychiatric disorders. Allosteric modulators that interact with sites distinct from glutamate, are of significant interest due to their ability to fine-tune receptor activity and potential for greater subtype selectivity. Positive allosteric modulators (PAMs) enhance, whereas negative allosteric modulators (NAMs) inhibit glutamate responses. Discovery programs commonly screen and classify ligands based on potency determinations, which lack sufficient rigor and can result in misinterpretation of activity. We apply rigorous analytical methods to investigate multiple measures of mGlu5 activation to dissect the structural basis and functional consequences of allosteric modulation in recombinant and primary cells. Using high-throughput assays for Ca^{2+} mobilisation, inositol phosphate accumulation and ERK1/2 phosphorylation, we have found that allosteric modulators can differentially activate and/or modulate distinct signalling pathways, referred to as biased agonism and biased modulation, respectively. For both mGlu5 PAMs and NAMs, distinct bias profiles can be linked to *in vivo* efficacy and may be predictive of adverse effect liability. Through structure-function analyses we are defining the key ligand-receptor interactions that govern these effects. Ultimately, our work will provide a better understanding of the mechanisms driving on-target therapeutic versus adverse effects and provide a framework for future rational discovery campaigns for biased modulators that can fine-tune receptor activity to the pathway level.

S6: Cannabinoid Receptor 2 Signalling Bias and Function in Human Primary Immune Cells

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Cannabinoid receptor 2 (CB_2) is a G protein-coupled receptor which is expressed on a range of immune cell types. Activation is thought to exert predominantly immunosuppressive effects and CB_2 is considered a promising target in a range of disorders with immune system involvement. However, to date the majority of the studies in this field have been performed on cell lines, rodent models, or stimulated primary cells.

We are studying CB_2-mediated signalling in human primary immune cells under conditions which closely mimic their *in situ* state. Our initial studies have revealed an unexpected cyclic-AMP signalling profile, wherein classically-expected $G\alpha_i$-mediated inhibition of cAMP synthesis occurs concurrently with $G\alpha_s$-mediated stimulation of cAMP synthesis, a phenotype not previously observed for CB_2. We have further characterised downstream signalling and measured an immunologically relevant functional outcome – cytokine synthesis.

We are now expanding this work to study CB_2 ligands which have been reported in cell lines to exert "bias" – i.e. stimulation of ligand-specific signalling profiles via activation of one receptor type. We will report on the various signalling fingerprints elicited by stimulating immune cells with cannabinoids, as well as the induced cytokine profile.

This work provides not only critical insight which will be important in the development of CB_2-targeted therapeutics, but highlights wider considerations for GPCR signalling studies and model validity.

S7: Targeting adenosine A1 receptor signalling to treat chronic pain.

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Chronic pain is a major global health burden that results in hypersensitivity to sensory input. Neuropathic pain, one of the most intense types of chronic pain, is caused by malfunction of the nervous system and involves persistent changes in signalling within pain pathways. We have shown that there is an increase in endogenous adenosine in the spinal cord in neuropathic pain states, which is accompanied by increased sensitivity of adenosine A1 receptors in spinal nociceptive neurons. These adaptations produce anti-nociceptive activity that can be further enhanced by positive allosteric modulation of the adenosine A1 receptor. In this talk, I will describe our work investigating the effects of the allosteric modulator MIPS521 on pain signalling from the level of the neuron, to the circuit and the whole animal. To understand the analgesic mechanism of MIPS521 within pain pathways we have used patch clamp electrophysiology to measure synaptic input into the spinal dorsal horn and changes in intrinsic activity of specific spinal neurons in both primate and rodent *ex vivo* spinal cord. These findings are supported by our *in vivo* data which show potential for the adenosine A1 receptor as a therapeutic target to reduce pathological pain.

S8: Exploring PACAP receptor signalling and pharmacology in sensory neurons.

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide hormone, which is hypothesised to play a pathophysiological role in migraine and pain. PACAP activates three related receptors; VPAC1, VPAC2 and PAC1. The PAC1 receptor has several known splice variants in the N-terminal domain, including PAC1n and PAC1s, and is believed to mediate the effects of PACAP in pain. In order to effectively target the PACAP system it is essential to determine which PACAP-responsive receptor mediates PACAP activity in sensory neurons.

mRNA arrays were preformed to determine the expression of G protein-coupled receptors in sensory neuron cultures. The pharmacology of PACAP38, PACAP27 and VIP and antagonists was profiled in sensory neuron cultures. This was compared to pharmacological profiles generated at PAC1n, PAC1s, VPAC1 and VPAC2 receptors in transfected Cos7 cells.

The PAC1 receptor the highest expressed Class B GPCR in sensory neuron cultures. The signaling profiles observed at PACAP receptors in Cos7 cells suggested the PACAP activates a similar signalling cascade at all four receptors. However, the agonist profiles displayed marked differences between the PAC1n and PAC1s splice variants. The agonist profiles observed in sensory neuron cultures were similar to those observed at transfected PAC1n receptors.

The pharmacology of sensory neuron cultures is consistent with a PAC1 receptor and suggests that this is a potential site for PACAP to induce migraine and pain. However, differences between the pharmacology of the PAC1n and PAC1s splice variants suggests that these receptors may be functionally distinct. Blocking the PAC1 receptor is a potential therapeutic strategy for migraine, however, the precise splice variant and agonist targeted should be considered in drug development efforts.

S9: Evaluation of G-protein biased opioids for the development of better, safer analgesics.

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The concept of biased agonism allows ligands binding to the same G-protein coupled receptor to signal via different pathways and thereby elicit different downstream cellular and behavioural effects. This has been applied to the development of opioid analgesics without abuse liability, tolerance or respiratory depressive effects. This is a significant because over 200,000 people overdosed on prescription opioids between 1999 and 2017 in the USA alone. Drugs targeting the mu-opioid receptor have high abuse potential and become ineffective following repeated use making them unsuitable for treating chronic pain. The desirable analgesic effects are attributed to activation of G-protein signaling pathways, whereby tolerance and respiratory depressive effects are mediated via recruitment of β-arrestin. Therefore, we utilized several mu-opioid receptor agonists with differing G-protein bias factors to evaluate whether G-protein bias correlates to superior efficacy and potency in preclinical models of acute and chronic pain and a reduction in side effects. Utilizing this same strategy we also evaluated a range of kappa opioid receptor agonists with differing bias factors to determine whether G-protein biased kappa opioid receptor agonists have anti-pain effects with reduced β-arrestin dependent side effects such as dysphoria and aversion. This research holds promise for the development of effective pain medications without abuse liability.

S10: Central sites controlling CGRP-induced light-aversive behavior

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A hallmark of migraine is photophobia. This photophobia in patients with PTH or migraine can be debilitating and treatments are lacking. The neuropeptide CGRP is a key player in migraine and induces photophobia-like behavior in mice. Although we know that CGRP is a player in migraine, it is still not known where it acts centrally to induce migraine-like phenotypes. In this study, we have focused on central cites of potential CGRP action that could act as sensory integration centers such as the posterior thalamic area (PTA) and the cerebellum. We therefore tested the central hypothesis that CGRP could act in these areas to induce light-aversive behavior in mice. We chose to use two targeted approaches. First, we injected CGRP into either the PTA or the cerebellum and then tested light sensitivity via the light dark assay and anxiogenic responses with the open field assay. Second, we used optogenetics to determine the role the neurons in these locations play in light-aversive behavior. `Injection of CGRP into the PTA sent wildtype mice into the dark, even with dim light, and without increased anxiety behavior in an open field assay. A similar phenotype was elicited by optogenetic activation of glutamatergic PTA neurons. In addition to the PTA, the injection of CGRP in the deep cerebellar nuclei induced significant light-aversive behavior, but this time with anxiety-like behavior as well. As a control, neither CGRP or optical stimulation of the hippocampus affected light aversion or open field behaviors. Thus, the PTA is likely to be a key integrator of light aversive signals that are modulated by CGRP in migraine. The cerebellum may contribute to light-aversive behaviors, but further testing will tease out its exact role.

S11: Towards neutral antagonism of the CB1 cannabinoid receptor

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Neutral antagonism of GPCRs remains relatively rare – indeed, a large majority of GPCR antagonists are actually inverse agonists[1]. Although this rarity is consistent with the constrains of structural biology and molecular dynamics[2], it belies the concept of "pure competition" which pervades many aspects of receptor theory, from Schild analysis to receptor depletion in studies interrogating receptor reserve. As part of our research programme into the molecular pharmacology of new generation synthetic cannabinoids, we characterised EG-018, a compound picked up in the US by the DEA, which shows notably low efficacy *in vitro*.

In pursuit of minimising ligand efficacy, collaborators have performed systematic SAR analysis and produced a family of 13 analogues of EG-018. The current study is therefore a comparative characterisation of EG-018 and its analogues, which also includes several other putative neutral antagonists of CB1. In HEK cells stably expressing human CB1, assays for inhibition of cAMP were performed by real time BRET biosensor (CAMYEL), and stimulation of pERK was characterised by AlphaLISA (PerkinElmer).

Signalling outcomes for the EG-018 analogues were highly variable, ranging from moderate efficacy agonism with high potency, to marginal agonism at lower potency. As predicted by differing pathway sensitivities to differences in ligand efficacy, several EG-018-based compounds were inactive in pERK alone – but were effective if applied as antagonists.

Developing neutral antagonists of the CB1 cannabinoid receptor has been a long-standing research goal, and such compounds would have utility both as research tools and in therapeutics. Although these results emphasise again the importance of system factors in determining signalling outcomes, some compounds characterised in this study are among the lowest efficacy agonists described to date and therefore promise that development of neutral antagonists is an achievable goal for CB1.

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2. Wacker, D., Stevens, R.C., Roth, B.L. *How ligands illuminate GPCR Molecular Pharmacology.* Cell. 2017;170(3):414-427.

S12: Mechanistic Insights into Ligand-Directed Signalling Bias – Characterization of an Adrenomedullin Alanine Library at Three Relevant Receptors.

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The adrenomedullin (AM) peptide plays a key role in the development and maintenance of the cardiovascular system. AM can activate three receptors. These receptors are created through the interaction of the class B GPCR, the calcitonin receptor-like receptor (CLR), with a receptor activity-modifying protein (RAMP). CLR/RAMP1 is known as the calcitonin gene-related peptide (CGRP) receptor, CLR/RAMP2 the AM_1 receptor, and CLR/RAMP3 the AM_2 receptor. Although the pharmacology of AM is best characterized through cAMP signalling, AM has been shown to regulate a number of intracellular pathways which may hold relevance in a physiological setting, such as improving cardiovascular health.

Biased signalling offers the potential to create novel therapeutics based on endogenous peptides. Investigations into molecular mechanisms of class B GPCR signalling have identified "bias hotspots" on receptors i.e. receptor regions which favour activation of some intracellular signalling molecules over others. However, the concept of "bias hotspots" in the peptide ligands themselves is relatively unexplored, and we have a limited molecular understanding of how ligands themselves drive bias. Therefore we investigated the molecular determinants of AM signalling by characterizing the signalling profiles of 15 alanine substituted analogues through a number of physiologically relevant pathways - cAMP, IP_1, pAkt, pERK, and pCREB at the CGRP, AM_1, and AM_2 receptors.

A small number of alanine substituted analogues had unique signalling profiles. The most pronounced effect was seen with AM G19A, which displayed higher potency at the CGRP receptor, and was uniquely able to activate pathways which the unmodified AM could not. These results identify possible "bias hotspots" in the AM peptide ligand, providing new insights into ligand-directed signalling bias for class B GPCRs.

S13: MRAP2: an essential GPCR regulatory protein for the control of energy and glucose homeostasis

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Accessory proteins are a family of single transmembrane proteins that interact with and regulate GPCRs trafficking and signalling. The Melanocortin Receptor Accessory Protein 2 (MRAP2) is on such protein expressed in the central nervous system and the pancreas. Deletion of MRAP2 causes severe obesity, thus suggesting an important role of MRAP2 in the regulation of energy homeostasis. We have shown that MRAP2 interacts with the Melanocortin-4 Receptor (MC4R), the ghrelin receptor (GHSR1a), the prokineticin receptor (PKR1) and the orexin receptor (OXR1), all important GPCRs for controlling food intake, energy expenditure and glucose homeostasis. Whereas the signals downstream of a subset of those receptors (MC4R and GHSR1a) are potentiated by MRAP2, others (PKR1, OXR1) are inhibited. The pharmacology of GHSR1a is drastically altered by MRAP2. Ghrelin is a hormone secreted by the stomach during starvation periods. Whereas in hypothalamic Agouti related Protein (AGRP) expressing neurons, activation of the ghrelin receptor promotes food intake, in the endocrine pancreas ghrelin blocks insulin secretion. Using in-vitro assays we found that MRAP2 almost completely eliminate the high constitutive activity of GHSR1a but very significantly increases its $G\alpha_{q/11}$-dependent response to ghrelin. In fact, in the absence of MRAP2, the ghrelin response is minimal. In addition, MRAP2 strongly inhibits ghrelin-stimulated β-arrestin recruitment and β-arrestin-dependent signalling downstream of GHSR1a. In-vivo, we found that MRAP2 is expressed in AGRP neurons and in β-cells of the endocrine pancreas. Deletion of MRAP2 results in a loss of both the hyperphagic response and the inhibition of insulin secretion triggered by ghrelin, thus confirming the requirement of MRAP2 for GHSR1a signalling in-vivo. Our studies so far demonstrate that MRAP2 is an important accessory protein for several GPCRs that regulate energy and glucose homeostasis and identify MRAP2 as an endogenous protein with the ability to bias GPCR signalling towards G-protein and away from β-arrestin.

S14: Leptin controls energy partitioning between fat and bone mass via a hypothalamic NPY relay

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Maintaining energy balance is important to ensure a healthy organism. However, energy partitioning, coordinating the distribution of sufficient energy to different organs and tissues is equally important, but far less is known about the control of this process. In obesity, increases in fat mass necessitates the production of additional bone mass to cope with the increase in bodyweight and processes need to be in place to communicate this new demand. However, it is now clear that this process is deregulated in obesity resulting in a detrimental effect on bone health with an increased risk of bone fractures and osteoporosis. Leptin and neuropeptide Y (NPY) are both critical to controlling fat as well as bone mass. Furthermore, they interact directly in the hypothalamus, an essential coordinating centre of energy homeostasis, although the exact mechanisms underlying their interaction are still unclear.

By specifically targeting the leptin receptor in NPY neurons, we show that chow-fed $Lepr^{lox/lox}$;$NPY^{Cre/+}$ mice exhibit significantly increased adiposity while bone mass is diminished, demonstrating a prominent role for leptin signaling in NPY neurons in the control of energy partitioning. Importantly, this occurred in the absence of changes in food intake or energy expenditure. Interestingly, this regulation in energy partitioning was reversed under conditions of positive energy balance induced by high fat diet (HFD) feeding. The obese phenotype of $Lepr^{lox/lox}$;$NPY^{Cre/+}$ mice was attenuated on HFD in both male and female mice. Furthermore, $Lepr^{lox/lox}$;$NPY^{Cre/+}$ mice were instead able to divert energy into the production of bone mass in response to the increase in bodyweight caused by HFD feeding, a change which was not evident in control mice.

Taken together, these results suggest that leptin signaling in NPY neurons is critical for coordinating energy partitioning between fat and bone mass especially under conditions of positive energy balance and this may have important therapeutic implications for both obesity and osteoporosis.

S15: Beneficial Effects of Leptin Antagonism on Glucose Homeostasis in DIO Mice

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Obesity is characterized by high circulating levels of leptin and a state of chronic low-grade inflammation. Recent studies have shown that pro-inflammatory signaling in the hypothalamus provokes a decrease of central leptin and insulin action associated with impaired glucose tolerance. Intriguingly, leptin not only regulates body weight and glucose homeostasis but also acts as a pro-inflammatory cytokine. Consequently, we hypothesized that hyperleptinemia may contribute to the manifestation of chronic low-grade inflammation leading to impairments in glucose tolerance. To test this hypothesis, we chronically administered different doses of a long-acting (pegylated) leptin antagonist (PESLAN) in mice fed a high-fat diet (HFD) to block excessive leptin action during diet-induced obesity (DIO). The initial use of a high dose of PESLAN further exacerbated the body weight gain induced by HFD and worsened glucose intolerance suggesting a substantial inhibition of leptin signaling and ongoing glucoregulatory action of the hormone during DIO. Using lower doses, chosen to block excessive leptin action while basic action is maintained, revealed an opposite effect and improved glucose tolerance significantly without affecting body weight. Immunohistochemical analysis of mouse brains treated with the low dose revealed a significant reduction in the number of both microglia and astrocytes in the arcuate nucleus of the hypothalamus. Our results suggest that excessive leptin action may increase pro-inflammatory signaling and thereby contributes to glucose intolerance during DIO. Lower doses of PESLAN partially but not fully restored glucose tolerance suggesting that removing hyperleptinemia is only one part of the puzzle to potentially treat DIO-induced glucose intolerance.

S16: Ghrelin at the crossroads between stress and reproduction

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An orexigenic gut-derived peptide ghrelin plays a multifaceted role in a number of physiological functions, including regulating stress responsivity and reproduction. It may therefore be pivotal in the integration of the hypothalamic-pituitary-adrenal (HPA) and –gonadal (HPG) axes. By using ghrelin-O-acyltransferase (GOAT) knockout (KO) mice that have no measurable levels of endogenous acyl ghrelin and chronically high levels of des-acyl ghrelin we have shown that an absence of acyl ghrelin does not prevent reproductive success, but that appropriate levels of acyl and des-acyl ghrelin may be necessary for optimal ovarian maturation. We then hypothesised that chronic stress would disrupt ovarian maturation and that this effect is mediated by a stress-induced increase in acyl ghrelin and activation of the growth hormone secretatogue receptor (GHSR). We subjected C57BL/6J female mice to 30 min of daily chronic predator stress for 4 weeks, used to model a posttraumatic stress disorder (PTSD), or no stress, and administered them daily with GHSR antagonist (D-Lys3-GHRP-6) or saline. Exposure to chronic predator stress reduced corticosterone and elevated acyl ghrelin levels. It did not affect the levels of circulating gonadotropins, but did deplete the primordial follicle reserve that was attenuated by GHSR antagonism. These findings suggest that chronic stress has subtle but potentially critical effects on female reproductive health and that at least some of these effects may be mediated by stress-induced acyl ghrelin.

S17: Small molecule agonists for class B G protein-coupled receptors: past, present and future

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Glucagon-like peptide-1 receptor (GLP-1R) belongs to the class B family of G-protein coupled receptors. The non-peptidic GLP-1R agonist Boc5 was shown to mimic a full spectrum of physiological actions of GLP-1. However, its druggability is hampered by poor oral bioavailability and difficulties in chemical synthesis. This led us to conduct structural biology studies of the receptor. Upon stabilization by negative allosteric modulators, two crystal structures of the human GLP-1R 7-transmembrane domain were determined in an inactive conformation, revealing a common binding pocket present in both GLP-1 and glucagon receptors. Molecular modeling and mutagenesis experiments indicate that agonist positive allosteric modulators (PAMs) target the same general region, but in a distinct sub-pocket which may facilitate the formation of an intracellular binding site that enhances G-protein coupling. The structure of human GLP-1R in complex with the G protein-biased peptide, exendin-P5, and Gs protein was also determined, offering insights into the structural basis of biased agonism. Allosteric modulation provides high selectivity, broad mimicry and less over-activation in terms of pharmacological properties. Based on the structural information, new efforts are being made to discover PAMs targeting the GLP-1R, with an ultimate goal of developing novel small molecule therapeutics to treat metabolic disorders.

S18 Control of α_{1A} adrenoceptor ligand binding kinetics

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There is limited information on sites that control ligand binding kinetics for α_1 adrenoceptors (ARs). We have observed differences in [^{3}H]-prazosin dissociation across the α_1AR subtypes, with dissociation from α_{1B} 8-fold slower than from α_{1A}ARs. The structural mediators of these effects remain unknown. This study aims to identify α_{1A}AR residues that control binding kinetics. In silico methods were used to identify interactions between allosteric compounds and the α_{1A}AR as well as subtype specific residues predicted to line the orthosteric binding trajectory. Identified residues were mutated to either alanine or the corresponding α_{1B} residue. Receptors were characterised using saturation, competition and kinetic radioligand binding assays and activation was measured using Fluo8AM calcium indicator. We identified a site on the α_{1A}AR, formed by residues at the top of helices 2 (S83, F86), and 3 (W102), that plays a role in orthosteric ligand binding kinetics. The W102A and F86A mutations increased [^{3}H]-prazosin dissociation and association rates, while these parameters decreased for S83A. [^{3}H]-prazosin dissociation was substantially slowed by the α_{1A} to α_{1B} mutation M292L but increased by F86M. S83, F86 are also involved in the negative allosteric modulation of [^{3}H]-prazosin dissociation by C9-bisacridine but not 5-(N,N-Hexamethylene)amiloride (HMA). HMA (10 μM) potentiated the norepinephrine maximum activation of F86A and S83A, while exerting no effect on wild-type norepinephrine efficacy or potency. In other GPCRs $D^{2.50}$ has been postulated to be a sodium binding site and contribute to HMA binding, however we found HMA's modulatory actions were not affected by the presence sodium or mutation of $D72^{2.50}$. The observed results suggest that the allosteric site for C9-bisacridine corresponds to the α_{1A}AR orthosteric vestibule while the binding site of HMA remains to be elucidated. In addition, subtype specific kinetic differences must be explained by a cooperative contribution of multiple non-conserved residues.

S19: Preassembled GPCR signalling complexes mediate distinct cellular responses to ultra-low ligand concentrations

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G protein–coupled receptors (GPCRs) are the largest class of cell surface signalling proteins, participate in nearly all physiological processes, and are the targets of 30% of marketed drugs. Typically, nanomolar to micromolar concentrations of ligand are used to activate GPCRs in experimental systems. We detected GPCR responses to a wide range of ligand concentrations, from attomolar to millimolar, by measuring GPCR-stimulated production of cAMP with high spatial and temporal resolution using FRET biosensors. Mathematical modelling showed that femtomolar concentrations of ligand activated, on average, 40% of the cells in a population provided that a cell was activated by one to two binding events. Furthermore, activation of the endogenous β_2-adrenoceptor (β_2AR) and muscarinic acetylcholine M_3 receptor (M_3R) by femtomolar concentrations of ligand in cell lines and human cardiac fibroblasts caused sustained increases in nuclear extracellular signal–regulated kinase (ERK) and cytosolic protein kinase C (PKC) activity, respectively. These responses were spatially and temporally distinct from those that occurred in response to higher concentrations of ligand and resulted in a distinct cellular proteomic profile. This highly sensitive signalling depended on the GPCRs forming preassembled, higher-order signalling complexes at the plasma membrane, as determined using GST pulldowns and by measuring acceptor photobleaching FRET at the plasma membrane. Recognising that GPCRs respond to ultralow concentrations of neurotransmitters and hormones challenges established paradigms of drug action and provides a previously unappreciated aspect of GPCR activation that is quite distinct from that typically observed with higher ligand concentrations.

S20: Distinct regulation of two CGRP receptors

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Migraine is a debilitating illness that affects around 15 % of the population. It is characterised by painful headaches and is associated with nausea and sensitivity to light and sound. A key mechanism in migraine pain is calcitonin gene-related peptide (CGRP) signalling through the trigeminovascular system. Drugs targeting CGRP or a CGRP receptor have proved clinically effective in the treatment of migraine, with three monoclonal antibody drugs recently receiving clinical approval and several small molecule drugs currently under clinical development.

Two CGRP responsive receptors have been identified in the trigeminovascular system; the CGRP receptor, a heterodimer of the calcitonin receptor-like-receptor and receptor activity-modifying protein 1 (RAMP1) and the AMY_1 receptor, a heterodimer of the calcitonin receptor with RAMP1. The role of each receptor in migraine is unclear, especially in terms of how the receptors may be regulated in response to elevated CGRP that is found in migraine patients.

The CGRP receptor undergoes internalisation in response to agonist stimulation, but there is no information available regarding the regulation of the AMY_1 receptor. Therefore, we have compared the internalisation properties of both receptors. In contrast to the CGRP receptor, the AMY_1 receptor remains at the cell surface in response to agonist stimulation. This work can help us understand the role of each receptor and could have implications for migraine treatment.

S21: Refining the model for ligand-dependent differential receptor:transducer coupling

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Receptor:transducer coupling is central to the function of G protein-coupled receptors. Ligand dependent differences in receptor:transducer interactions underlie the phenomenon of differential efficacy and are presumed to underlie biased signaling. We have demonstrated that divergent efficacy of two calcitonin receptor (CTR) ligands (salmon & human calcitonin) is a consequence of differences in nucleotide handling at the Gαs transducer, rather than the absolute receptor:transducer affinity. In protein-population biophysical assays the average conformation of Gαs bound to the CTR differs according to the bound ligand. We observe a ligand-dependent difference in apparent GTP affinity and GTP on-rate that are correlated with the conformation of the receptor-bound Gαs. We interpret these disparities as ligand-dependent receptor conformational sampling being communicated to the transducer, resulting in ligand-dependent differences in Gαs conformational sampling. The variation in nucleotide handling could be due to absolute differences in Gαs conformation or to divergent exchange rates between Gαs-transducer conformations. In the former case, this would point to distinct absolute conformations being important for determining efficacy, whereas the latter would suggest that receptor:transducer dynamics determine efficacy. To refine our model we have developed a novel bi-orthogonal bi-arsenical labeling approach to label the G protein hetero-trimer with a Cy3 / Cy5 FRET (Förster resonance energy transfer) pair. We are using this strategy in single molecule FRET-FLIM (Fluorescence lifetime imaging) to distinguish ligand-dependent differences in either absolute or dynamic Gαs conformation and to understand the transitions between nucleotide free and bound Gαs conformations. We believe this data will inform approaches to designing agonists with particular efficacy profiles.

S22: New tools to dissect G-protein effector interactions in cells

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To transduce a signal, cell surface receptors like receptor tyrosine kinases (RTKs) and G-protein coupled receptors need to couple to effector proteins inside the cell. One type of effector system, the phosphatidylinositol 3 kinases (PI3Ks), amplify the signal by phosphorylating phosphatidylinositol lipids embedded in the cells plasma membrane. There are four class I PI3Ks called PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ and they catalyse the formation of PI(3,4,5)P_2 when activated cell surface receptors are detected. The change in lipid phosphorylation state then recruits other kinases to the membrane where they become activated and can continue the signalling cascade. The PI3Kβ and PI3Kγ enzymes both couple to G-protein coupled receptors by interacting with G$\beta\gamma$ subunits released by activated GPCRs. Of these two enzymes the PI3Kβ enzyme can also couple to RTKs. Genetic dissection of these two receptor inputs has uncovered new functions for the G$\beta\gamma$-PI3Kβ axis in breast cancer cell metastasis. We are interested in discovering new tools that can be used to chemically dissect the input of GPCRs into lipid kinase activation via the G$\beta\gamma$-PI3Kβ interaction. The approaches to studying this interaction currently include myristoylated peptides and some small organic molecules like M119/gallein. To expand the tool box of peptide reagents, we have explored peptides based on the G$\beta\gamma$ binding surface of a PI3K effector protein, and also developed a new type of cell permeable peptide. Here, I will present some of our data.

S23: Chromenopyrazole-based chemical tools to study cannabinoid type 2 receptor

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The cannabinoid type 2 receptor (CB_2R) plays an important role in many human pathophysiological conditions such as immunological and neurological disorders. The precise role of CB_2R in these disease conditions remains to be thoroughly investigated, and a toolbox of selective chemical probes to study this receptor in the native cell environment would be of great benefit. We sought to use the previously reported chromenopyrazole scaffold [1] as a starting point for the development of CB_2R chemical tools. We designed and synthesised chromenopyrazole derivatives with varied physicochemical properties and fluorescent ligands with different fluorophores appended at various positions of the scaffold. These chromenopyrazoles were evaluated using a competition radioligand binding assay with HEK-293 hCB_2R or HEK-293 hCB_1R cells and [^{3}H]CP55940. The functional nature of these compounds was determined using a cAMP BRET assay in HEK-293 hCB_2R cells. Several high affinity CB_2R chromenopyrazole ligand-linker conjugates and two high affinity fluorescent ligands were obtained. Of particular note were polar dipeptide containing chromenopyrazoles and a Cy5-fluorescent ligand that exhibited high affinity and selectivity for CB_2R (pK_i = 7.38 ± 0.05 at hCB_2R; 5.26 ± 0.11 at hCB_1R). Widefield imaging experiments in live HEK-293 hCB_2R cells showed specific binding of this Cy5 chromenopyrazole fluorescent ligand to hCB_2R with little non-specific binding.

1. Cumella, J.; Hernandez-Folgado, L.; Giron, R.; Sanchez, E.; Morales, P.; Hurst, D. P.; Gomez-Canas, M.; Gomez-Ruiz, M.; Pinto, D.; Goya, P.; Reggio, P. H.; Martin, M. I.; Fernandez-Ruiz, J.; Silva, A. M. S.; Jagerovic, N., ChemMedChem 2012, 7 (3), 452-463.

Summary of Abstracts for the Poster Session Template

No.	Title	Presenter	Institutions
S24	Exploring Expression of CGRP Receptors in Rat Brain; Implications for Migraine	E. Hendrikse	School of Biological Sciences, University of Auckland.
S25	Lipidation of calcitonin gene related peptide (CGRP) peptide antagonists as a strategy to develop therapeutics for the treatment of metabolic disease.	A. Jamaluddin	School of Biological Sciences, University of Auckland.
S26	Understanding the CGRP receptor in endosomes	T. Alexander	School of Biological Sciences, University of Auckland.
S27	Pharmacological Characterisation of PACAP-responsive receptors reveals signalling bias and probe-dependent antagonism	Z. Tasma	School of Biological Sciences, University of Auckland.
S28	Novel synthetic cannabinoids possess signalling profiles distinct from THC at the type 1 cannabinoid receptor	M. Patel	Department of Pharmacology and Toxicology, University of Otago.
S29	Differential internalisation of the CGRP and AMY_1 receptor	T. Rees	School of Biological Sciences, University of Auckland.
S30	G Protein-Coupled Receptor Kinases Enhance and Inhibit Recruitment of β-arrestins to the type-1 Cannabinoid Receptor.	J.J. Manning	Department of Pharmacology and Toxicology, The University of Otago.

S24; Exploring Expression of CGRP Receptors in Rat Brain; Implications for Migraine

Hendrikse ,E.R.[1,2], Wookey, P.J.[3], Walker, C.S.[1,2], Hay, D.L.[1,2],

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Migraine is a debilitating and intensely painful neurological disorder affecting one in ten people worldwide. While the exact pathophysiology of migraine remains difficult to untangle, it is clear that the peptide hormone calcitonin gene-related peptide (CGRP) is a key player. Blocking CGRP action has therapeutic benefit in migraine, as shown by the various CGRP-inhibiting treatments emerging, with four antibody-based therapies recently approved for clinical use.

CGRP is present in the brain and sensory nervous system, where it acts at specific heterodimeric class B G protein-coupled receptors. The canonical CGRP receptor comprises the calcitonin-like receptor (CLR) associated with receptor activity-modifying protein 1 (RAMP1). RAMP1 can also couple to the calcitonin receptor (CTR), forming the AMY_1 receptor. The AMY_1 receptor is potently activated by CGRP *in vitro*, but its role in CGRP activity *in vivo* is not well understood. In particular, there is currently limited information about the localization of the AMY_1 receptor in cells and tissues relevant for CGRP biology, such as the brain.

To address this, antibodies were validated for CLR, CTR and RAMP1, and used to probe areas of the rat brain for protein expression using immunohistochemistry. This allows expression of the CGRP and AMY_1 receptor components in relevant brain regions to be compared. The focus was the brainstem, where many pain-sensing pathways are present. This work gives novel insights into CGRP receptor expression, particularly the spatial relationships between CGRP and its receptors in physiologically relevant tissues. This will improve understanding of the possible role of the AMY_1 receptor in CGRP biology and could maximise the therapeutic potential of CGRP-based migraine treatments.

S25: Lipidation of calcitonin gene related peptide (CGRP) peptide antagonists as a strategy to develop therapeutics for the treatment of metabolic disease.

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CGRP is a neuropeptide that initiates cellular signalling through binding and activation of CGRP receptors. As CGRP activity is implicated in pain, migraine, and metabolic diseases, blocking CGRP activity is an active area of research. Small molecule and monoclonal antibody CGRP receptor antagonists or blockers, such as Telcagepant and Erenumab, have shown clinical efficacy for migraine treatment.

Another potential therapeutic strategy is to develop peptide antagonists. However, because of the short half-life of peptides in vivo, their utility as therapeutics for clinical use is limited. Fortunately, modifications to the peptide through either lipidation or PEGylation can extend peptide half-life. $CGRP_{8-37}$ is a truncated form of the CGRP peptide lacking the first seven residues. $CGRP_{8-37}$ is an antagonist at both the CGRP receptor and the Amylin 1 receptor (AMY_1) which is also responsive to CGRP. The objective of this study is to elucidate whether lipidation of peptides based on $CGRP_{8-37}$ display similar or improved potency to $CGRP_{8-37}$ at these receptors. Our results show that modification of certain $CGRP_{8-37}$ analogues via lipidation improved antagonism across both CGRP and AMY_1 receptors. We also observed retention of binding to the CGRP receptor when compared to unmodified $CGRP_{8-37}$. A lipidated $CGRP_{8-37}$ analogue also had comparable activity to unmodified $CGRP_{8-37}$ upon screening for its ability to attenuate capsaicin-induced dermal vasodilation in vivo. These findings suggest that it is possible to lipidate CGRP peptide antagonists thereby offering the opportunity to further characterise their potential as therapeutics.

S26: Understanding the CGRP receptor in endosomes

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Calcitonin gene-related peptide (CGRP) has been demonstrated to play an integral role in migraine pathogenesis. CGRP activates the CGRP receptor which is a Class B G protein-coupled receptor (GPCR). It is composed of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). One way in which the CGRP receptor is regulated is through internalisation, where the receptor is trafficked from the cell surface to intracellular endosomes. Previously, it was thought that CGRP receptor signalling was confined to the cell surface but recent findings show that signalling from endosomes can also occur. There is limited information about the CGRP receptor within endosomes, what proteins it interacts with, and how endosomal signalling is regulated. We have investigated this using multiple approaches; visualisation of CGRP receptor internalisation through fluorescence microscopy, isolation of early endosomes that contain the CGRP receptor for proteomic analysis, and identification of interacting proteins through cross-linking and western blotting.

Stimulation of the receptor with [Cy5[3]]-hαCGRP allowed visualisation of CGRP in endosomes. Internalisation of almost all the CGRP was seen 15 minutes post stimulation. Sucrose density centrifugation was used to isolate early endosomes followed by FLAG affinity purification to purify the CGRP receptor. The CGRP receptor was successfully purified from the early endosomal fraction for further analysis using mass spectroscopy. To support this work, chemical cross-linking followed by immunoprecipitation and western blotting is being used to link and identify proteins that are bound to the CGRP receptor. So far β-arrestin has been identified to bind to activated CGRP receptors. This work will provide a greater understanding of the regulation of the CGRP receptor potentially leading to improved treatments for migraine.

S27: Pharmacological Characterisation of PACAP-responsive receptors reveals signalling bias and probe-dependent antagonism

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Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide involved in nociceptive behaviours and migraine-associated pain. Three different G protein-coupled receptors are potently activated by PACAP; the PAC_1 receptor, the $VPAC_1$ receptor and the $VPAC_2$ receptor. The PAC_1 receptor has several known splice variants in the N-terminal domain, which is important for peptide recognition. These variants include the PAC_{1n} receptor (full N-terminus) and the PAC_{1s} receptor (21 amino acid N-terminal deletion). To develop new pain treatments targeting PAC_1, it is essential to understand how PACAP signals and how this signalling is antagonised. Here we pharmacologically characterised the signalling capabilities of PACAP-responsive receptors and how these responses were blocked by antagonists.

The signalling profiles of the human PAC_{1n}, PAC_{1s}, $VPAC_1$ and $VPAC_2$ receptors were examined in transfected Cos7 cells. cAMP, IP_1, pAkt, pERK and pCREB were measured. The ability of antagonists to block multiple agonists at PACAP-responsive receptors was studied using cAMP assays.

PACAP-responsive receptors coupled to all signalling pathways measured. Limited signalling bias was observed at the PAC_{1n} and $VPAC_1$ receptors. The PAC_{1s} receptor displayed a distinct agonist profile where VIP was 20-fold more potent than at the PAC_{1n} receptor. Probe dependent antagonism was observed at the PAC_{1n} and PAC_{1s} receptors. VIP and PACAP-27 were both more potently antagonised by $PACAP_{6-38}$ and M65 compared to that of PACAP-38.

The PAC_{1s} receptor displayed a distinct pharmacological profile to that of the PAC_{1n} receptor, which may play a unique role in pain responses. Differences in antagonist pharmacology at PACAP-responsive receptors suggests the effectiveness of blocking a signalling pathway can be influenced by which endogenous agonist is present. This probe dependent effect has potential implications for the development and effectiveness of anti-PAC_1 receptor drugs for pain therapy.

S28: Novel synthetic cannabinoids possess signalling profiles distinct from THC at the type 1 cannabinoid receptor

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Synthetic cannabinoids (SCs) represent the most rapidly proliferating class of "designer drugs". Initially developed for pharmacological interrogation of the endocannabinoid system, SCs have been diverted to recreational drugs in an attempt to create "legal" or unregulated alternatives to cannabis. However, SC abuse is being increasingly associated with severe toxicity and death, with more than 50 deaths in NZ attributed to SC (mis)use between May 2017 and 2018. Little is currently known of the molecular pharmacology or mechanisms underpinning their toxicity in humans, although the effects are believed to be mediated by the type 1 cannabinoid receptor (CB_1). In this study, we aimed to characterise the activity of a structurally diverse set of novel SCs at CB_1 in comparison to traditional cannabinoids, such as THC, in order to identify whether compounds associated with toxicity would exhibit distinct molecular signalling profiles, which may help explain their effects.

Using real-time BRET biosensor assays, we found that the SCs behaved as potent, efficacious agonists in cAMP inhibition and β-arrestin 2 translocation. This was supported by the complete internalisation of cell surface CB_1 upon stimulation with novel SCs, evaluated using immunocytochemistry. The SCs were also found to be less efficacious and potent at recruiting β-arrestin 1, in comparison to β-arrestin 2; suggesting reduced intrinsic efficacy in this pathway. Quantification of bias revealed that SCs activities were relatively balanced in the pathways tested, compared to the well-established reference cannabinoid, WIN55,212-2. However, the SCs illustrated markedly distinct potency and efficacy profiles to the phytocannabinoid, THC, which is generally considered 'safe' in humans. In particular, while the majority of SCs demonstrated robust β-arrestin translocation, THC failed to elicit a measurable response. Further study is required to delineate the role of β-arrestins in the effect and toxicity of SCs in humans.

S29: Differential internalisation of the CGRP and AMY_1 receptor

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The neuropeptide calcitonin-gene related peptide (CGRP) plays a key role in migraine pathogenesis and is known to potently activate two receptors, the CGRP and Amylin 1 (AMY_1) receptor. These receptors are expressed in the trigeminal ganglia, which is important for craniofacial pain and migraine. These receptors display different pharmacology, the CGRP receptor responds to CGRP and the AMY_1 receptor responds equally to CGRP, amylin and pramlintide, an amylin mimetic. The CGRP receptor is known to internalise in response to CGRP, however little is known about the internalisation of the AMY_1 receptor. In this study we aimed to characterise whether the AMY_1 receptor internalises and how this compares to the CGRP receptor.

To study these receptors novel Cy5-labelled [$Cy5^3$]-hαCGRP and [$Cy5^{21}$]-pramlintide were synthesised then pharmacologically characterised in transiently transfected Cos-7 cells. Receptor internalisation was studied using high content imaging with the Cy5-labelled peptides and using cell-surface ELISA in transiently transfected HEK293S and Cos-7 cells.

Pharmacological characterisation of [$Cy5^3$]-hαCGRP and [$Cy5^{21}$]-pramlintide suggested they had similar cAMP signalling to the unmodified peptides. Internalisation was then quantified using [$Cy5^3$]-hαCGRP and [$Cy5^{21}$]-pramlintide. The [$Cy5^3$]-hαCGRP had significantly greater internalisation at the CGRP receptor than the AMY_1 receptor. This was mirrored by the loss or retention of cell surface expression of the receptors over time in response to unmodified hαCGRP. Similarly to [$Cy5^3$]-hαCGRP, [$Cy5^{21}$]-pramlintide did not induce AMY_1 receptor internalisation.

The receptors displayed different internalisation profiles. The AMY_1 receptor does not appear to internalise in response to hαCGRP and pramlintide while the CGRP receptor internalises robustly to hαCGRP as expected. These data suggest that CGRP activity could be regulated by distinct internalisation profiles, with implications for physiology and drug treatment.

S30: G Protein-Coupled Receptor Kinases Enhance and Inhibit Recruitment of β-arrestins to the type-1 Cannabinoid Receptor.

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Contemporary interest in GPCR molecular pharmacology is in targeting subsets of the complement of signal transduction molecules activated by a receptor. This is proposed to 'bias' the signalling response and result in unique functional outcomes. Popular candidate effectors for the discovery of biased ligands for the type-1 cannabinoid receptor (CB1) are β-arrestin-1 and -2. These scaffold proteins canonically interact with phosphorylated GPCRs post-stimulation, playing a key role in desensitisation and internalisation. β-arrestins are also postulated to act as mediators for different second messengers, therefore altering recruitment of either β-arrestin relative to other signal transducers may be a desirable drug design approach.

Little is known about the regulation of β-arrestin recruitment to CB1. This study has characterised the six G protein-coupled receptor kinases (GRKs) to determine their influence on receptor-arrestin interactions. Here, Bioluminescence Resonance Energy Transfer has been used to identify real-time differences in β-arrestin-1 and -2 recruitment to CB1 with GRK1-6 for three cannabinoid ligands of varying efficacy (MDMB-FUBINACA, CP55,940, trans-Δ9-tetrahydrocannabinol - THC).

All GRKs, with the exclusion of GRK4, enhance recruitment of β-arrestin-1 and inhibit recruitment of β-arrestin-2 for MDMB-FUBINACA and CP55,940. In contrast, THC-mediated recruitment of either arrestin with most GRKs is enhanced.

The 'barcode' hypothesis in GPCR pharmacology suggests that different combinations of phosphorylated sites on the receptor can cause differences in signalling outcomes. These data argue against the existence of such a phenomenon for β-arrestin recruitment through CB1, as GRK-mediated phosphorylation causes near-identical effects between GRK subtypes. However, further characterisation is needed to determine if different GKRs results in altered signalling downstream of arrestin recruitment.