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## The light activated chloride channel GtACR2 is a novel potential therapeutic to treat chronic pain.

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Chronic pain affects 1 in 5 New Zealanders costing our society approximately $14 billion in 2016. Opioids are used to treat chronic pain but show poor long-term efficacy and high rates of addiction. Innovations for effective, personalised chronic pain treatments are desperately needed. Aberrant pain signalling, arising from disinhibition of spinal cord pain projection neurons, is proposed to underlie chronic pain. Altered chloride transporter expression in these neurons disrupts their chloride balance eroding the effectiveness of inhibitory input.

We propose that expressing and activating the light-activated chloride channel *Guillardia theta* anion channel rhodopsin 2 (GtACR2) in spinal cord pain projection neurons will restore their chloride balance, re-establish inhibition, and reduce pain. Here, we produced a lentiviral vector encoding a red fluorescence tagged GtACR2 construct and confirmed its expression and function *in vitro*.

We successfully targeted GtACR2 expression to the cell body of iPSC-derived, cultured human neurons. This subcellular localisation is required to influence chloride balance solely in the cellular compartment where it is altered in chronic pain. Whole-cell patch clamp electrophysiology of GtACR2-expressing neurons showed light-induced ionic photocurrents (N = 15 cells) and transient silencing of action potential activity. Illumination of GtACR2-expressing cultures successfully silenced neuronal network activity for the entire 30 second illumination period, as detected using a genetically encoded fluorescent calcium indicator (N = 3 wells, 21 recordings across 4 days).

Our successful generation and *in vitro* validation of this unique optogenetic tool, and its viral delivery vector, paves the way for its *in vivo* testing in an animal model of chronic pain. Our results provide strong evidence for the novel use of GtACR2 to restore inhibition of spinal cord pain projection neurons. GtACR2 is promising as a specific, light-tuneable therapeutic to re-establish normal pain signalling and alleviate chronic pain.

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## Microparticles produced from human papillomavirus type 16 E6 and E7 expressing keratinocytes regulate antigen-presentation by Langerhans cells.

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Persistent infection by human papillomavirus (HPV), such as HPV16, initiates around 5% of all human cancers. The progression from persistent infection to cancer is associated with the over-expression of HPV16 E6 and E7 (E6/E7), and modulation of antigen-presenting cells (APCs). Microparticles (MPs) are small (100 to 1000 nm), cell membrane-derived vesicles. This study aimed to understand the role of MPs produced from HPV16 E6/E7-expressing cells on antigen-presentation by Langerhans cells (LCs), the only APCs that are co-located with HPV-infected keratinocytes.

To measure the effects of MPs on T cell proliferation in response to LC-presented ovalbumin (OVA), E6/E7 or control (Ctrl) MPs purified from E6/E7 expressing or control C57Bl/6 mouse keratinocytes were incubated with bone marrow-derived LCs, pulsed with OVA and co-cultured with CD8 T cells. CD8 T cell proliferation was increased following

incubation with Ctrl MPs (76.23 ± 15.68, mean percent ± SD) compared to no MPs (39.31 ± 16.43, $P < 0.01$, Kruskal-Wallis, Dunn's multiple comparisons). CD8 T cell proliferation was reduced following incubation with E6/E7 MPs (48.80 ± 15) compared to Ctrl MPs (N = 8 mice, Kruskal-Wallis, Dunn's multiple comparisons). When LCs derived from Transporter associated with Antigen Processing 1 (TAP1) knockout mice were used, CD8 T cell proliferation was significantly reduced in E6/E7 (22.84 ± 5.46, $P < 0.001$) and Ctrl (19.95 ± 7.47, $P < 0.0001$) MP cultures (N = 8 mice, Two-way ANOVA, Sidak's multiple comparisons).

Here we show that MPs increase proliferation of CD8 T cells upon incubation with LC-pulsed OVA, and that E6/E7 MPs suppresses this effect. Additionally, the effects of MPs were ablated in the absence of TAP1 supporting the involvement of the antigen-presentation pathway of LCs. HPV16 E7 has been previously reported to repress TAP1. The measurement of TAP1 expression in LCs could indicate a potential mechanism for the E6/E7 MP suppression of antigen-presentation.

## An in vitro investigation of the effect of environmental contaminants nitrate, nitrite, and N-nitrosodiethylamine on colorectal cancer.

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Epidemiological studies have correlated elevated concentrations drinking water nitrate with an increased risk of developing colorectal cancer (CRC). Nitrate is rapidly absorbed *in vivo*, where 0.02 – 0.04% is converted to N-nitrosodiethylamine (NDEA), a class 2A carcinogen. How such environmental contaminants affect CRC cells is not well understood. This project investigated the potential for nitrate, nitrite, and NDEA at concentrations relevant to drinking water, to affect CRC cell viability *in vitro*. The effect of NDEA on CYP2E1 expression was also assessed as a source of reactive oxygen species which may serve as a mechanism of NDEA toxicity.

The cell viability of HT-29 and Caco-2 CRC cells following 72 hours exposure to nitrate (0 – 400 mg/L), nitrite (0 – 20 mg/L), or NDEA (0 – 200 nM) was determined using the sulforhodamine B assay (N = 3). Western immunoblotting was used to assess the expression of CYP2E1 at 24 and 72 hours in both cell lines exposed to NDEA (200 nM and 200 μM, N = 1).

While no concentration of nitrate and nitrite significantly altered cell viability in either cell line, NDEA at 100 nM and 200 nM significantly increased cell viability in HT-29 cells by 13% (± 3.18) and 12% (± 3.79) respectively (mean ± SD, $p < 0.05$, ANOVA, Bonferroni adjustment), with no effect observed in Caco-2 cells. Initial Western immunoblotting indicates that NDEA at 200 nM and 200 μM increases CYP2E1 expression in both Caco-2 and HT-29 cells at 24 hours, however two further biological replicates are required to confirm this.

Toxic NDEA as a product of drinking water nitrate may increase the cell viability of CRC cells accounting for correlations between elevated drinking water nitrate and incidence of CRC. However, toxicokinetic studies would be required before extrapolating these *in vitro* results to true carcinogenic risk from these environmental contaminants.

## $Ca^{2+}$-imaging in rat adrenal slices reveals chromaffin cell heterogeneity.

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The ability to undergo physiological adaptations to stress is critical to life. An important aspect of this stress response is the release of catecholamines from adrenal medullary chromaffin cells into the circulation. This secretion is caused by a rise in intracellular $Ca^{2+}$ ($[Ca^{2+}]i$) in response to several physiological stimuli. Increasing evidence suggests that the adrenal medullary anatomical organisation and cell interconnectivity contributes greatly to this $[Ca^{2+}]i$.

The current study thus aimed to characterise this stress response by examining the stimulus-dependent $[Ca^{2+}]i$ changes in individual chromaffin cells within rat adrenal slices. Adrenal vibratome sections were prepared from adult male Sprague Dawley rats, loaded with the $Ca^{2+}$-indicator Fluo-4, and changes in $[Ca^{2+}]i$ recorded from individual cells using fluorescence microscopy.

Nicotinic stimulation, mimicking the acute stress response, induced a sharp $[Ca^{2+}]i$ rise which, in some cells (N = 17/66), returned to basal in the continued presence of nicotine. In contrast to studies on isolated cells, most recorded cells (N = 49/66) entered a sustained, elevated plateau phase for the duration of ncotinic exposure. Pituitary adenylate cyclase-activating peptide (PACAP), mimicking a persistent stress response, increased $[Ca^{2+}]i$ in approximately half of the recorded cells (N = 33/67), but this response was delayed by approximately 10 min after exposure. Histamine, a non-neuronal chromaffin cell stimulator, induced a rapid $[Ca^{2+}]i$ rise in most cells (N = 50/55). These responses varied greatly between cells and consisted of multiple complex peaks, differing in duration and magnitude. Despite intercellular differences, histaminergic responses were highly reproducible within an individual cell.

Each examined stimulus produced heterogeneous responses with novel $[Ca^{2+}]i$ characteristics not reported in isolated chromaffin cells. The results, therefore, provided evidence for contributions of cell-cell interactions to

$Ca^{2+}$-signalling and subsequent secretory output from the chromaffin cells, while highlighting avenues for future research into cellular and functional heterogeneity of the adrenal medulla.

## Access to dietetic services for inflammatory bowel disease patients in New Zealand – a patient view.

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International guidelines recommend that inflammatory bowel disease (IBD) patients should have access to specialised dietitian support. This patient group is at high risk of malnutrition and nutrition interventions can reduce disease activity and improve quality of life. Anecdotal reports suggest that New Zealand (NZ) IBD patient access to dietitians is variable.

This research aimed to investigate factors associated with patient access to a dietitian and whether access meets patients' expectations. In early 2020, an anonymous electronic survey was disseminated to patients (and parents) by Crohn's and Colitis NZ and IBD health professionals via email and social media, with a reach of approximately 2000 people. Quantitative responses were analysed via chi-square and Fisher's exact tests and qualitative responses were analysed using a general inductive approach.

The respondents (N = 407) were mostly female (74%) and NZ European (91%), 5% identified as Māori. While 95% of respondents had topics they would like to discuss with a dietitian, only 52% had ever seen a dietitian and 45% had never been referred. Of those who had seen a dietitian, 37% had self-funded their appointment, many because they were unable to access publicly funded appointments. Patients more likely to have seen a dietitian were: younger ($P < 0.001$); diagnosed with Crohn's disease ($P = 0.001$); had previous IBD surgery ($P < 0.001$); on biologic therapy ($P = 0.005$). Common themes identified through general inductive analysis identified that there is a lack of publicly funded dietetic services, that dietitians need specialist IBD knowledge, that patients/respondents want routine referrals to dietitians and to have ongoing dietitian access.

Results indicate that there is inequitable and inadequate access to dietetic services for IBD patients in NZ, with variable referral rates and substantial numbers of patients required to pay for dietitian appointments. Poor access increases malnutrition risk in this vulnerable patient group, potentially leading to worse health outcomes and negative impacts on quality of life.

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## RFamide-related peptide neurons modulate chronic glucocorticoid-induced reproductive suppression.

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Globally, one in four couples are affected by infertility. Chronic stress is often a result, but also a possible cause of reproductive dysfunction. Regulation of gonadal function by luteinizing hormone (LH) from the pituitary gland is suppressed during chronic stress. However, the mechanisms are poorly understood. We investigated whether hypothalamic RFamide-related peptide (RFRP) neurons mediate this suppressive effect of glucocorticoids.

Cre recombinase-dependent DREADDs (designer receptors exclusively activated by designer drugs), activated by the synthetic DREADD agonist clozapine-n-oxide (CNO, 2 mg/kg), were used to selectively manipulate RFRP activity in the transgenic mice. Immunohistochemical staining of the neuronal activity marker Fos-related antigen confirmed downregulated RFRP neuronal activity in the inhibitory DREADD-expressing mice ($P < 0.0001$) and upregulated activity in the excitatory DREADD-expressing mice ($P < 0.0001$) compared to non-DREADD controls in both sexes (t-tests). The effect on LH pulse frequency (mean ± SEM) was measured by tail tip blood sample collection every 6 mins for 3 hours in awake, freely-moving mice.

As expected, control mice had a marked reduction in LH pulse frequency in response to 4 days of subcutaneous ~100 mg glucocorticoid (corticosterone) implant treatment (males: 1.38 ± 0.19 before and 0.92 ± 0.06 pulses/h after corticosterone, $P = 0.030$, N = 8; females 1.54 ± 0.15 pulse/h before and 0.38 ± 0.11 pulses/h after corticosterone, $P = 0.0001$, N = 7). In contrast, LH pulse frequency was rescued from these glucocorticoid effects in RFRP-silenced females (1.23 ± 0.10 before and 1.074 ± 0.12 pulses/h after corticosterone, $P = 0.120$, N = 9), but not males (1.29 ± 0.15 before and 0.86 ± 0.12 pulses/h after corticosterone, $P = 0.030$, N = 8) (2-way ANOVA with corticosterone treatment and RFRP inhibition as factors and Holm-Sidak's multiple comparison post-hoc test). Supporting a sexually-dimorphic role for RFRP neurons in LH suppression, upregulation of RFRP neuronal activity reduced LH pulse frequency in female mice (controls: 1.7 ± 0.01, N = 5; RFRP-excited: 0.6 ± 0.19 pulse/h, N = 3; $P = 0.004$) but no effect was observed in male mice (controls: 1.1 ± 0.11, N = 5; RFRP-excited: 1.3 ± 0.15 pulses/h, N = 5; $P > 0.999$) (t-tests).

These results reveal a novel sex-specific requirement of RFRP neurons in modulating

suppressive effects of stress steroids on LH secretion and highlight complexities in neuronal signaling associated with reproductive dysfunction.

## oCOm-21 has an anti-inflammatory effect via the NLRP3 inflammasome.

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During cardiac bypass procedures, the heart is subjected to repeat cycles of ischaemia reperfusion injury (IRI). Pro-inflammatory signalling involving the NLRP3 inflammasome, has been identified as a key contributor in the pathogenesis of cardiac remodelling. Recent research highlighted the capability of carbon monoxide (CO) releasing compounds to reduce injury via the NLRP3 inflammasome. This study aimed to test whether a novel organic CO releasing molecule, oCOm-21, produces an anti-inflammatory effect by reducing NLRP3 levels within hearts undergoing IRI.

Male CYP1a1-Ren2 rats were induced to develop either non-hypertrophic or moderately hypertrophic hearts before being randomly allocated to vehicle or oCOm-21 (1 – 10 μM) treatment groups prior to IRI (N = 3/group). Hearts were sectioned for histology to evaluate the damage from IRI and immunofluorescence staining for determination of NLRP3 expression. Ventricles were homogenised for Western blotting to determine protein expression levels. Results were analysed using a One-way ANOVA with a post-hoc Bonferroni analysis.

Untreated controls in both normotrophic and hypertrophic groups sustained greater histological damage than oCOm-21 treated hearts. Immunofluorescence examination showed a 2- and a 3-fold decrease in NLRP3 within non-hypertrophic hearts treated with 3 μM and 10 μM oCOm-21, respectively compared to vehicle ($P < 0.01$). Moderately hypertrophic hearts treated with 3 μM of oCOm-21 had a 1.96-fold decrease in NLRP3 staining compared to the vehicle ($P < 0.05$). No significance was observed between vehicle and 1 μM oCOm-21 for both hypertrophic groups. Western blotting indicated no significance between vehicle and oCOm-21 treated hearts ($P > 0.05$).

These preliminary results strengthen the hypothesis that IRI evokes an inflammatory response, resulting in cardiac damage, which may be attenuated with oCOm-21 via the inhibition of NLRP3. Although the immunohistochemical results demonstrates a promising link between CO and NLRP3 inhibition, the lack of reproducibility with Western blotting requires additional research. The current research performed has provided evidence that CO can reduce myocardial IRI through the NLRP3 inflammasome within a diseased heart model similar to healthy hearts.

## Epicardial adipose tissue morphology diversity in Māori, Pacific and New Zealand/European post-mortem cases.

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Obesity is the leading cause of morbidity and heart disease in New Zealand (NZ), and Māori and Pacific people are disproportionately affected. Obesity is characterized by adipose tissue expansion. The fat surrounding the heart, Epicardial Adipose tissue (EAT), has recently been identified as a diagnostic tool for cardiovascular disease. Recent discoveries show that obesity-induced morphological changes to EAT are dissimilar to changes in subcutaneous or visceral adipose tissues. While adipocyte size in subcutaneous, appendicular and pericardial adipose tissues increased in relation to body mass index (BMI), EAT adipocyte size did not. Additionally, the well-established relationship between increased EAT thickness and BMI was not observed in Māori, nor Pacific people. Our study aimed to investigate how obesity affects EAT morphology and EAT localization in diverse ethnic New Zealand populations.

In post mortem cases of NZ/European (N = 57), Māori (N = 16) and Pacific (N = 6) people, adipocyte size was determined in histological slices of different adipose depots, whereas myocardial adipose infiltration and fibrosis were determined in histological slices of the left ventricle. In subcutaneous, appendicular and pericardial adipose tissue, the adipocyte size positively correlated with BMI in NZ/European and Māori populations, while not in Pacific cases. Additionally, there was no correlation between EAT adipocyte size and BMI in any cases ($P = 0.706$). Furthermore, EAT adipocyte size positively correlated with myocardial fibrosis in NZ/European ($P = 0.0015$, $r^2 = 0.5263$) however not in Māori/Pacific ($P = 0.2954$). Finally, a positive correlation was observed between EAT adipocyte size and myocardial adipocyte infiltration, but only in Māori/Pacific ($P = 0.023$, $r^2 = 0.5455$).

In conclusion, the differences in adipocyte morphology between Māori, Pacific people, and NZ/European, highlight possible physiological ethnic variances, which may associate with diverse risk factors and disease characteristics. This should trigger discussions on the validity of current diagnostic and potential treatment strategies for cardiovascular disease and obesity in NZ populations.

## Characterisation of the inflammatory response to injury in an *ex vivo* rodent model of spinal cord injury.

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Spinal cord injury (SCI) is a significant injury, and a burden to the patient, their family and to the healthcare system. The inflammatory response that follows the injury exasperates tissue damage and scarring, which prevents neuronal regeneration and patient recovery. Current *in vivo* models are problematic due to the extreme suffering of animals receiving the injury. This study aimed to assess the extent and source of inflammation in the *ex vivo* model that has the potential to reduce animal usage and suffering.

Spinal cord segments were dissected from adult Sprague-Dawley rats (7 weeks post-natal), with random segments receiving a compression injury after 24 hr culture (T0). Segments were harvested immediately after dissection (T-1), or 24 hr (T1) and 7 days (T7) after injury. Quantitative PCR and immunohistochemistry were performed to assess the mRNA levels of inflammatory regulators and the distribution of resident immune cells, respectively.

Interleukin (IL)-6 expression was upregulated by 1400- and 4200-fold, respectively, for uninjured (UI) and injured (I) tissue at T1 compared to T-1, indicating that an inflammatory response to injury is occurring (N = 2). In addition, IL-1β was upregulated by 1.9- and 6.5-fold, for UI and I tissue at T1 compared to T-1, again indicating that an inflammatory response to injury is occurring (N = 2). Also, at T1, increased activation of macrophages through calprotectin staining was observed while increased expression of resident microglia through mannose staining was observed.

These results suggest that pro-inflammatory signalling by resident immune cells occurs within the *ex vivo* model of SCI at 24 hr post-injury. This model may therefore have utility for testing therapeutics aimed at reducing SCI-induced inflammation.

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