

Understanding the mechanisms of G protein-coupled receptor mediated nutrient sensing in enteroendocrine cells

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Abstract

Nutrient sensing is the process by which cells detect and respond to dynamic metabolite fluctuations. In gastrointestinal enteroendocrine cells (EECs), this process facilitates the post-prandial secretion of anorectic gut hormones. As these elicit diverse impacts on physiology, appetite and metabolism, they present potential targets for anti-obesity therapeutics. Short chain fatty acids (SCFAs) are produced as by-products of the fermentation of non-digestible carbohydrates by the gut microbiota, predominantly in the colon. SCFAs induce EECs to release anorectic hormones GLP-1 and PYY by acting as ligands at G protein-coupled receptors (GPCRs) FFAR2 and FFAR3. Thus far, it has been confirmed that FFAR2 and FFAR3 couple to $G\alpha_{i/o}$ but FFAR2 alone activates $G\alpha_{q/11}$ calcium mobilisation. However, FFAR2 is seemingly only able to induce calcium signals in EECs when activated by synthetic FFAR2 ligands, not SCFAs. It is plausible that crosstalk between FFAR2 and FFAR3 could be preventing FFAR2 coupling to $G\alpha_q$ in response to SCFAs. This has been assessed by combining genome editing and functional signalling assays. Conversely, the results demonstrate that co-expression of FFAR2 and FFAR3 in heterologous systems enhances propionate-mediated calcium mobilisation. Furthermore, functional knockout of FFAR3 has no impact on propionate signalling in EECs. Interestingly, crosstalk with FFAR4 may represent a mechanism for the lack of SCFA-induced $G\alpha_q$ signalling in EECs. The impact of SCFAs on the expression of other GPCR signalling machinery remains poorly characterised. This project has, to date, demonstrated propionate and butyrate robustly upregulate transcripts encoding the umami taste receptor, via a $G\alpha_{i/o}$ independent mechanism. We show that overnight exposure of EECs to butyrate enhances the calcium mobilisation in response to L-Alanine when IMP is present. Therefore, I propose an alternative, indirect mechanism by which SCFAs enhance gut hormone release; namely, by inducing a hypersensitivity of EECs to L-amino acids by promoting expression of taste signalling components.

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List of abbreviations

4-CMTB	4-chloro- α -(1-methylethyl)-N-2-thiazolylbenzeneacetamide
AC	Adenylyl cyclase
AgRP	Agouti-related peptide
AMPK	5' AMP-activated protein kinase
ANS	Autonomic nervous system
AR	Adrenoreceptor
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
AU	Arbitrary units
BBB	Blood brain barrier
BCAA	Branched chain amino acid
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
CCK-R	Cholecystokinin receptor
cDNA	Complementary DNA
CNS	Central nervous system
CpG	Cytosine-phosphate guanine
CRD	Cysteine-rich domain
CREB	cAMP response element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CVD	Cardiovascular disease
DAG	Diacylglycerol

DMEM	Dulbecco's modified Eagle's Medium
DMN	Dorsomedial nucleus
DPP-IV	Dipeptidyl peptidase IV
DSB	Double stranded break
EC	Extracellular
EEC	Enteroendocrine cell
ENS	Enteric nervous system
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
FFAR	Free fatty acid receptor
FRET	Fluorescent resonance energy transfer
FSHR	Follicle stimulating hormone receptor
GDP	Guanosine di-phosphate
GEF	Guanine exchange factor
GI	Gastrointestinal
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GMP	Guanosine monophosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine tri-phosphate
HCA	Hydroxycarboxylic acid receptor
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HF	High-fidelity
HPA	Hypothalamic pituitary axis

HR	Homologous recombination
HTRF	Homogenous Time Resolved Fluorescence
IBMX	3-isobutyl-1-methylanthine
IC	Intracellular
IMP	Inosine monophosphate
Indels	Insertions/ deletions
IP ₃	Inositol (3,4,5) triphosphate
KD	Knock-down
KO	Knock-out
L-AA	L-Amino acid
L-Ala	L-Alanine
L-Asp	L-Aspartame
LBD	Ligand binding domain
LCFA	Long chain fatty acid
L-Gln	L-Glutamine
L-Glu	L-Glutamate
LHA	Lateral hypothalamic area
LHR	Luteinising hormone receptor
L-Leu	L-Leucine
L-MSG	L-Monosodium glutamate
L-Phe	L-Phenylalanine
L-Trp	L-Tryptophan
MAPK	Mitogen activated protein kinase
MCFA	Medium chain fatty acid
mGluR	Metabotropic glutamate receptor
MUFA	Monounsaturated fatty acid
NAc	Nucleus accumbens

NAM	Negative allosteric modulator
NDC	Non-digestible carbohydrates
NHEJ	Non-homologous end joining
NLS	Nuclear localisation sequence
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarii
OXM	Oxyntomodulin
PAM	Positive allosteric modulator
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC- β	Phospholipase C β
PM	Plasma membrane
POMC	Pro-opio-melanocortin
PTM	Post-translation modification
PTX	Pertussis toxin
PUFA	Polyunsaturated fatty acid
PVN	Paraventricular nucleus
PYY	Peptide tyrosine-tyrosine
qPCR	Quantitative PCR
REE	Resting energy expenditure
RGS	Regulator of G-protein signalling
RNP	Ribonucleoprotein
SCFA	Short chain fatty acid
SFA	Saturated fatty acid

SGLT-1	Sodium/ glucose co-transporter member 1
sgRNA	Synthetic guide RNA
T2DM	Type II Diabetes Mellitus
TAS1R	Taste receptor family 1
TAS2R	Taste receptor family 2
TM	Transmembrane
tracrRNA	Transactivating RNA
TrpM5	Transient receptor potential cation channel subfamily M member 5
VFT	Venus-flytrap
VMN	Ventromedial nucleus
VTA	Ventral tangential area
WHO	World Health Organisation
WT	Wild-type
YR	PYY receptor
B-OHB	β -hydroxybutyrate

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Chapter 1: Introduction

1.1 Obesity

One of the most pressing challenges faced by modern society is the alarmingly fast increase in the prevalence of obesity, with approximately a third of the world's population now classified as obese (WHO, 2018), thus, having a body mass index (BMI) of $> 30 \text{ kgm}^{-2}$.

Weight gain is a consequence of consuming more energy than expended. As a basic survival mechanism, the storage of adipose tissue provides an emergency resource when an animal experiences a nutrient scarce time period. However, for the vast majority in modern society, an excess of energy-dense, high-calorie food products are highly abundant and easily accessible as well as being marketed in an engaging manner. The reverberations of this are reflected in statistics published by the World Health Organisation (WHO), which suggest that since 1975 global obesity percentages have nearly tripled. Globally, nearly 650 million adults are obese and 381 million children are overweight or obese (WHO, 2018).

High BMI has been shown to be implemented in many dangerous chronic conditions such as cardiovascular disease (CVD; Sowers et al., 2003), type II diabetes mellitus (T2DM; Leong et al., 1999), musculoskeletal disorders, particularly osteoarthritis and bone fractures (Wearing et al., 2006) and some cancers (Calle et al., 2004). Additionally, obesity is closely correlated with mental health issues such as depression, anxiety and substance abuse (Simon et al., 2006). These are amongst the maladies that trigger 2.1 million obesity-related deaths per year (WHO, 2017), with obesity decreasing average life expectancy by five to twenty years (Bluhner et al., 2019). The co-morbidities associated significantly lessen patients' quality of life and it has been estimated that in Europe and North America up to 15% of disability adjusted life years are lost directly due to obesity (Allender et al., 2007). Rising obesity rates load a massive economic and social strain onto community services. This is due, firstly, to absenteeism decreasing productivity and secondly, by the increased prevalence and earlier onset of weight-related disabilities lowering the retirement age (Hammond et al., 2010). By 2050, it is predicted that the widely reaching implications of obesity on society will cost the UK £50 billion, with the direct cost on the NHS hitting a £9.7 billion (Public Health England, 2017).

The traits associated with development of obesity can broadly be classified into five categories: differences in behaviour, metabolism, energy balance, the gut microbiome and the constituency of adipose and skeletal muscle tissue. In many cases, the effect of a singular dysregulation does not have a major impact on the probability of developing obesity, but a combination of these susceptibility traits can be catastrophic (Ghosh et al., 2017). Social and environmental factors can increase the tendency for overconsumption and sedentary behaviour, but the weight gained from identical energy input and outputs varies between individuals. The effects of long-term overfeeding on body weight and adiposity of monozygotic twins demonstrates a significant similarity between twin pairs when compared to those who were genetically different (Bouchard et al., 1990). Such examination of identical twins has resulted in estimates that BMI has a heritability of approximately 40–70% (Maes et al., 1997).

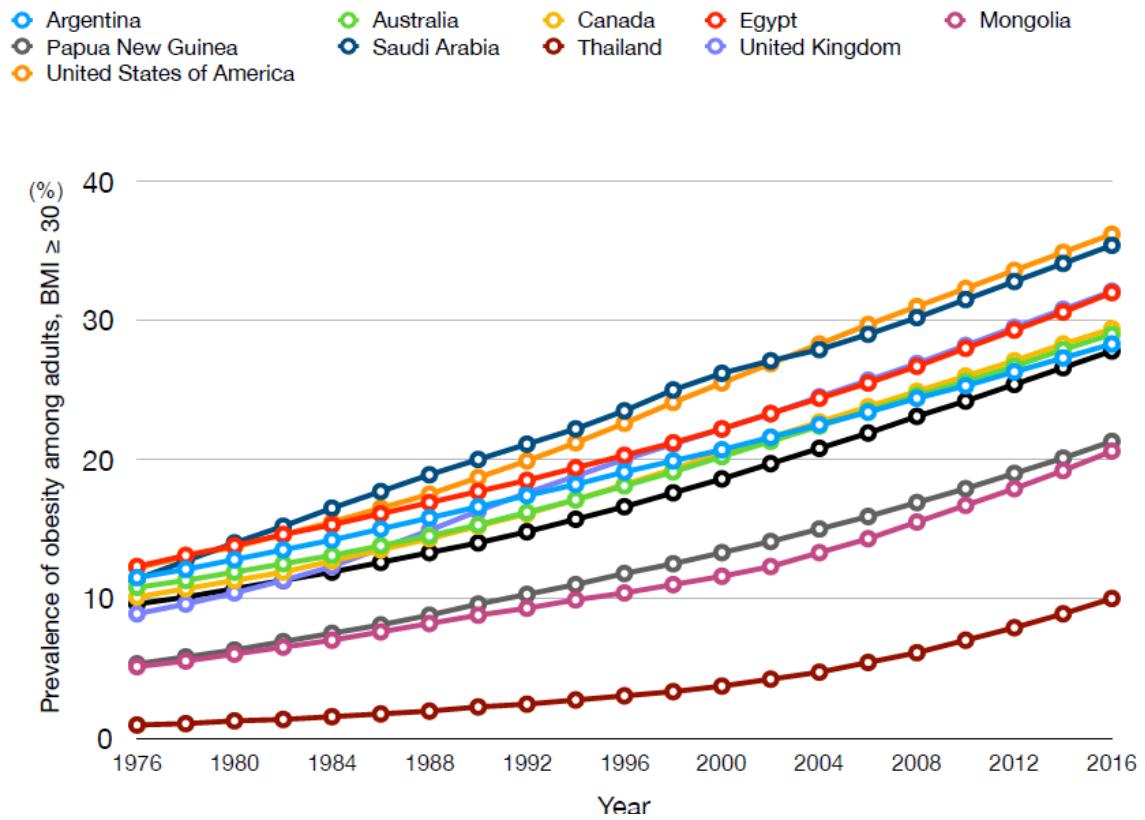


Figure 1.1 Trends in adult obesity globally from 1976 to 2016 for 11 selected countries Data is expressed as the percentage of the adult (>18 years) population with a body mass index (BMI) equal or more than 30 kgm^{-2} , and therefore classified as obese. Data taken from the World Health Organisation annual report (2018)

1.2 Homeostatic mechanisms of food intake

Obesity is caused by a chronic imbalance between the energy consumed and energy expended. It seems to result from an increased appetite (the drive to eat). Appetite regulation is controlled by a complex array of central and peripheral signalling pathways which converge to elicit a single behaviour (Yu et al., 2012).

1.2.1 The gut-brain axis

The gut-brain axis is a bi-directional neurohumoral communication pathway, including the central, autonomic and enteric nervous systems (CNS; ANS; ENS), the hypothalamic pituitary adrenal (HPA) axis as well as neuroendocrine and immunological systems. The gut-brain axis plays a key role in communicating sensory information via neuronal, humoral and immunological signals (Bliss et al., 2018). On consumption of a meal, the fluctuations in macromolecule concentration in the gut lumen are detected by “nutrient sensing” via a large range of receptors (Norton et al., 2017), predominantly G protein-coupled receptors (GPCRs), ion channels and macromolecule transporters. The gastrointestinal (GI) tract is densely innervated with over 500 million neurons, including neurons of the ENS and the sensory afferents of the vagal nerve, which communicate bi-directionally. These sensory afferents bundle together to form the vagus nerve which forms a physical link between the ENS and the brain stem, where it terminates. As well as being responsive to dynamic signals from the ENS, the vagal sensory afferents express a diverse array of chemoreceptors and mechanoreceptors, able to detect the nutritional and physical state of the gut independently (Breit et al., 2018). The GI tract also qualifies as the largest enteroendocrine organ of the body, able to secrete over 20 different peptides which signal via humoral and neuronal routes to various brain areas including the hypothalamus, brain stem and midbrain. The crosstalk between these brain regions integrates the complex signals from the gut-brain axis with peripheral signals of long-term energy supplies, controlling appetite.

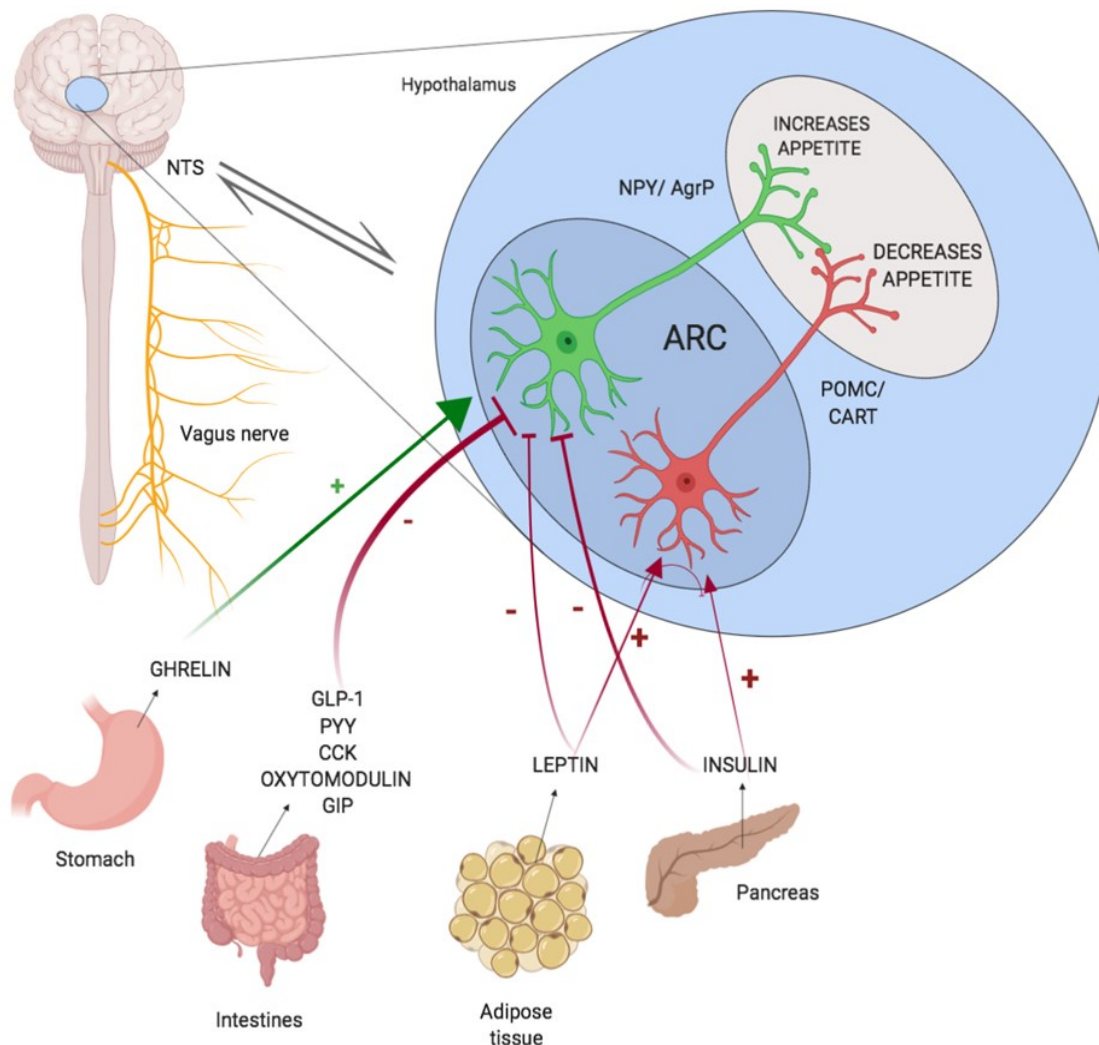


Figure 1.2 Simplified diagram depicting peripheral and central mechanisms of appetite. The hypothalamus is essential for the homeostatic regulation of appetite. It signals bi-directionally with the brain stem, at which the vagus nerve terminates at the nucleus tractus solitarius (NTS), but also communicates with higher brain regions. Key hypothalamic nuclei, such as the arcuate nucleus (ARC) and the paraventricular nucleus (PVN), are essential for appetite control. The ARC contains two subsets of neurons: the orexigenic neuropeptide Y/agouti-related protein (NPY/AgrP) neurons and the anorexigenic proopiomelanocortin/cocaine-and amphetamine-regulated transcript (POMC/CART) neurons. Both feed into the PVN and act to increase and decrease food intake respectively. Satiation is also moderated by circulating endocrine factors. These provide information about the short-term nutritional state of the gastrointestinal tract and the quantity of long-term energy stores in peripheral tissue. Short term energy signals include gastrointestinal hormones. Of these, ghrelin is the only one which has orexigenic properties and is secreted from the stomach. Anorexigenic signals include those from distinct sites in the intestine, which secretes peptides such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide tyrosine tyrosine (PYY), oxyntomodulin (OXM) and also signals of long-term energy supplies, related to adiposity, such as insulin from the pancreas and leptin from adipose tissue. The ability of these hormones to moderate neuronal activity at three sites: the vagus nerve, brainstem and hypothalamus allows the complexity of multiple signals to be entwined into a signal response. + indicate orexigenic signals and – indicate anorectic signals. *Image created with BioRender.*

1.2.2 Brain regions involved in appetite control

The hypothalamus, although small, is a fundamental brain region for modulating homeostatic and endocrine pathways that are crucial for survival. Situated at the base of the brain, just beneath the thalamus, the hypothalamus plays a critical role in energy homeostasis; the process by which energy stores are maintained at a level adequate to meet the environmental demands.

The hypothalamus, consisting of a complex communication network of anabolic and catabolic neurocircuits between several small nuclei, plays a key role in regulating food intake. These nuclei include the lateral hypothalamic area (LHA), which increases hunger (Anand and Brobeck., 1951) and the paraventricular area (PVN), which acts to instil satiety (Sims et al., 1986). Other hypothalamic nuclei involved include the arcuate nucleus (ARC), LHA, PVN, ventromedial nucleus (VMN), and the dorsomedial nucleus (DMN), each of which has a unique role in nutrient and endocrine sensing and signalling which intercalate to provide a homeostatic regulatory effect (Yu et al., 2012).

The ARC is taken to be the primary sensor of peripheral signals, due to its proximity to the median eminence. This has a specialised blood brain barrier (BBB) formation, facilitating hormones, nutrients and other signalling molecules to gain access to the ARC (Rodríguez et al., 2010). Bundles of second-order neurons projecting from the ARC communicate these signals to other nuclei, such as the PVN and LHA (Yu et al., 2012). Some projections have strong anorexergic reverberations when activated, expressing neuropeptides pro-opiomelanocortin (POMC; Cone et al., 2005) and cocaine- and amphetamine-regulated transcript (CART; Murphy et al., 2005). Conversely, other neurons projecting from the ARC co-express orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), promoting hunger (Sohn et al., 2015).

The hypothalamus has a densely innervated bi-directional communication pathway with the brain stem, particularly the nucleus tractus solitarius (NTS). The NTS is not only perfectly positioned to receive peripheral humoral signals but is also the termination site of the vagal afferent nerve, the principle neuronal connection between the gut and the brain. Therefore, the NTS can receive and integrate detailed signals concerning the nutritional state of the GI tract and can cross-talk intricately with hypothalamic regions (Travagli et al., 2006).

The mid-brain is also involved in mediating the rewarding or hedonic component of food intake. We have always had a constant and powerful urge to survive and reproduce which, throughout our development as a species, has been fuelled by calorific consumption. Consequently, a strong correlation between nutritional intake and the sensation of reward has evolved. The mesolimbic dopamine pathway consists of dopaminergic projections from the ventral tegmental area (VTA) to other areas of the limbic system, especially the nucleus accumbens (NAc). This is thought to mediate the rewarding sensations of palatable foods, typically foods high in fat or sugar (Hernandez et al., 1988). Metabolic and endocrine signals of satiety can act to diminish the strength of dopaminergic reward signals, but, conversely, dopaminergic induced food cravings can override homeostatic signals of satiety (Yu et al., 2012).

1.2.3 Adiposity signals

Adiposity signals have been proposed to act as a specialised negative feedback, to help elicit a fine control of energy balance and to allow the calorie consumption to adapt to energy stores throughout the body (Figlewicz, 2003).

Leptin, expressed predominantly in adipocytes from the *ob* gene, is a cytokine with many effects on energy homeostasis, endocrine function and metabolism. Plasma concentrations of leptin correlate

with adipose tissue mass and therefore, any fall in the plasma leptin level acts as a signal of energy depletion, increasing appetite (Montzoros et al., 2011). However, a day of starvation can induce drops in plasma leptin levels of 50%, which increases to 80% after three days, before any noticeable change in BMI (Chan et al., 2015). The longest isoform of the leptin receptors (LEPR-b) is expressed in the ARC, where leptin increases the activity of anorexigenic POMC neurones (Cowley et al., 2001) and in the NTS and VTA, where leptin's action also contributes to satiety signals and curtails food reward (Montzoros et al., 2011).

Insulin is secreted peripherally by pancreatic β -cells of the Islets of Langerhans in the pancreas in response to blood glucose levels, but plasma concentrations are also correlative with adiposity (Rocha et al., 2011). The receptors for insulin are found throughout the CNS, through which insulin signals to diminish appetite. For example, insulin receptors are expressed in the ARC, where insulin acts to decrease the activity of orexigenic NPY/AgRP neurones (Figlewicz et al., 2003).

1.2.4 The gastrointestinal tract as an endocrine organ

In addition to its role in absorbing water, ions and essential nutrients, the GI tract also has a key role as an endocrine organ. The concept that the GI is able to secrete signalling peptides in response to the physiological state of the gut has been documented for some time, since the observation that changes in acidity of the intestinal lumen influences pancreatic secretory activity via a signalling molecule, thereby named secretin (Bayliss and Starling, 1902).

Specialised secretory cells, enteroendocrine cells (EECs), make up about 1% of the total luminal lining and release over 20 different gut hormones which signal via humoral and neuronal routes (Egerod et al., 2012). EECs gain a detailed profile of the nutritional state of the gut via nutrient sensors expressed on the extensions of their apical surface. The modern human diet consists of a large variety of nutrients and macromolecules, the breakdown of which is carried out physically and chemically via enzymatic catalysis from the moment they reach the oral cavity. This results in a complex assortment of metabolites in fluctuating concentrations throughout the GI tract such as sugars, amino acids, triglycerides, long chain fatty acids (LCFAs; $C > 12$), each of which possess a unique uptake and signalling profile (Psichas et al., 2015). This dynamic range of nutrients and bioactive metabolites in the gut lumen results in electrochemical changes, ion and solute influxes and the activation of various effector molecules key for signalling, resulting in the exocytosis of gut hormone containing dense-core vesicles through the wider basolateral membrane (Psichas et al., 2017). This sensitivity to fluctuating conditions is vital for creating an optimal digestion environment, such as the stimulation of gastric acid secretion, activation of gut motor responses and conversion of pepsinogen into pepsin (Taylors et al., 1982). Luminal macromolecule sensing is also important for communicating the nutritional state of the gut to hypothalamus nutrient centres and other neuromodulatory pathways (Blouet et al., 2010).

EECs have been categorised into around 15 distinct subtypes, dependent on the expression of peptide precursors which are alternatively spliced, processed and post-translationally modified into key peptide hormones (Aaboe et al., 2008). Of interest, are proglucagon derivatives glucagon-like-peptide 1 (GLP-1) and Peptide Tyrosine-Tyrosine (PYY) secreted from L cells, and cholecystokinin (CCK), secreted from enterochromaffin/ I-type EECs. Other types of EECs include G cells, X/A gastric cells, D cells, and K cells, which secrete gastrin, ghrelin, somatostatin, gastric inhibitory polypeptide (GIP) respectively. The EEC subtypes, although categorised in terms of the hormone they predominantly secrete, are versatile to express a whole spectrum of gut hormones with many overlaps between subtypes, so that each can adapt with high plasticity to environmental conditions (Habib et al., 2012).

Gut hormones contribute to gut-brain axis communication, by signalling the oscillating metabolic condition of the GI tract to key brain areas involved in control of food intake, such as the hypothalamus and hindbrain (Weltens et al., 2018). They act via various mechanisms; in a paracrine fashion such as modulation of vagal nerve afferents and in an endocrine fashion, entering the circulation and reaching peripheral targets. All the named gut hormones have anorexigenic properties, discounting appetite-stimulating ghrelin.

GLP-1 is synthesised by post-translational modification of a pre-pro-glucagon precursor into two biologically active forms: GLP₇₋₃₆ and GLP₁₇₋₃₇. It is secreted post-prandially from L-type EECs, localised within the colon and distal small intestine in response to dietary nutrients (Gribble et al., 2016). To this date, there is only one known GLP-1 receptor (GLP-1R), a stimulatory class B GPCR (Sato et al., 2000). GLP-1R is expressed in the CNS and in peripheral organs, such as the heart, kidneys and pancreas (Cho et al., 2014).

GLP-1 has well-established metabolic activity and is characterised most extensively as an incretin (VilSBoll et al., 2003). The “incretin effect”, a concept introduced in the early 1900s, describes the more prominent production of insulin by the pancreas in response to intake of oral glucose, as opposed to intravenously injected or infused glucose (Bayliss et al., 1901). When GLP-1 binds to GLP-1R expressed on pancreatic Islets of Langerhans β -cells, an increase in adenylyl cyclase (AC) activity causes cellular cyclic adenosine monophosphate (cAMP) concentrations to rise. Subsequently, a complex signalling cascade promotes insulin expression and secretion. This gives rise to GLP-1's incretin effect in a manner which is dependent on glucose as a co-factor, reducing the potential risk of hypoglycaemia on activation of the GLP-1R (VilSBoll et al., 2007). This phenomenon is estimated to cause 50-70% of the total insulin secretion from the pancreas (Kazakos et al., 2011). Consequently, GLP-1 analogues have been examined for potential utilisation in the treatment of diabetes (Breen et al., 2013).

Aside from its incretin properties, GLP-1 elicits actions such as reducing gastric motility, regulating gastric acid secretion, inhibiting glucagon release and regulating weight and appetite (Cho et al., 2014). The effects of GLP-1 on appetite seem to be due to the expression and signalling of GLP-1R in key hypothalamic nuclei such as the ARC and PVN (Shughrue et al., 1996), in the midbrain VTA and NAc (Merchenthaler et al., 1999) and in the NTS, a secondary site of GLP-1 synthesis (Larsen et al., 2007). Studies have shown that intracerebroventricular injections of Ex-9, a GLP-1 antagonist, increase the weight of rats fed *ad libitum* approximately twofold (Turton et al., 1996).

The observation that 90% of gut-synthesised GLP-1 does not reach the systemic circulation, coupled with the extremely short half-life of GLP-1 (<2 mins), due to its rapid degradation by dipeptidyl peptidase IV (DPP-IV; Kazakos et al., 2011), has led to doubts in the endocrine abilities of GLP-1 and suggestions that its actions are mediated chiefly in a paracrine fashion. However, large increases in plasma GLP-1 concentrations are observed after carbohydrate intake, an effect which is attenuated in obese individuals (Ranganath et al., 2008). The intravenous administration of synthetic GLP-1 analog, liraglutide, has showed some efficacy in the management of T2DM and the observed weight loss has led to trials for their use to treat non-diabetic obese patients (Rosenstock et al., 2010; Behary et al., 2019).

Co-released with GLP-1 from L-type EECs in response to calorific intake and specific nutrients, PYY also functions as a post-prandial regulator of energy homeostasis, as well as modulating gastric motility and regulating gastric and pancreatic secretory processes (Manning et al., 2014).

There are two bioactive forms of PYY; PYY₁₋₃₆ and the DPP-IV cleavage product of this, PYY₃₋₃₆. These

isoforms have remarkably opposing effects on energy homeostasis, regarded to be due to their differing affinities and efficacies when acting at the five PYY receptor (YR) subtypes (Walther et al., 2012). Intravenous peripheral administration of PYY₃₋₃₆ attenuates appetite, slowing weight gain, in rodents (Batterham et al., 2002) and diminishes caloric intake in both lean and obese humans (Batterham et al., 2003). It is generally regarded that these strong anorexergic reverberations are mediated primarily via PYY₃₋₃₆ acting at YR2. This receptor is expressed in key hypothalamic and hindbrain nuclei (Manning et al. 2014) and in brain centres involved in food rewards (Weise et al., 2012). Such administrations also enhance glucose tolerance in mice, but it has not been ascertained whether PYY₃₋₃₆ signals directly in pancreatic islets to induce insulin secretion or whether its glucoregulatory effects are a result of an indirect mechanism, such as GLP-1 signalling potentiation or extra-islet YR2 signalling (Manning et al., 2014).

CCK is thought to be secreted from more proximal regions of the GI tract than PYY and GLP-1, from mucosal I-type EECs in the jejunum and duodenum, particularly in response to dietary fats and amino acids (Buchan et al., 1978). The post-translational processing of pre-pro-CCK is complex, resulting in the release of several bioactive forms of CCK, all with an identical heptapeptide C-terminal sequence. Through this conserved sequence, the biological effects of CCK are mediated via action at two receptor isoforms, CCK1 and CCK2. The differing N-terminal polypeptide sequences of CCK impact the selectivity and potency of receptor binding, a phenomenon also moderated by sulfation of a tyrosine residue at position 7 (Rhefeld et al., 2017).

The 33 amino acid form of CCK was the first gut hormone reported to curtail appetite (Gibbs et al., 1973). This effect is thought to be mediated, firstly, in a paracrine fashion via CCK1R expressed in vagal afferent nerves that terminate in the NTS, causing an activation which is dulled in obesity (Covasa et al., 2010). It has been suggested that CCK1 activation in vagal afferents is able to modify the vagal afferent plasticity and susceptibility to signals from other messengers by fine-tuning gene expression, such as increasing YR2 expression, increasing CART expression and reducing expression of cannabinoid (CB1) receptors. (Dockray et al., 2012). Although vagotomy significantly impairs CCK anorexergic effects (Smith et al., 1981), observations that peripheral CCK administration to rodents can directly stimulate anorexergic CART-neurons of the PVN suggests that CCK can function, secondly, in an endocrine fashion (Peter et al., 2010). As well as acting as a satiety cue, other actions of CCK include promoting secretions of pancreatic enzymes, although the incretin properties are weak in comparison to other gut hormones such as GLP-1, inducing contractions of the gallbladder and slowing gastric emptying (Dockray et al., 2012).

1.3 The gut microbiota

Thriving within the human GI tract, principally the colon, is an incredibly diverse and dense microbial population of over 1000 different species; the gut microbiota. The commensal relationship between the host and gut microbiota has a significant impact on host homeostasis and physiology (Krishnan et al., 2015). It has been suggested that the composition of the gut microbiota may contribute to the pathogenesis of obesity as it plays an extensive role in aiding digestion and nutrient extraction (Al-Assal et al., 2018); regulating immune function and inflammation (Pindjakova et al., 2017); influencing host behaviour and appetite through the gut-brain axis (Moran and Shanahan, 2014) and altering fatty acid oxidation in the liver (López et al., 2017).

The gut microbiota composition is flexible, fluctuating throughout lifespan in response to many environmental factors such as diet, exercise, antibiotic exposure, disease states, chemical exposure and birth method. The gut microbiota bacteria can be grouped into 6 phyla, with the two most predominant being *Firmicutes* and *Bacteroidetes*. Discrepancies in gut microbiota profiles have been

correlated with obesity. This was first observed when comparing the gut microbiota profiles of genetically engineered obese mice (*ob/ob*), to their lean sibling littermates (+/+ or *ob/+*). Obese mice had higher proportions of *Firmicutes* compared to *Bacteroidetes*, independent of diet constitution (Ley et al., 2005). This disrupted ratio is also observed in obese versus lean humans, although weight loss via calorie restriction has been shown to restore the ratio somewhat (Ley et al., 2006). As well as this discrepant ratio, it has repeatedly been observed that obese individuals lack bacterially diverse ecosystems (Al-Assah et al., 2018). The richness of the microbiome can negatively be correlated with prevalence of markers of metabolic disease (Le Chatelier et al., 2013). Studies transplanting the so-called “obese-microbiome” into lean counterparts have shown that this, alone, is enough to induce metabolic disease and increase in adiposity (Tremaroli et al., 2015). One explanation for this is that the obese microbiome harvests energy more efficiently, increasing the bioavailability of energy-dense nutrients for absorption (Turnbaugh et al., 2006). Additionally, microbially-produced metabolites can act as signalling molecules which have a great impact on physiology (Al-Assal et al., 2018).

A typical Western diet, consisting of highly processed fatty and sugary foods and a lack of fibre, leads to permanent bacterial taxa extinction (Sonnenberg et al., 2016) as well as break-down of mucosal integrity triggering inflammation and decreased immunity (Desai et al., 2016). It is estimated that only 9% of the UK population reach the recommended daily intake of fibre (30 g per day for an adult; Roberts et al., 2018). This is worrisome as fibre in the diet moulds the environmental microbial niche, allowing certain species to flourish and increasing the microbial diversity, a property correlated with improved metabolic parameters (Deehan et al., 2017). Fibre is also the principle source of short chain fatty acids (SCFAs). The non-digestible carbohydrates (NDCs) found in high-fibre foodstuffs cannot be broken down by the enzymatic machinery encoded by the human genome. Therefore, the undigested fibrous material enters the colon in relatively whole structures. Here, the host is completely reliant on the gut microbiota to break down NDCs via a saccharolytic fermentation process which produces SCFAs as a by-product (Hijova et al., 2007).

1.3.1 Short chain fatty acids

SCFAs, carboxylic acids with fewer than 6 carbons in the chain, are principle gut microbiota derived metabolites. 95% of the SCFAs produced in the colon consist of acetate (C=2), propionate (C=3) and butyrate (C=4) in a 3:1:1 ratio (Hijova et al., 2007), reaching concentrations of 10^{-1} M (Stevens et al., 1998). SCFAs diffuse readily across the epithelium, but this movement is also assisted by transporter proteins expressed on the apical and basolateral membranes. This transport can be via an electroneutral process, such as with proton-coupled MCT1 and MCT4 or sodium-coupled SMCT2, or electrogenic in nature such as with sodium-coupled SMCT1 (Spivaprakasam et al., 2011). The uptake allows SCFAs to reach concentrations of 10^{-5} M in the peripheral plasma (Cummings et al., 1987). SCFAs are, principally, an energy source. They deliver 6-9% of the body's energy demands. Butyrate alone provides 60% of the energy requirements of the colon (Stevens et al., 1998). Aside from their role as a fuel source, SCFAs act as bioactive signalling molecules in multiple cell types throughout the body. In the colon, SCFAs alter gut motility, modulate secretory processes and impact the division, differentiation, adhesion and growth of cells, playing a fundamental role in the maintenance of sound gut integrity. In adipocytes, SCFAs increase lipogenesis and triglyceride storage (Al-Assah et al., 2018). The effects of SCFAs on both the innate and adaptive immune systems have also been documented, such as their role in regulating the complex inflammatory processes involved in arthritis, asthma and colitis (Maslowski et al., 2005). SCFAs also have a significant impact on resting energy expenditure (REE), with a recent study in fasting humans demonstrating that REE and lipid oxidation was significantly increased by oral administration of propionate (Chambers et al., 2018).

Investigations in humans have provided evidence that there is a clear correlation between fasting concentrations of circulating SCFAs and metabolic health parameters. Particularly, higher circulatory SCFAs are associated with a reduction in free fatty acids, triacylglycerol and glycerol in the blood, the latter being used as an indicator of whole-body lipolysis. Conversely, elevated levels of circulatory SCFAs held a positive association with plasma concentrations of GLP-1 (Müller et al., 2019).

The wide-range of reported effects of SCFAs are likely to be a direct result of two principle mechanisms: firstly, the SCFAs are ligands to GPCRs, fatty acid receptor 2 and 3 (FFAR2; FFAR3; Brown et al., 2003) and secondly, butyrate and propionate are inhibitors of histone deacetylases (HDACs), with potential to modulate gene expression and cell fate (Sanderson et al., 2004).

1.4 G protein-coupled receptors

Expressed ubiquitously, GPCRs are the most extensive and diverse family of proteins in the human genome. GPCRs are highly druggable targets due to their extensive actions and sensitivity to a dynamic range of ligands. Such ligands include neuropeptides, hormones, neurotransmitters, metabolites and chemokines, which can act in endocrine, autocrine and paracrine fashions. Signalling can impact physiology by promoting exocytosis and subsequently secretion, regulating gene expression, altering cell metabolism, guiding cell migration and gating cell excitability (Stevens et al., 2013). Consequently, approximately a third of all FDA-approved drugs target GPCRs to treat a wide range of diseases (Hauser et al., 2017).

Over 800 different human GPCRs have been identified to date, which are grouped broadly into five major families; Class A (Rhodopsins), Class B (Adhesion and Secretin), Class C (Glutamate) and TAS2 (Frizzled/ Taste receptor 2). Further analysis of sequence similarities has led to additional in-depth sub-categorisation (Stevens et al., 2013).

1.4.1 GPCR structure

GPCRs are membrane-embedded protein structures and, thus, it is extremely challenging to obtain their high-resolution structures. The rhodopsin receptor, a light-activated photoreceptor found predominantly in the retinal rod cells, was the first to be crystallised successfully (Palczewski et al., 2000), providing valuable insights into GPCR structure and function. However, it was when the first ligand-activated GPCR, the β 2-adrenoreceptor, was crystallised in its carazolol-bound state (Cherezov et al., 2007), that valuable knowledge of orthosteric binding sites was gained, enabling an advancement in drug discovery and design. Since then, utilisation of stabilising ligands and the induction of stabilising point mutations has combated some of the issues with using traditional techniques but can disrupt the reliability of whether the resulting structures are physiologically relevant (Topiol et al., 2018). Nevertheless, it has become evident that GPCRs can spontaneously adopt several different conformations; active, inactive and intermediate; and flip continuously between these states (Dror et al., 2011).

All GPCRs share the same basic structure; seven α -helical membrane-traversing domains, an extracellular (EC) N-terminal domain, an intracellular (IC) C-terminal domain, and three hydrophilic loops on each side of the plasma membrane (PM) respectively. The most striking variability between GPCR subtypes is in the N-terminal domain, which forms a large orthosteric ligand binding domain (LBD) for some subtypes, whereas other GPCRs bind orthosteric ligands via a ligand binding pocket that forms in the crevices between the α -helical transmembrane (TM) domains (Park et al., 2008). Regardless of the orthosteric ligand binding mechanism, the EC domains are sensitive to changes in the extracellular environment and the presence of stimuli is communicated across the 30 Å PM distance via subtle variations in the three-dimensional conformation. These variations are likely to share similarities independent of location of orthosteric binding site (Park et al., 2008).

Also essential to the signal transmission is the intracellular coupling to cytosolic and membrane-bound signalling proteins. The specificity is reliant on the precise architecture of the IC region, and slight changes in this can lead to biased agonism. The frequency by which these interactions occur is dependent on intrinsic activity of the GPCR as well as being induced by stimulation from stimuli (Topiol et al., 2018). The primary mechanism of signal transduction from GPCRs are via the heterotrimeric G proteins, consisting of $G\alpha$ (45 kDa), $G\beta$ (35 kDa) and $G\gamma$ (15 kDa) subunits. When inactive, the $G\alpha$ subunit is in its guanosine-di-phosphate (GDP) bound state, complexed with membrane-associated $G\beta$ and $G\gamma$ subunits (Duc et al., 2017).

1.4.2 GPCR activation

As mentioned previously, GPCRs cycle spontaneously and dynamically between active, intermediate and inactive conformations with underlying basal activity. However, extracellular ligands can stabilise certain conformations in the GPCR structure, altering the transition time between different states. Each ligand has a unique structure affecting these properties, and determining the efficacy, affinity and pharmacology (Wootten et al., 2013).

Orthosteric ligands, those associating with the ligand binding site, are further classified as full, partial or inverse agonists. Full agonists induce the maximal response from the receptor, whereas partial agonists can elicit a scale of responses, typically less than the maximal, even when fully occupying all available binding sites. In contrast, inverse agonists are ligands which diminish basal activity of the GPCR. Allosteric ligands, those binding at structural regions distinct from the ligand binding site, can enhance (positive allosteric modulators; PAMs) or decrease (negative allosteric modulators; NAMs) the activity of an agonist, but have no intrinsic efficacy when used alone. Several forms of physiological molecules; ions, amino acids, peptides, lipids; can act allosterically, fine-tuning the susceptibility of the GPCR to signalling. They do this by, firstly, rescaling the intrinsic efficacy, affinity, binding kinetics of orthosteric ligands and, secondly, by altering the intrinsic ability of the GPCR to shift dynamically between active and inactive conformations (Park et al., 2008). Allosteric modulators can be targeted for drug design as their inability to alter the GPCR signalling when used in isolation allows physiological spatial and temporal parameters to be maintained, limiting potential side effects and the possibility of over-activation and tachyphylaxis. They can also be designed with greater selectivity at divergent subtypes of GPCRs which may share similar orthosteric ligand binding sites (Wootten et al., 2013).

On activation, drastic shifts in conformation in the transmembrane α -helices alter the IC protein domain environment, allowing the GPCR to act as a guanine exchange factor (GEF). The conformational change allows a novel $G\alpha$ /GPCR interface to form. This destabilises the interactions between GDP and the key residues of the α -subunit nucleotide-binding pocket, enabling GDP to dissociate. The lost GDP is replaced with guanosine triphosphate (GTP), a molecule that helps stabilise highly flexible regions of the $G\alpha$ subunit. This shift in $G\alpha$ subunit structure causes it to disassociate from the $G\beta$ and $G\gamma$ heterodimer. Both the $G\alpha$ and $G\beta\gamma$ complex are then at liberty to activate several distinct effector molecules, depending on the type of G protein, triggering a diverse range of intracellular signalling cascades. The intrinsic GTP-ase activity of $G\alpha$ removes the γ -phosphate from GTP via hydrolysis, converting it back to GDP. This allows the G protein heterotrimer to reform, terminating activation of effector molecules (Duc et al., 2017). This is the classical model of G protein activation, but there is evidence to suggest that G proteins can be activated in other ways such as via structural rearrangements of G protein complexes (Gales et al., 2006). This could be by cytosolic proteins with GPCR-independent GEF activity which promote GDP to GTP conversion independently of the GPCR (Tall et al., 2006) or by cytosolic proteins with Regulators of G Protein Signalling (RGS) domains which have GTPase activity and thus, terminate GTP-bound $G\alpha$ effects. It has also been shown that some peptides can induce conformational shifts in the structure of the $G\alpha$ or $G\beta\gamma$ subunits independently of nucleotide exchange, and that these shifts alone are adequate to influence G protein activity (Ghosh et al., 2003).

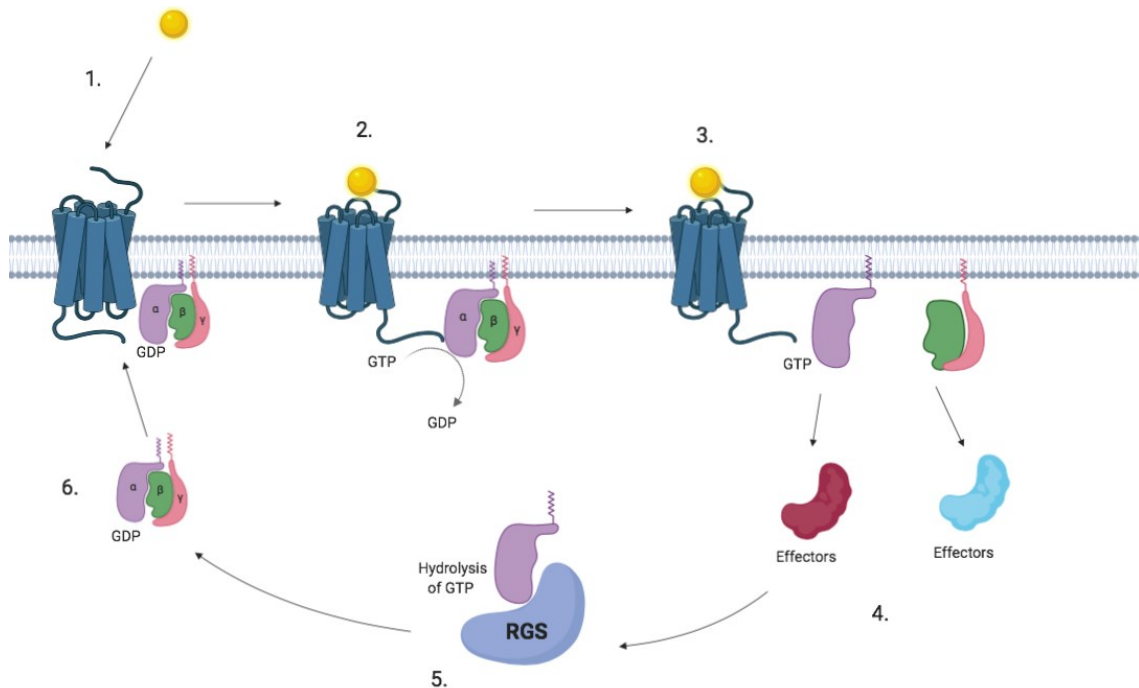


Figure 1.3 GPCR and heterotrimeric G protein activation and deactivation cycle. (1) The receptor is in an inactive conformation. In this state the $G\alpha$ subunit is associated with guanosine diphosphate (GDP) and the heterotrimer is bound in complex, which can be associated with the GPCR. (2) On ligand binding, the receptor takes its active conformation. This conformational change induces the receptor to act as a guanine exchange factor (GEF), facilitating the loss of GDP from the $G\alpha$ subunit, which is immediately replaced by free cytosolic guanosine triphosphate (GTP). (3) The GTP binding to $G\alpha$ subunit induces the dissociation of the heterotrimer, facilitating the release of the $G\beta\gamma$ heterodimer. (4) Both the $G\alpha$ and $G\beta\gamma$ are now free to bind their range of effectors and activate intracellular signalling cascades. (5) Intrinsic GTPase activity of the $G\alpha$ subunit, aided by regulator of G protein signalling (RGS) domain, catalyzes the hydrolysis of GTP back to GDP. (6) The heterotrimeric G protein can then re-associate with the receptor, which returns to an inactive basal state. **Image created with BioRender.**

1.4.3 G protein signalling pathways

Although there are over 20 $G\alpha$ subtypes in the human genome, the signalling elicited from these has been grouped into four $G\alpha$ canonical G protein effector pathways; namely, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$.

Adenylyl cyclase (AC) is a PM-bound enzyme which catalyses the cyclisation of adenosine triphosphate (ATP) to cAMP, a second messenger able to diffuse and activate other intracellular targets such as protein kinase A (PKA). $G\alpha_s$ and $G\alpha_{i/o}$ activate or inhibit AC respectively. In contrast, $G\alpha_{q/11}$ couples to phospholipase C β (PLC- β), which cleaves PM lipid phosphatidylinositol phosphate (PIP₂) to produce the second messengers inositol (3,4,5) triphosphate (IP₃) and diacylglycerol (DAG). IP₃ mobilises intracellular calcium stores by interacting with its receptor, a ligand gated cation channel. Concurrently, DAG engages with and activates calcium dependent Protein Kinase C (PKC) at the PM (Neves et al., 2002). $G\alpha_{12/13}$ subunits can control the activation state of the monomeric GTPase, RhoA, by coupling to Rho-guanine exchange factors (Rho-GEFs). Where PKA and PKC activate downstream targets primarily by phosphorylation, RhoA acts by catalysing GTP hydrolysis (Suzuki et al., 2007). It is now commonly realised that the classical G protein signalling routes are not

the sole method of signalling utilised by GPCRs (Flordellis et al., 2012). GPCRs have also been shown to activate other pathways previously thought of as G protein independent. For example, β -arrestins can mediate signalling to mitogen activate protein kinase (MAPK) pathways (Daaka et al., 1998).

Additionally, there are a further 11 $G\gamma$ and 6 $G\beta$ subunits in the human genome, which, as well as negatively regulating $G\alpha$ interactions, possess their own signalling functions. The earliest evidence of this was the observation that $G\beta\gamma$ subunits actively moderate ion flux through muscarinic potassium (K^+) channels (Logothetis et al., 1987). Since, it has been realised that the targets of $\beta\gamma$ subunits are a large and seemingly structurally unrelated group of effectors, many of which are modulated by direct interactions. This includes known targets of $G\alpha$ subunits such as AC, PLC- β , voltage-activated calcium channels and MAPK (Smrcka et al., 2008). However, $G\beta\gamma$ subunits also interact with proteins not related to classical GPCR signalling pathways such as phosphoinositide-3 kinase (PI3K), GEFs, PDZ-domain containing proteins and several cytosolic Ser/Thr kinases, providing intricate crosstalk mechanisms with innumerable cellular signalling cascades (Dupré et al., 2009).

1.4.4 Signal Bias

Each GPCR may have multiple orthosteric or allosteric binding sites, each able to associate with several ligands with varying affinity and efficacy. Chemically diverse ligands can stabilise the activated GPCR in specific conformations, each with a slight preference to couple to certain intracellular molecules. This tendency leads to preferential selection of certain signalling cascades over others, a concept known as “signal bias” (Seyedabadi et al., 2017). Allosteric modulators can also modify signal bias (Wootten et al., 2013). To add further complexity, signal bias has a level of plasticity within itself; it is cell-type and context dependent (Mailman et al., 2007). Signal bias also can impact desensitisation and internalisation properties (Seyedabadi et al., 2017). This has opened doors for the design of ligands which favour certain signalling pathways, potentially reducing unwanted side effects (Flores-Otero et al., 2014).

1.4.5 Desensitisation and internalisation

After prolonged ligand exposure, GPCRs can undergo a desensitisation mechanism, reducing the response and avoiding potentially toxic over-excitation. Acute desensitisation results in inactivation of receptors at the plasma membrane. Following chronic ligand exposure, the GPCR can also be internalised, where it is either recycled to the PM or targeted for degradation as part of the overall mechanism of receptor downregulation. There are 2 major families of proteins mediated receptor desensitisation; Ser/Thr kinases and arrestins.

The phosphorylation of intracellular Ser/Thr residues of the GPCR can occur via both homologous and heterologous desensitisation pathways, which induce desensitisation and internalisation via discrete signalling routes (Hausdorff et al., 1989). Homologous desensitisation; a rapid process by which the signalling from the agonist bound GPCR is curtailed in a self-regulating feedback mechanism; is mediated via GPCR kinases (GRKs). There are 7 GRKs, all of which share a kinase domain, RGS domain and carboxyl terminal domain, key for positioning the GRK adjacent to the membrane. The classical kinase activity of GRKs produces specific phosphorylation patterns of specific GPCR residues, primarily in the C-terminus but also on the IC loops (Shukla et al., 2011), facilitating the high affinity binding of β -arrestins. These block engagement of G proteins, acutely terminating G protein signalling (Kelly et al., 2008). Aside from kinase activity, the GRK recruitment can attenuate signalling via steric hindrance of the GPCR and via direct interactions with $G\alpha_{q/11}$ subunits (Carmen et al., 1999; Day et al., 2003). Heterologous desensitisation, on the other hand, can occur independently of agonist occupancy of the receptor, such via PKA or PKC mediated

phosphorylation of the receptor at sites distinct from GRK (Kelly et al., 2008).

There are four types of arrestins, two of which, β -Arrestin 1 and 2, are ubiquitously expressed. These are scaffolding proteins with a complex array of interacting partners including kinases, ubiquitin ligases, cell surface receptors, signal transduction proteins and deubiquitinases (Shekela et al., 2011). As well as sterically hindering the association of G proteins and, thus, terminating the signalling, β -arrestins are promiscuous in their interactions and signalling properties; regulating the MAPK cascade, transactivating tyrosine receptors, inducing internalisation, impacting trafficking and contributing to transcriptional profiles (Rajagopal et al., 2017). The β -arrestin-dependent internalisation pathway is mediated via its association with AP2 and clathrin, which together induce membrane curvature. This leads to endocytosis of the receptor from the PM into small vesicles known as endosomes. When in these endosomes, certain receptors persist in signalling via β -arrestin (Luttrell et al., 1999) and via G proteins (Thomsen et al., 2016). However, these signalling properties are temporally and spatially distinct from canonical pathways. There is growing evidence to suggest GPCRs can also be internalised via clathrin-independent routes and that GPCRs undergo constitutive internalisation in the absence of agonist stimulation (Scarselli et al., 2009).

On entering the complex endosomal network, the GPCR can either be targeted for lysosomal degradation or recycled back to the PM to reinitiate sensitivity to EC stimuli and canonical interactions (Calebiro et al., 2018). This sorting process is complex, dependent on the spatial and temporal signalling environment (Rajagopal et al., 2017). An example of this regulation is via post-translational modifications (PTMs), such as ubiquitination. The covalent linkage of ubiquitin molecules to GPCR lysine residues act to target the GPCR for degradation, leading to the long-term termination of receptor signalling (Marchese et al., 2001). This degradation of GPCRs is essential for maintaining the turnover of GPCRs and allowing cellular adaptations to chronic stimuli, such as with drug administration (Hanyaloglu et al., 2008).

1.4.6 GPCR oligomerisation

The idea that GPCRs can crosstalk with each other, in a manner not necessarily dependent upon physical interactions has added a further layer of complexity to GPCR signalling properties. Furthermore, it has now become evident that they can also form quaternary structures of two or more subunits, with themselves (homo-oligomers) and with other receptor types (hetero-oligomers; Milligan et al., 2018). This interaction is thought to alter trafficking, signal bias, ligand specificity, and functional selectivity of each of the component parts, diversifying the signalling possibilities and functional output. Although most preliminary studies documented the formation of oligomers in heterologous expression systems, it has now become evident that oligomer formation holds significant physiological value, for example in endocrine systems (Jonas and Hanyaloglu, 2017).

1.4.7 Mechanisms of GPCR signalling mediated gut hormone release

The mechanisms of nutrient-induced gut hormone release from EECs are dependent, at least in part, on the activation of a diverse array of nutrient-sensitive GPCRs expressed on the apical membrane, a selection of which are shown in **Table 1.1**. L-type EECs are electrically active, susceptible to ion fluxes and membrane potentials that can cause exocytosis of gut hormone containing dense core vesicles, stored in close proximity to the basolateral membrane (Psichas et al., 2015).

Glucose can stimulate electrochemical fluctuations independently of GPCR signalling, and this is thought to be the primary mechanism of glucose-stimulated gut hormone secretion. This is reliant on the sodium concentration gradient maintained by the active sodium/potassium ATPase pump. This allows the passive diffusion of sodium down its concentration gradient from the lumen into the

epithelial cells, via the sodium/ glucose co-transporter member 1 (SGLT-1). The entry of glucose into the cell induces calcium channel opening and calcium influxes, prompting exocytosis of vesicles containing GIP (Sykes et al., 1980) and GLP-1 (Shima et al., 1990) and their subsequent secretion from EECs.

When considering the mechanisms of GPCR-dependent gut hormone release, two primary mechanisms have been proposed (**Figure 1.4**). Firstly, via $G\alpha_{q/11}$ -mediated PLC- β activation, which produces IP_3 , able to diffuse and release calcium from intracellular stores such as the endoplasmic reticulum (ER). The rise in cytosolic calcium promotes vesicle exocytosis (Psichas et al., 2015). Secondly, $G\alpha_s$ activation of AC induces intracellular cAMP accumulation which can independently induce GLP-1 secretion. cAMP can regulate ATP-sensitive potassium channels, the background conductance of which have a hyperpolarising effect on cells. The closure of these potassium channels augments calcium responses, increasing the number of cells responding to glucose (Simpson et al., 2007). cAMP induced activation of PKA can also directly induce the transcription of the preproglucagon gene (Drucker et al., 1997). Some GPCRs known to induce gut hormone release couple to other downstream G proteins, such as $G\alpha_{i/o}$ which inhibit AC. The underlying mechanisms

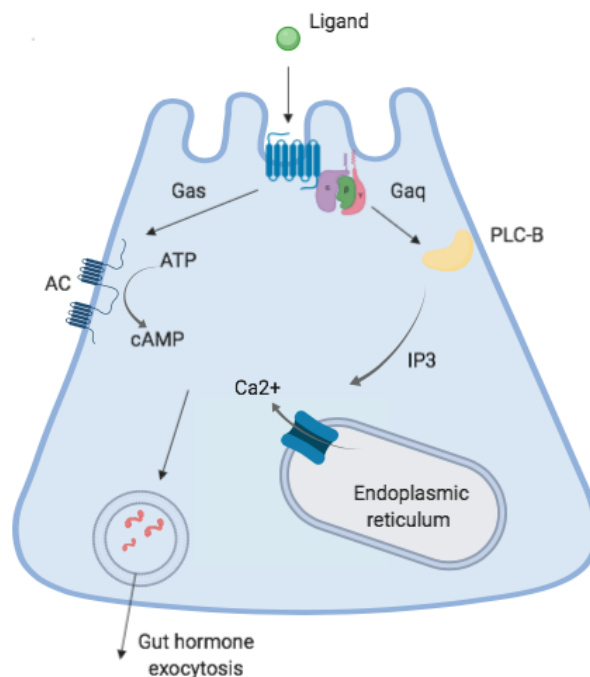


Figure 1.4 Nutrient sensing GPCR pathways leading to gut hormone secretion in enteroendocrine cells There are a large repertoire of GPCRs that sense nutrients in EECs. There have been two proposed pathways for which GPCR stimulation can lead to gut hormone secretion. Firstly, $G\alpha_{q/11}$ coupling can activate phospholipase C- β (PLC- β) which cleaves membrane lipids to form diffusible inositol triphosphate (IP_3). IP_3 binds and activates its receptor, a ligand gated ion channel on the membrane of the endoplasmic reticulum, leading to the release of calcium (Ca^{2+}) from intracellular stores. The rise in cytosolic calcium causes exocytosis of dense core gut peptide containing vesicles. PLC- β activity also synthesises plasma membrane diacylglycerol (DAG), able to calcium-dependently activate Protein kinase C (PKC), an enzyme with multiple downstream targets. The alternative pathway, likely to act in tandem, is mediated via $G\alpha_s$ activation of adenylyl cyclase (AC), which catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). cAMP moderates potassium channels, hyperpolarising the cell and can augment calcium responses. cAMP can also activate protein kinase A (PKA) and directly moderate gene expression. **Image created with BioRender.**

linking the diminished cAMP accumulation with gut hormone release are yet to be determined.

There is a highly complex signalling environment throughout the GI tract. The number of diet-derived nutrients and microbiota-synthesised metabolites is countless but, in addition to this, the spatiotemporal concentrations of these are continuously oscillating sporadically. Questions remain as to how the signalling from the dynamic mixtures of nutrients and metabolites, as well as non-nutrient stimuli such as paracrine gut hormone signalling, can interact with each other. The known plasticity of GPCR signalling, coupled with the magnitude of intracellular targets and the ability of these to crosstalk with each other creates a vast GPCR signalling network which is integrated to elicit a single response. For example, in L-type EECs it has been suggested that FFAR1-mediated GLP-1 secretion, thought to act predominantly via $G\alpha_q$ -induced calcium influxes, is dependent upon high basal concentrations of cAMP in the cell which results from the $G\alpha_s$ signalling from other GPCRs, such as TGR5 or GPR119 (Hauge et al., 2015; Hauge et al., 2017). Further unpicking the details of these interactions may allow the identification of certain synergistic effects which can be utilised for therapeutic augmentation of gut hormone release (Norton et al., 2017).

Free Fatty Acid Receptors				
GPCR	G protein signalling pathways	Metabolite ligands	Expression	Function
FFAR1	Gα _q	Unsaturated LCFAs, PUFAs, omega-3 fatty acids, omega-6 fatty acids, MUFAs	L-type, I-type and K-type EECs	Stimulates GLP-1, GIP and CCK secretion
FFAR2	Gα _{i/o} Gα _q	SCFAs (acetate > propionate > butyrate)	L-type EECs, I-type, D-type EECs	Stimulates GLP-1 secretion, inhibits somatostatin secretion (propionate)
FFAR3	Gα _{i/o}	SCFAs (propionate/ butyrate > acetate)	L-type, I-type, D-type EECs	Stimulates GLP-1 secretion
FFAR4	Gα _{i/o} Gα _q	Unsaturated LCFAs, PUFAs, omega-3 fatty acids, omega-6 fatty acids	D-type, L-type, I-type and K-type EECs	Inhibits ghrelin and somatostatin secretion
GPR119	Gα _s	N-acylethanol-amines, 2-monoacyl-glycerole	L-type, K-type EECs	Stimulates GLP-1 and GIP secretion, may act in synergy with FFAR1
HCA1	Gα _{i/o}	Hydroxycarboxylic Acids	D-type EECs	Inhibits ghrelin and somatostatin secretion
TGR5	Gα _s	Bile acids	L-type cells	Stimulates GLP-1 secretion, nitrous oxide release and suppression of gastric motility
Amino Acid Receptors				
GPCR	G protein signalling pathways	Metabolite ligands	Expression	Function
CaSR	Gα _i Gα _q	Ca ²⁺ , L-amino acids, oligopeptides	D-type EECs, ghrelin cells, parietal cells, G-type EECs	Stimulates GLP-1 secretion
TAS1R1-TAS1R3	α-gustducin, α-transducin, βγ mediates PLC-β	L-amino acids, particularly L-glutamate and L-arthinine, enhanced by IMP or GMP	I-type EECs, L-type EECs	Stimulates CCK secretion

GPRC6a	Gα _q	Basic amino acids, L-arginine, L-lysine, and L-ornithine, calcium ions	L-type EECs	Stimulates GLP-1 secretion
GPR93	Gα _i Gα _q	Peptones	L-type EECs, I-type EECs	Stimulates GLP-1 and CCK secretion
GPR142	Gα _i Gα _q	Aromatic amino acids, L-tryptophan, L-phenylalanine	L-type EECs, K-type EECs, ghrelin cells	Stimulates GLP-1 and GIP secretion
Taste receptors				
GPCR	G protein signalling pathways	Metabolite ligands	Expression	Function
TAS1R2-TAS1R3	α-gustducin, α-transducin	Wide range of chemically different compounds, including sugars, artificial sweeteners, sweet amino acids and sweet proteins	L-type EECs, G-type EECs, I-type EECs, K-type EECs with α-gustducin	Maybe stimulates GLP-1, GLP-2, GIP, PYY and CCK secretion
TAS1R1-TAS1R3	α-gustducin, α-transducin, βγ mediates PLC-β	L-amino acids, particularly L-glutamate and L-arginine, enhanced by IMP or GMP	I-type EECs, L-type EECs	Stimulates CCK secretion
TAS2Rs	α-gustducin, α-transducin	Large range of bitter compounds including caffeine, denatonium benzoate and phenylthiocarbamide	L-type EECs, I-type EECs with α-gustducin	Stimulates CCK, PYY and GLP-1 secretion

Table 1.1 A selection of the GPCRs known to be involved in nutrient sensing and expressed throughout the gastrointestinal tract (Husted et al., 2017; Kinnamon et al., 2017, Norton et al., 2017, Psichas et al., 2015; Free fatty acid receptor (FFAR), Enteroendocrine cell (EEC), Hydroxycarboxylic acid receptor (HCA), G-protein receptor (GPR), G protein-coupled receptor (GPCR), Calcium-sensing receptor (CaSR), Taste receptor family 1 (TAS1R), Taste receptor family 2 (TAS2R), Glucagon-like peptide 1 (GLP-1), Glucagon-like peptide 2 (GLP-2), Cholecystokinin (CCK), Gastric inhibitory polypeptide (GIP), Inosine monophosphate (IMP), Guanosine monophosphate (GMP), Long chain fatty acid (LCFA), Medium chain fatty acids (MCFAs), Short chain fatty acids (SCFAs), Polyunsaturated fatty acids (PUFAs), Phospholipase C-β (PLC-β)

1.5 Free fatty acid receptors

Non-esterified fatty acids contain terminal carboxylic acid groups connected to aliphatic chains of unspecified length and varying saturation, diversifying the properties greatly. They are broadly grouped, depending on how many carbons make up this chain length, into SCFAs ($C < 6$), medium-chain fatty acids (MCFAs; $6 < C < 12$) and LCFAs ($C > 12$). These are also categorised depending on degree of saturation, as carbon-carbon double bonds (usually of *cis* conformation) can occur in the aliphatic chains. Those with exclusively single bonds are saturated (SFAs), those containing a singular double bond are monounsaturated (MUFAs) and those with multiple double bonds are polyunsaturated (PUFAs),

In addition to intracellular targets, fatty acids act upon a family of Class A GPCRs, collectively termed free fatty acid receptors (FFARs). Of these, FFAR1 and FFAR4 are activated by MCFAs and LCFAs; obtained predominantly from the diet, whereas FFAR2 and FFAR3 interact with SCFAs; bioactive microbiota metabolites. Human FFAR1, FFAR2 and FFAR3 receptors are expressed in tandem on chromosome 19 and share 30-40% homology, giving them similar structural identities. FFAR4, however, has its own distinct chromosomal locus and displays more distinct characteristics (Milligan et al., 2016).

1.5.1 Free fatty acid receptor 1

FFAR1 (also GPR40) is activated, with modest potency, by a broad spectrum of MCFAs and LCFAs, predominantly coupling to $G_{\alpha_{q/11}}$ (Briscoe et al., 2003), with evidence of G_i and G_s interactions in certain cell models (Milligan et al., 2016). Expressed in L-type, I-type and K-type EECs, FFAR1 has been linked with GIP, GLP-1 and CCK secretion in response to dietary LCFAs, suggesting FFAR1 plays a role in dietary nutrient sensing (Edfalk et al., 2008; Liou et al., 2011). However, it has also been suggested that in order to induce GLP-1 release, luminal LCFAs must first be absorbed into the vasculature (Christensen et al., 2015). A role for FFAR1 has also been highlighted in the pancreas, where its expression on β -cells has been linked with glucose-dependent insulin secretion (Itoh et al., 2003). This led to the development of a library of synthetic FFAR1 agonists, some with encouraging results in rodent models of T2DM (Mancini et al., 2013). Of note is TAK-875, a highly selective FFAR1 agonist, which showed promising ability to improve glycaemic control and lower fasting glucose concentrations in Phase II trials with T2DM patients, without the danger of hypoglycaemia (Burant et al., 2012). However, TAK-875 was not developed for commercial use in T2DM patients due to the accumulation of drug metabolites in bile acids causing unacceptable inhibition of bile acid transporters. This led to intolerable hepatotoxicity profiles (Marcinak et al., 2018).

1.5.2 Free fatty acid receptor 4

FFAR4 (GPR120) is also responsive to LCFAs, especially PUFAs. FFAR4 plays essential roles in metabolism, as demonstrated by the adiposity, obesity and impaired glucose tolerance observed in FFAR4 KO mice models (Ichimura et al., 2012).

In the GI tract, FFAR4 transcripts have been found expressed in X/A-type, L-type, I-type and K-type EECs. FFAR4 has been linked with post-prandial declines in ghrelin secretion, as stimulation of FFAR4 with natural LCFAs and with synthetic agonists has been shown to attenuate orexigenic ghrelin secretion *in vitro* and *in vivo* from gastric secretory cells (Gong et al., 2014). This was noted to be via a $G_{\alpha_{i/o}}$ inhibitory mechanism (Engelstoft et al., 2013), although FFAR4 seemingly prefers to couple to $G_{\alpha_{q/11}}$ in heterologous systems (Milligan et al., 2017). PUFAs, especially α -linoleic acid, also stimulate GLP-1 release from EECs *in vitro* via a mechanism that is impaired on FFAR4 knockdown (KD). This translated *in vivo*, with the significant elevation of plasma GLP-1 concentrations inducing insulin

secretion in mice (Hirasawa et al., 2005). It remains to be established whether this holds true in human models as observations of fluctuating gene expression add another layer of complexity. For example, in humans, BMI positively correlates with relative transcripts of FFAR4 (Little et al., 2014), whereas BMI negatively correlates with gut hormone concentrations (Ranganath et al., 1996). It has been reported that increases in dietary energy intake upregulates FFAR4 (Little et al., 2014), maybe explaining the association between transcript levels and body weight. How this contributes to the prototypically impaired metabolism and energy regulation of obese individuals remains to be determined. FFAR4 has been shown to be expressed in mouse hypothalamic nuclei, where linoleic and oleic unsaturated fatty acids induced signalling cascades, possibly contributing to their ability to reverse hypothalamic inflammation, including improving insulin/ leptin resistance and stimulating anorexergic POMC/CART expression (Cintra et al., 2012). Several FFAR4 specific agonists have been developed; largely directed at the therapeutic potential to alleviate T2DM; although none yet have been trialled clinically (Milligan et al., 2017).

1.5.3 Short chain fatty acid receptors FFAR2 and FFAR3

Pharmacology and signalling

FFAR2 (GPR43) and FFAR3 (GPR42), both activated by SCFAs, share 52% sequence similarity (Brown et al., 2003). Although both are activated to some extent by all SCFAs, there are differences in the sensitivities to the aliphatic chain lengths, which also vary between species orthologues, as demonstrated in **Table 1.2** (Bolognini et al., 2016). Acetate and propionate both show close to optimal activations profiles at human FFAR2 (hFFAR2), but where propionate acts almost equipotently at human FFAR3 (hFFAR3), acetate is roughly ten times as potent at hFFAR2 than hFFAR3, a result replicated via several functional assays (Milligan et al., 2016). Despite these apparent distinctions, it is still inadequate to use acetate as a truly selective ligand. Rodent orthologues show a different rank order of SCFA selectivity; acetate no longer preferentially activates FFAR2 over FFAR3, but propionate shows significant selectivity for FFAR3. Attempts are underway to uncover the precise mode of ligand binding for each receptor isoform and the key amino acid residues involved, to aid in the design and synthesis of novel selective ligands (Schmidt et al., 2011). Negatively charged amino acid residues on EC loop 2 are thought to form ionic bonds with a positively charged arginine. This helps to mould the structure of the ligand binding pocket to determine ligand binding and basal activity of the receptor. These negatively charged residues differ between species orthologues, offering clues to the molecular mechanism underlying ligand preferences (Hudson et al., 2012). Key residues within the TM domains of hFFAR2 have also been demonstrated to be crucial for its function, and this structural knowledge has been used to develop compounds specific to the orthosteric binding site of hFFAR2 (Hudson et al., 2013). This was the first report of orthosteric ligands, but other compounds, such as 4-chloro- α -(1-methylethyl)-N-2-thiazolylbenzeneacetamide (4-CMTB), have allosteric ability to modulate FFAR2 activity (Smith et al., 2011). CATBP is a highly selective hFFAR2 antagonist but is rendered ineffective by replacements of certain residues in the rodent orthologue (Hudson et al., 2012).

FFAR2 and FFAR3 have slightly different signalling profiles, with FFAR2 reportedly able to couple to $G\alpha_q$ or $G\alpha_{i/o}$ but FFAR3 only able to couple to the latter (Brown et al., 2003). Additionally, the recruitment of β -arrestins seems to be exclusively to FFAR2 (Hudson et al., 2012 ; Hudson et al., 2013), with little evidence of association with FFAR3 (Milligan et al., 2016).

	Human	Mouse
FFAR2	$C2 = C3 \approx C4 > C5 > C6 \approx C1$	$C3 > C2 > C4$
FFAR3	$C3 \approx C4 \approx C5 > C6 > C2 > C1$	$C3 > C4 > C2$

Table 1.2 Differences in ligand potencies between species orthologs of FFAR2 and FFAR3 (C2=acetate, C3=propionate, C4= butyrate, free fatty acid receptor (FFAR); Bolognini et al., 2016)

The physiological relevance of FFAR2 and FFAR3 signalling

FFAR2 and FFAR3 are expressed in several peripheral organs such as the GI tract, pancreas, adipose tissue, immune system, liver and nervous system, playing a key role in many different metabolic and physiological processes. It has been extremely difficult to isolate the functional roles of the two receptors due to their overlapping expression profiles and similar ligand sensitivities (Milligan et al., 2016).

SCFAs are known widely for their ability to modulate inflammatory properties, mediated in part via FFAR2 signalling in neutrophils, intestinal T-regulatory cells, eosinophils, macrophages and dendritic cells, playing a role in cell motility and secretory profiles. For example, in neutrophils FFAR2 is fundamental for SCFA-induced chemotaxis, thought to be mediated via $G\alpha_{i/o}$ signalling (Vinolo et al., 2011) and in mononuclear cells acetate activation of FFAR2 inhibits TNF and IL-17 secretion (Masui et al., 2013). In the GI tract, FFAR2 is expressed in enteric leukocytes, where it is thought to play a role in facilitating the infiltration necessary to protect against inflammatory conditions (Nohr et al., 2013). In colitis, FFAR2 expression in T-regulatory cells mediates the anti-inflammatory protective properties of propionate (Smith et al., 2013).

The effect of SCFAs on glucose homeostasis is complex. In the pancreatic β -cells, FFAR2 can couple to $G\alpha_q$ calcium responses that contribute directly to glucose stimulated insulin secretion (McNelis et al., 2015). However, coupling of FFAR2 or FFAR3 to $G\alpha_{i/o}$ in β -cells seems to have the opposite effect (Tang et al., 2015). It has also been shown that acetate and propionate can act via FFAR2 to, firstly, induce insulin release via PKC and PLC- β and, secondly, protect pancreatic islets from apoptosis (Pignatelli et al., 2016). Further evidence for FFAR2 in glucose homeostasis comes from experiments in cultured hepatocytes, where propionate activation of FFAR2 leads to a downstream signalling cascade resulting in 5' AMP-activated protein kinase (AMPK) phosphorylation, crucial for propionate-mediated suppression of hepatic gluconeogenesis and inhibition of glucose production (Yoshida et al., 2019).

SCFAs have a fundamental role in the physiology of adipose tissue. SCFAs have been shown to increase glucose uptake into adipocytes (Kimura et al., 2013). In mice, acetate and propionate both decrease lipolysis, reduce plasma triglycerides (Ge et al., 2008) and increase adipogenesis (Hong et al., 2005) via a mechanism fully dependent upon the expression of FFAR2. Examination of the body composition of FFAR2 KO mice demonstrates that they display much higher adiposity compared to wild-type (WT) counterparts, even when fed a low fat diet. This is thought to be a direct result of the loss of FFAR2 inhibition of fat-accumulating insulin signals (Kimura et al., 2013). It has also been suggested that FFAR2/3 signalling may promote leptin secretion from adipocytes, although this

remains to be further clarified (Milligan et al., 2016). FFAR2 is also involved in the effects the gut microbiota composition has on host adiposity, as when colonising germ-free WT and FFAR2 KO mice with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii*, FFAR2 KO mice show significantly less fat accumulation than WT, despite similar calorific intake (Samuels et al., 2008).

FFAR3 is expressed in the sympathetic ganglia, and propionate induced FFAR3 coupling to $G\alpha_{i/o}$ increases the activation of sympathetic nerves, causing increased heart rate, energy expenditure and thermogenesis. Interestingly, β -hydroxybutyrate (β -OHB) is a metabolite derived from fatty acids, produced by the liver as an alternative energy source to glucose in periods of fasting or intense exercise. β -OHB has been reported to antagonise FFAR3, decreasing sympathetic activity and reserving energy in times of energy depletion (Kimura et al., 2011). It has been observed that FFAR3 can also inhibit calcium signals through modulation of N-type calcium channels in subsets of sympathetic ganglia neurons most associated with vasoconstriction (Colina et al., 2018).

FFAR3 is also expressed in the enteric nervous system and has been suggested to facilitate SCFA modulation of vagal nerve activity (Cook et al., 2019). Observations that SCFAs induce phasic contractions of colonic smooth muscle, in a potency order consistent with FFAR3 sensitivity (propionate > butyrate > acetate), have suggested that SCFA modulation of FFAR3 on enteric nerves is responsible for this (Tazoe et al., 2009) although whether SCFAs directly induce these contractions in humans remains to be ascertained (Chambers et al., 2014).

FFAR2 and FFAR3 in enteroendocrine cells

The observation that both FFAR2 (Karaki et al., 2008) and FFAR3 (Tazoe et al., 2009) are expressed in the human colonic mucosa is not surprising considering the high concentrations the SCFAs can reach at this site of their biogenesis. Importantly, these receptors are not only expressed in enterocytes, but also in EECs, where their expression is co-localised with PYY (Karaki et al., 2008; Tazoe et al., 2009) and GLP-1 (Tolhurst et al., 2012). Diets high in fibre have been shown to increase plasma GLP-1 concentrations both in rodents (Zhou et al., 2008) and humans (Freeland et al., 2010) and SCFA action at FFAR2/3 was postulated to be the signalling mechanism facilitating this. In primary murine colonic cultures, acetate and propionate both significantly increase GLP-1 secretion in a manner which is decreased in FFAR2 or FFAR3 KOs (Tolhurst et al., 2012). These findings can also be extended to human L-cells in culture, with propionate stimulating PYY and GLP-1 release (Chambers et al., 2015). The rise of GLP-1 was mirrored *in vivo* when mice were administered with a glucose load but whereas in FFAR2 KO mice the glucose mediated GLP-1 release was reduced by 40%, there was no significant decrease in GLP-1 secretion in the FFAR3 KO mice. Additionally, the development of a transgenic mouse model that expresses fluorescent Venus protein downstream from the preproglucagon promoter has allowed the L-type EECs visualised. This model has facilitated the identification of calcium responses that are L-type specific and has led to results suggesting that the gut hormone release is mediated via FFAR2 $G\alpha_q$ coupling (Tolhurst et al., 2012). Further work has demonstrated that intra-colonic injections of propionate doubles plasma concentration of both PYY and GLP-1 in WT mice, whereas FFAR2 KO mice had a significant attenuation of propionate induced release to 1.2-fold and 1.3-fold respectively (Psichas et al., 2015). The synthesis of an inulin-propionate ester, which releases propionate on fermentation, has allowed efficient and targeted delivery of propionate directly to the human colon. Supplementation of dietary components with this novel inulin-propionate ester significantly increased plasma GLP-1 and PYY concentrations 4 hours after ingestion compared to control. A 24-week trial with overweight adults demonstrated benefits in body weight and composition (Chambers et al., 2015). It has also been shown that FFAR2 signalling, as well as promoting PYY release from pre-existing PYY positive cells, is a catalyst for the

expansion of the population of PYY-expressing cells in mice, with the inulin ester able to increase the number of this cell type by 87% (Brooks et al., 2017).

To help differentiate the roles of FFAR2 from FFAR3, chemogenetic techniques have recently been employed to create a genetically modified hFFAR2 receptor that is exclusively activated by sorbic acid but is unresponsive to endogenous SCFAs. This receptor coupled to the same downstream signalling G proteins and showed similar pharmacological profiles. Therefore, when expressed in FFAR2 KO mice downstream of the *ffar2* promoter it could be used to examine the functional role of FFAR2. On activation with sorbic acid, there was significant increase in GLP-1 release, which was attenuated with pharmacological inhibition of $G\alpha_q$ but not $G\alpha_{i/o}$, (Bolognini et al., 2019).

FFAR2/FFAR3 interactions and trafficking

Although co-immunohistology staining studies have shown that FFAR2 and FFAR3 are not co-localised in L-type EECs (Tazoe et al., 2009), more recent single cell RNA sequencing analysis of preproglucagon positive cells from the small intestine of mice has revealed that cells, particularly those with high prototypical L-cell preproglucagon and PYY expression, do show overlapping expression profiles in FFAR2, FFAR3 as well as FFAR1 and GPR119 (Glass et al., 2017). The co-expression of these GPCRs in the same cell implies that GPCR crosstalk and oligomerisation is feasible. FFAR2-FFAR3 heteromers have been observed innately via proximity ligation assays in primary human monocytes and macrophages. When co-expressed in a heterologous system, transfected human embryonic kidney (HEK-293) cells, fluorescent resonance energy transfer (FRET) analysis was utilised to demonstrate evidence of this interaction. Furthermore, it was reported that FFAR2-FFAR3 heterodimerisation could have significant effects on receptor signalling profiles. Firstly, heteromers demonstrated a 1.5-fold magnification in the SCFA-mediated cytosolic calcium signalling when compared to the calcium signalling induced by monomeric FFAR2 alone. Secondly, the acetate and propionate induced inhibition of cAMP, observed robustly in cells expressing FFAR2 and FFAR3 monomers, was completely diminished with heteromer expression. Thirdly, the β -arrestin-2 recruitment profile of heteromers mirrored that of monomeric/homomeric FFAR2, 30 times greater than that of FFAR3, even in response to a FFAR3 specific agonist. The heteromer expression was also linked with an increase in p38 MAPK phosphorylation (Ang et al., 2017). This dimerisation was not observed in human enterocytes, but no studies to date have looked specifically at this in EECs.

1.6 Taste receptors

In the gustatory system, dietary compounds are thought to elicit one of five perceptual taste qualities: sweet, umami, bitter, salty and sour. The former three of these taste sensations are detected via GPCRs which can be grouped into two major families: taste receptor family 1 (TAS1Rs) and taste receptor family 2 (TAS2Rs; Depoortere et al., 2014). In Type II taste cells in the oral cavity, taste receptors are known to signal primarily via taste-specific G protein subunits: α -gustducin, α -transducin or $G\alpha_{14}$; $G\beta_3$ or $G\beta_1$; and $G\gamma_{13}$. The $\beta_3\gamma_{13}$ subunits dissociate and activate PLC- β_2 , initiating IP₃-dependent calcium mobilisation into the cytosol. The increase in cytosolic calcium opens the PM monovalent cation channel, transient receptor potential cation channel subfamily M member 5 (TrpM5), causing cellular depolarisation. The influx of calcium, coupled with membrane depolarisation, results in action potentials and ATP release through pannexin hemi-channels. ATP is the primary messenger signalling taste perception, acting as an autocrine and paracrine messenger to amplify the response (Brehens et al., 2010).

Only recently has it been discovered that some of the key GPCRs, as well as downstream signalling components, responsible for taste sensations in the oral cavity are also expressed in extra-gustatory tissues such as in the GI tract, testes, respiratory epithelia, brain, immune system and kidney (Behrens et al., 2010). The expression profile of taste signalling proteins throughout the GI tract is dynamic and non-conserved throughout various EEC subtypes in rodent and humans (Sternini et al., 2010). For example, α -gustducin is found co-expressed with GLP-1 in L-type EECs (Rozengurt., et al., 2006), with ghrelin in P and D1 cells (Janssen et al., 2010) and CCK in I-cells (Sternini et al., 2008) in rodents and humans. The presence of taste signalling components suggests they may contribute to the nutrient sensing mechanisms involved in promoting gut hormone release, demonstrated by several studies (Jang et al., 2007; Rozengurt et al., 2006; Gerspach et al., 2011; Kok et al., 2018; Yu et al., 2015; Yoshida et al., 2017).

1.6.1 Taste receptor family 1

TAS1Rs; TAS1R1, TAS1R2, TAS1R3; are Class C GPCRs. They have definitive and large extracellular bi-lobed Venus-Flytrap (VFT) domains, able to take open or closed conformations (Muto et al., 2007). These are connected to the TM domains via a cysteine rich domain (CRD), resembling the well-studied metabotropic glutamate receptor 1 (mGluR1). Structural analysis has identified two key cavities in the VFT domain with the ability to accept ligands, and many amino acid residues within these cavities are conserved throughout this family, perhaps representative of their fundamental value for ligand association (Morini et al., 2005). Taste receptor monomers are fully dependent upon heterodimerisation for activation by a wide range of sweet and umami taste stimuli (Nelson et al. 2001; Nelson et al., 2002).

Sweet taste receptor

The heterodimerisation of TAS1R3 with TAS1R2 is sensitive to a selection of sweet tasting compounds. Heterologous co-expression of these subunits induces robust calcium responses in HEK-293 cells in response to sucrose, fructose, saccharin, some non-calorific natural and artificial sweeteners and some D-amino acids in a way that was fully abolished when expressing the monomers in isolation. Interestingly, the TAS1R2-TAS1R3 did not induce calcium responses to some key dietary sugars such as glucose, maltose, fructose and galactose (Nelson et al., 2001). Two cavities in the VFT domains are thought of as the key ligand binding domains in mGluR1, and it is generally accepted that equivalent regions of TAS1R2 dictate sensitivity to sweeteners such as aspartame and neotame (Xu et al., 2004). However, the wide scope of sweet ligands able to activate

the receptor suggests different compounds may associate with other distinct portions of heteromers, such as external regions of the activated receptor conformation (Kojima et al., 2011). For example, the sweetener cyclamate and the sweet taste inhibitor, lactisone, bind the C-terminal TM region of TAS1R3 (Xu et al., 2004) and the sweet proteins monellin and brazzein associate with regions in the CRD of TAS1R3 (Jiang et al., 2004).

Mouse and human L-type EECs express TAS1R2-TAS1R3 alongside α -gustducin, suggesting this receptor may play a role in glucose induced GLP-1 secretion (Jang et al., 2007). EECs secrete GLP-1, GIP, CCK and PYY in response to glucose, but this is likely to be mediated, at least in part, by glucose stimulated sodium influx from the SGLT1 (Reimann et al., 2008). However, α -gustducin KO mice have impaired glucose induced GLP-1 secretion as well as dysregulation in insulin secretion and blood glucose homeostasis. In human L-type cell lines, glucose and non-calorific sweeteners induce GLP-1 secretion *in vitro*, an effect which was attenuated by pre-treatment with lactisone, a human and primate specific antagonist of the sweet taste receptor. This suggests that the taste receptor signalling pathway plays a fundamental role in gut hormone release (Jang et al., 2007). Similar observations have also been made *in vivo*, as intra-gastric or intra-duodenal nutrient infusions have demonstrated that administration of glucose elicits robust increases in plasma GLP-1, PYY, and CCK concentrations in healthy human volunteers. The GLP-1 and PYY secretion was significantly attenuated (by 25% and 15% respectively) when glucose was co-infused with lactisone, signifying that the taste receptor signalling pathway does play a role in L-cell nutrient sensing. In contrast, CCK secretion remained unchanged in the presence of lactisone, perhaps indicative of a glucose-sensing pathway independent of taste receptor signalling in I-type EECs (Gerspach et al., 2011).

Umami taste receptor

Umami is the Japanese word for “delicious” and is the flavour associated with savoury, protein-rich foodstuffs. The umami taste receptor is formed by the heterodimerisation of structurally similar TAS1R1 with TAS1R3. Both subunits, like with the sweet taste receptor heteromers, contain large VFD domains and CRDs which, when in complex, are sensitive to L-amino acids (L-AAAs) such as L-Glutamate (L-Glu) and L-Aspartate (L-Asp). The co-expression of human TAS1R1 and TAS1R3, but not the expression of monomers in isolation, in HEK-293s causes robust calcium responses to L-Glu, L-Glutamine (L-Gln) and L-Monosodium glutamate (L-MSG), but not their D-enantiomers. The human isoform shows great selectivity for these ligands, which elicit a response ten times stronger than other L-AAAs. The ligand induced calcium responses are synergised in a dose-dependent manner by the presence of purinic ribonucleotides, such as inosine monophosphate (IMP) and guanosine monophosphate (GMP), which, alone, do not elicit any signals (Nelson et al., 2002). Genetic modification techniques, coupled with molecular modelling, has shed light on the cooperative ligand binding mechanism underlying this positive allosteric modulation. The orthosteric binding site, where L-Glu associates, resides near the hinge of the VFD. IMP associates nearby, stabilising the ligand bound VFD in closed conformation and preventing ligand dissociation (Zhang et al., 2008).

Human and mouse taste receptors share approximately 70% homology (Nelson et al., 2001), inducing differences in ligand sensitivities between the human and mouse isoforms of the umami receptor. Where the human isoform shows great propensity to be activated solely by L-Glu, L-Gln and L-MSG, the mouse TAS1R1-TAS1R3 heterodimer is responsive, in at least some extent, to a broad spectrum of the twenty essential L-amino acids in the millimolar range, but not to their D-enantiomers or to L-Trp (Nelson et al., 2002). Site directed mutagenesis of hTAS1R1 has shown that ligand activation of the human receptor is dependent upon 12 key residues at the orthosteric binding site within the VFD domain of hTAS1R. In contrast, the broader sensitivity of the mouse umami receptor is facilitated by ligand binding allosterically, at positions distinct from the

orthosteric and IMP binding site. It has become evident that residues inside the orthosteric site are instrumental to determine ligand selectivity, but residues elsewhere play a role in determining basal receptor activity in a species-specific manner (Toda et al., 2013).

Amino acids are known to induce CCK secretion from I-type EECs in humans, monkeys, dogs and rodents. As the umami receptor is expressed predominantly in the proximal intestine, with α -gustducin (Dyer et al., 2005; Jang et al., 2007), it is feasible that it could play a role in L-AA nutrient sensing. It has been shown that L-AAs, L-Phenylalanine (L-Phe), L-Glu, L-Leucine (L-Leu) and L-Tryptophan (L-Trp) can induce CCK secretion from murine STC-1 cell line *in vitro*. Addition of IMP potentiates the CCK secretion induced by all amino acids except L-Trp, which does not show any efficacy at the mouse umami taste receptor. The use of RNA silencing showed that this CCK secretion was attenuated with TAS1R1 or TAS1R3 KD. These results were mirrored by the pharmacological inhibition of TAS1R3 by gurmarin, providing evidence of its functional role in amino acid induced CCK secretion *in vitro* (Daly et al., 2012). The umami taste receptor has also been shown to play a role in ghrelin secretion from mouse gastric cell lines. Here, L-MSG and L-Alanine (L-Ala) promote octanoyl ghrelin secretion via intracellular calcium signalling, via a process which is curtailed by pharmacological inhibition with gurmarin (Vancleef et al., 2015). However, there is a lack of knowledge about whether the gastrointestinal nutrient sensing properties of the umami taste receptor is conserved in humans as although high protein diets are known to promote satiety (Anderson et al., 2004), there are other candidate amino acid sensors which may play a more instrumental role (Norton et al., 2017).

1.6.2 Taste receptor family 2

The TAS2Rs are an extensive family of GPCRs, with over 25 different isoforms in humans, and many more in rodents. Having evolved as a protective mechanism against toxins, they detect a large and dynamic range of structurally distinct compounds, which are perceived as bitter tasting. All members of the bitter taste receptor family are structurally distinct, with the majority sensitive to a relatively narrow selection of agonists of certain structures. However, there are some members of the family; TAS2R10, TAS2R14, and TAS2R46; which are greatly flexible, able to be activated by a whole array of structurally diverse ligands (Brehens et al., 2018). Structural analysis has suggested this is a result of an extremely malleable orthosteric agonist binding site, which adapts sterically to fit many ligands (Brockhoff et al., 2010).

Bitter taste receptors have been found to be expressed in EECs alongside α -gustducin (Depoortere et al., 2014) and bitter compounds have been shown to influence EEC secretory profiles in a number of clinical and preclinical studies, modulating the release of ghrelin, CCK, PYY and GLP-1 (Kok et al., 2018; Yu et al., 2015; Yoshida et al., 2017; Pham et al., 2016). For example, stimulation with bitter compounds, such as denatonium and phenylthiocarbamide, has been shown to elicit calcium responses and reduce intracellular cAMP concentrations, inducing CCK secretion from mouse EEC cell lines (Chen et al., 2006). Similar calcium responses have been shown to elicit PYY and GLP-1 secretion from *in vitro* human EEC cell lines in a way which was diminished in TAS2R2, TAS2R3 and TAS2R43 KD by RNA interference (Kim et al., 2014). In clinical studies performed on healthy volunteers, administration of bitter tasting quinine tablet one hour before an *ab libitum* meal significantly reduced calorific intake, thought partly to be mediated by the increased plasma CCK concentrations observed. Interestingly, the satiating impact of quinine was dependent upon individual genetic polymorphisms which determine phenylthiocarbamide tasting status (Andreozzi et al., 2015). In addition to quinine, studies have shown that administration of encapsulated bitter secoiridoids can acutely reduce appetite, diminishing the daily calorific intake by a third compared to placebo (Mennella et al., 2016).

1.7 Current therapeutic options for treatment of obesity

Despite extensive research, there are limited treatment options available for obesity. Lifestyle advice and counselling is the most common form of therapy, reliant on the theory that obesity is caused by the disparity between energy expended and consumed, but advice remains contradictory, unclear and inefficacious (Ryder et al., 2019). For patients, complying and persisting with strict diet modulations can be challenging. Due to the high recidivism observed in many weight-loss programs, novel therapies are required to demonstrate that the effects are long-lasting and durable (Grattan et al., 2012).

For over 50 years, the search for a pharmacological agent to treat obesity has been underway, with only limited success. Many of the early weight loss therapies yielded severe and unacceptable side-effects. The first drug group introduced for obesity were centrally acting sympathomimetics (Wilding et al., 2007) but harmful side-effects such as abuse potential and cardiovascular risks led to a decline in their use. Since then, a range of serotonin releasing agents, dual-monoamine reuptake inhibitors, serotonin receptor agonists, cannabinoid receptor antagonists and opioid receptor antagonists have been utilised in attempts to combat excessive weight gain, but each drug group has its own range of drawbacks which need to be overcome, partly due to the brain's complexity and widespread, uncharacterised expression of many receptor subtypes (Rodgers et al., 2012).

Orlistat is the only approved pharmacological intervention available in the UK. It inhibits pancreatic lipases, which play an instrumental role in dietary triglyceride hydrolysis. This reduces the bioavailability and subsequent absorption of energy-dense free fatty acids and monoacylglycerides by 35% for an average meal, causing a calorie deficit of approximately 200 calories (Guercioli et al., 1997). However, the excess accumulation of fats in the gut lumen can contribute to gastrointestinal upset and side effects.

At present, the most effective treatment for sustained long-term weight loss is gastric bypass surgery. Due to economic strains, surgical procedures are only available on the NHS to morbidly obese patients ($\text{BMI} > 40 \text{ kgm}^{-2}$), or obese patients with a severe co-morbidity if they have attempted all other treatment options. It is accepted that at least some of the anorexergic effects are mediated via surgical influence on EEC peptide hormone secretion (Meek et al., 2016). Evidence suggests that there is a significant amplification in postprandial GLP-1, PYY (Holdstock et al., 2003) and anorexergic oxyntomodulin (LaFerrere et al., 2010) secretion in patients who have undergone gastric bypass surgery compared to pre-surgical rates whereas plasma ghrelin concentrations fall (Geloneze et al., 2003). The direct intravenous administration of analogues of these gut hormones has been suggested as a method to pharmacologically mirror the effects of gastric by-pass surgery. This led to the development of gut hormone synthetic analogues being developed for obesity management. The use of these thus far, however, has been hindered by the intolerable emesis, nausea and challenging pharmacodynamics but specifically targeted manipulations of the analogue structure have come some way to resolve these issues (Rangwala et al., 2019). Human clinical trials are underway in trialling the effects of subcutaneous administration of combinations of different analogues, such as using GLP-1, PYY and oxyntomodulin together, which have been shown to be safe and tolerable, reduce body weight and have glucomodulatory effects in obese and pre-diabetic patients (Behary et al., 2019). Oral administration of peptide analogues is challenging, as the acidic properties of the stomach and the hydroxylation by peptidases cause protein denaturation and degradation. Several attempts have tried to overcome this. For example, a recent Phase IIIa clinical trial has demonstrated oral administration of GLP-1 analogue, seraglutide, was associated with a higher rate of weight loss over 26 weeks in T2DM patients compared to subcutaneous liraglutide

(Pratley et al., 2019). Recently, a novel approach has been to encompass GLP-1 analogues into lipid coated nanoparticles, which, when consumed orally, take a dual pronged approach to firstly, elicit endogenous GLP-1 secretion and secondly, increase the delivery and bioavailability of the GLP-1 analogues, synergising the beneficial effects on insulin resistance and glucose tolerance (Xu et al., 2019).

It has been proposed that the structural alterations brought about by the invasive surgery increases the proportion of intact macromolecules reaching the lower ileum (Nausheen et al., 2013) and, consequently, the sensors embedded in the luminal membrane of L-type EECs. There are several current studies underway to mirror the effects of bariatric surgery in a non-invasive fashion (Norton et al. 2017), such as the development of carefully designed diet products high in protein (Van der Klaaw et al., 2013) or fibre (Polyviou et al., 2016). It has been postulated that these diet interventions may potentiate the signalling of nutrient sensing receptors, activating the intracellular pathways that result in gut hormone secretion (Norton et al., 2017). As well as improving appetite ratings and metabolic parameters, dietary interventions have been shown to improve gut microbiota profiles and diversity (Deehan et al., 2017). The discrepancies between lean and obese microbiomes is very complex, and, although it is simplistic to think that one species of bacteria can be held responsible, there are negative correlations observed between obesity, adiposity, metabolic disease and inflammation markers and *Akkermansia muciniphila*. Supplementing obese individuals with this has recently been shown to improve some metabolic biomarkers in a safe and tolerable fashion (Depommier et al., 2019).

It is evident that more research is needed to further quantify how specific diet formulations can hijack complex nutrient sensing mechanisms to curtail appetite, alter metabolism and modulate physiology. A deeper understanding of the GPCR signalling network, and how this influences EEC secretory profiles is fundamental to design dietary formulations which provide the optimal nutrients, metabolites and bioactive signalling molecules, which can work in synergy to augment gut hormone release. However, due to the multi-factorial nature of obesity, it is highly likely that there will not be a one-size-fits-all solution and that treatment programs will need to be personalised in nature to suit the individualistic genetic, environmental, metabolic and physiological differences.

1.8 Hypothesis and Aims

In such a dynamic and complex signalling environment as found within the GI lumen, interactions can occur between different nutrients that can work in synergy or antagonistically to affect the complex GPCR signalling networks.

To address this hypothesis I will determine if SCFAs can alter the gene expression of key components of other GPCRs and if this alters the sensitivity of enteroendocrine cells to the ligands of these GPCR signalling pathways and ultimately regulate gut hormone secretion. The ability of SCFAs to regulate this expression via FFAR2, FFAR3 and receptor-independent mechanisms will be assessed. Furthermore, the potential role of crosstalk between FFAR2 and FFAR3 and its impact on $G\alpha_q$ and $G\alpha_{i/o}$ signalling when EECs are activated by SCFAs will be examined.

Chapter 2: Materials

2.1 Primary antibodies

Antibody	Isotype	Manufacturer	Catalogue no.	Dilution IF (FACS/ Confocal)
Anti-FLAG M1	Mouse	Sigma	F3040	1:500
Anti-FLAG M2	Rabbit	Sigma	F2555	1:500
Anti-HA mouse	Mouse	Abcam	ab18181	1:500

2.2 Secondary antibodies

Antibody	Isotype	Reactivity	Manufacturer	Catalogue no.	Dilution WB	Dilution (FACS/ Confocal)
Alexafluor 488	Goat	Mouse	Thermo Fisher	A11001	N/A	1:1000
Alexafluor 488	Goat	Rabbit	Thermo Fisher	A11008	N/A	1:1000
Alexafluor 647	Goat	Mouse	Thermo Fisher	A21235	N/A	1:1000
Alexafluor 647	Goat	Mouse	Thermo Fisher	A21245	N/A	1:1000

2.3 Plasmid constructs

DNA Construct	Source
Flag-LHR	Aylin Hanyaloglu
Flag-FFA2	Natarin Caengprasath
Flag-FFAR3	Matilda Shackley
HA-FFAR3	Matilda Shackley

2.4 Quantitative-PCR primers

Gene	Forward (5'-3')	Reverse (5'-3')
FFAR1	ACATTGGATTGGCCCAACCGCA	TCCGCGATGCTTTCGTGATCTGT
FFAR2	CGAGAACTTCACCCAAGAGC	TGAGGGAACTGAACACCACA
FFAR3	CAATACTCTGCATCTGTGAC	CAGGTAGACGGAAAAGAAA
FFAR4	ACTTTGCTCCCTCTACGCA	GGATGGCTTGGTACCCGAAG
CCK	AGCGCGATACATCCAGCAG	ACGATGGGTATTCGTAGTCCTC
Preproglucagon	TTACTTTGTGGCTGGATTGCTT	AGTGGCGTTTGTCTTCATTCA
TAS1R1	ACCCTGGGGTATGAACTGTATG	TGGAGGAGTGGTTGCGAAGA
TAS1R2	CAGGCGAGGACACTCCATTG	GGAGGGTAAAGAGGCCACC
TAS1R3	TGCTGCCTACTGCAACTACAC	CCGGTCACTTAGCCGATCC
TASR2(107)*	TGCTCGGAGTTTATAGGGACA	CCAGAGGTAAGCATGTGTGGAA
TASR2(108)*	TGACACGTCATTGACCTCAG	GCTGGTCTGTTTCTCTGCAT
TASR2(138)*	TCATTTCTGTTCTTTCAGCCAT	CAGCAGTGCGATGTCACAGT
PYY	ACGGTCGCAATGCTGCTAAT	GACATCTCTTTTCCATACCGCT
α -gustducin	TAGGAGCCGAGAGGACCAAG	GCTGGTATTCAGATGCCCTTTC
β -actin	GGCTGTATTCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

* Murine TAS2R isoforms of interest

2.5 Synthetic guide RNA sequences

gRNA_ffar3_KO	Sequence (5'-3')
gRNA_ffar3_KO_1	AAGAAAGAAGCTTGTCCTCA
gRNA_ffar3_KO_2	GAGGAACACCAACAGGTAGA
gRNA_ffar3_KO_3	TTTCTTTCCGTCTACCTGT

2.6 Pharmacological compounds

Inhibitor/Activator	Target	Manufacturer	Catalogue Number	Final concentration
IBMX	PDE inhibitor	Sigma	I5879	500 μ M
PTX	G α_i inhibitor	Tocris	3097	200 ng/ μ L
FSK	AC activator	Cayman	Cay11011	3 μ M
TAK-875	FFAR1 agonist	Cayman	Cay17335	10 μ M
TUG-891	FFAR4 agonist	Cayman	Cay17035	10 μ M
4-CMTB*	FFAR2 agonist	Tocris	4602	10 μ M
AR420626	FFAR3 agonist	Cayman	Cay17531	10 μ M

* 4-Chloro- α -(1-methylethyl)-N-2-thiazolyl-benzeneacetamide

2.7 Chemical Reagents

Reagent	Manufacturer
Absolute ethanol	VWR
Ampicillin	Sigma
Agarose Powder	Sigma
Bovine serum albumin (BSA)	Sigma
Bovine serum albumin fatty acid free	Sigma
Bromophenol blue	Sigma
Chloroform	VWR
Deoxynucleotide triphosphate (dNTPs)	Life Technologies
Dimethyl sulfoxide (DMSO)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glycerol	Sigma
Hydrochloric acid	Sigma
5'-Inosine-mono-phosphate	Sigma
Isopropanol	VWR
L-Alanine	Sigma
Lithium chloride	Sigma
Lysogeny broth or Luria Bertani broth (LB)	MP Biomedicals
Methanol	VWR
5'-Monophosphate glutamine	Sigma
Phosphate buffered saline (PBS)	Sigma
PBS with Ca ²⁺ /Mg ²⁺	Sigma
Sodium chloride	Sigma
Sodium acetate	Sigma
Sodium butyrate	Sigma
Sodium propionate	Sigma
Sodium Hydroxide	Sigma
Trizol	Life Technologies

2.8 Kits

Reagents	Manufacturer
cAMP Dynamic 2	Cisbio
Dnase I, Rnase-free	Thermo Fisher
Fluo-4 Direct Calcium Assay	Thermo Fisher
Genomic DNA Extraction Kit	Thermo Fisher
HiSpeed Plasmid Maxi	Qiagen
IP-One	Cisbio
Qiaprep Spin Mini	Qiagen
Qiaquick Gel Extraction	Qiagen
Quick Ligation	NEB
Quikchange II Site-Directed Mutagenesis	Agilent
Superscript TM IV First-Strand Synthesis System	Invitrogen

2.9 Cell culture reagents

Reagents	Manufacturer
Dulbeccco's modified eagles medium (DMEM) ± phenol red	Sigma
Electroporation Buffer L	Lonza
Fetal bovine serum	Sigma
Geneticin (G418)	Thermo Fisher
Lipofectamine 2000	Thermo Fisher
Opti-MEM I reduced serum media	Thermo Fisher
Penicillin/Streptomycin	Thermo Fisher
Trypsin-EDTA solution	Thermo Fisher

2.10 Other reagents

Miscellaneous	Manufacturer
DEPC Treated Water	Sigma
DNA loading dye	Bioline
Glass bottomed petri dishes (35mm)	Mattek Corporation
Microscope slides	Appleton Woods
Nuclease-Free Water	Ambion
Plasticware	Corning
Power SYBR® Green PCR Mastermix	Applied Biosystems
Primers	Life Technologies
Rnase Zap	Sigma
SYBR Safe DNA Gel Stain	Invitrogen

2.11 Buffers

All buffers used at room temperature, unless otherwise stated.

General

Phosphate buffered saline (PBS): 140 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4 , pH 7.2, 1.5 mM Na_2HPO_4 , pH 7.2

Immunofluorescence

Flow cytometry (FACS) buffer: 2% (v/v) Fetal bovine serum (FBS) in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$

Blocking solution and antibody incubation: 2% (v/v) FBS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$

Bacterial media

LB Broth: 1% (w/v) Bacto Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl. Made up in sterile water and autoclaved

LB Agar: LB Broth + 1.5% (w/v) Agar

Chapter 3: Methods

3.1 Molecular Biology

3.1.1 Generation of chemically competent *Escherichia coli*

The *Escherichia coli* (*E.coli*) DH5 α strain was used for plasmid amplification. To generate chemically competent *E. coli*, -80 °C stocks were streaked onto LB Agar plates and incubated at 37 °C overnight. Colonies were then picked and used to inoculate a 5 mL LB culture which was grown overnight at 37 °C + 200 rpm shaking. 1 mL of the overnight culture was used to inoculate a 200 mL LB culture, which was grown at 37 °C + 200 rpm shaking until reaching an optical density (OD) of 0.2-0.4. At this point, the culture was transferred to a 250 mL centrifuge flask (Corning™) and centrifuged at 4500 rpm for 15 minutes. The pellet was then subjected to a series of washes as detailed below.

Wash number	Wash solution	Wash volume (mL)
1	MgCl ₂ (1 M)	100
2	CaCl ₂ (1 M)	200
3	CaCl ₂ (85 mM) / 15% glycerol (v/v)	50

The final re-suspension was prepared with 500 μ L of ice-cold 85 mM CaCl₂ containing 15% glycerol (v/v) solution. Cells were separated into 50 μ L aliquots in pre-cooled 1.5 mL Eppendorf tubes and used immediately in transformation reactions or stored at -80 °C.

3.1.2 Transformation of chemically competent *Escherichia coli*

Cells for transformation were used directly after preparation or stored aliquots were thawed on ice for ~ 5 minutes. To each reaction tube, 10-100 ng of the appropriate plasmid was added followed by incubation on ice for a further 20 minutes. The samples were then subjected to a 42 °C heat shock for 30 seconds. After a further 2 minutes on ice, 950 μ L of LB media was added to each tube and the samples were recovered at 37 °C + 200 rpm shaking for 1 hour. For transformations using purified plasmid, recovered cells were then plated by spreading 20 μ L of undiluted culture onto antibiotic selective LB Agar plates and incubated overnight at 37 °C. For ligation reactions, recovered cells were pelleted by centrifugation at 13000 rpm for 1 minute. The supernatant was discarded and the pellet was re-suspended in 20 μ L of LB media before being spread onto antibiotic selective LB agar plates and incubated overnight at 37 °C.

3.1.3 Plasmid extraction

Plasmid Mini/Maxi-Prep kits (QIAGEN) were used to extract plasmid samples according to manufacturer's instructions. Concentrations were subsequently measured on a Nanodrop Spectrometer.

3.1.4 High-fidelity polymerase chain reaction

All high-fidelity (HF) polymerase chain reactions (PCR) were performed with Q5® High Fidelity Polymerase (NEB) according to the manufacturer's instructions. All samples were purified using a QIAquick PCR Purification kit (QIAGEN) according to manufacturer's instructions and final DNA concentrations were measured on a Nanodrop Spectrophotometer.

3.1.4 Primer design

All primers were designed with aid of SnapGene Viewer. Forward and reverse primers were designed to be 21 bp in length, with similar T_m and with 40-80% GC content. All primers were ordered from SigmaAldrich.

3.1.6 Gel electrophoresis

DNA fragments and plasmids were analysed via gel electrophoresis. DNA samples to be analysed were diluted with Loading Buffer (NEB). 0.8% agarose supplemented with SYBRSafe Gel Stain (ThermoFisher Scientific) is run at 140 V, 50 mA for 30 minutes. Gels were subsequently imaged with a SynGene Box Imager. Gel extractions were performed using a Qiagen Gel Extraction kit according to the manufacturer's instructions.

3.1.7 Sanger Sequencing

All Sanger Sequencing was performed by Genewiz. Data was analysed using SnapGene Viewer.

3.1.8 Synthesis of FLAG-FFAR3 plasmid construct

A plasmid construct was synthesised in a pcDNA3.1 backbone consisting of N-terminally FLAG tagged FFAR3 protein with a prolactin signal peptide under the control of a CMV enhancer (all FFAR genes/proteins utilised in this project were murine isoforms; hereby named FLAG-FFAR3). Primers TS01 (5'-CGAGCGCTGATGGGGACAAGCTTCTTTC-3') and TS02 (5'-TAGGGCCCTTAGCTCGGACACTCCTTG-3') were used to amplify the FFAR3 gene from the Myc-DDK-tagged Ffar3 template (Origene, MR622214) using Q5 HF-polymerase, annealing temperature of 63 °C and extension time of 30 seconds. TS01 and TS02 were designed with 5' overhangs containing the AfeI or ApaI restriction site respectively. A pcDNA3.1 backbone and the PCR-amplified FFAR3 construct were digested with AfeI and ApaI restriction enzymes (NEB) according to the manufacturer's instructions. After gel extraction to purify the correct bands, a T4 Quick Ligation (NEB) reaction was performed using a 3:1 ratio of insert to backbone. The plasmid was amplified in *E. coli* and Sanger sequencing was used to confirm correct sequence.

3.1.9 Synthesis of HA-FFAR3 plasmid construct

Gibson Assembly was used to assemble the HA-tagged FFAR3 construct (all FFAR genes/proteins utilised in this project were murine isoforms; hereby named HA-FFAR3) using primers TS37 (5'-CTCCGATTACAAAGATGATGATGATAAGATGGGGACAAGCTTCTTCT-3') and TS38 (5'-TAGGTGACACTATAGAATAGGGCCCTTAGCTCGGACACTCCTTGG-3') to amplify the pcDNA3.1 backbone from the FLAG-FFAR3 construct. Oligonucleotides TS39 (5'-AGCCATCCAAGGAGTGTCCGAGCTAAGGGCCCTATTCTATAGTGT-3') and TS40 (5'-AATTGCCAAGAAAGAAGCTTGTCCCCATCTTATCATCATCTTTGT-3') were annealed together in a thermocycler by heating to 95°C and slowly cooling at a rate of 5 °C/minute until reaching 25 °C, to synthesis the HA-tag encoding insert. Gibson Assembly was used to clone this fragment into the PCR-amplified vector; a 3 molar excess of insert fragment to linearised backbone fragment was used. Fragment and backbone DNA samples were made up to 10 µL with dH₂O and incubated with 10 µL of 2x GA MasterMix (NEB) for 20 minutes at 50 °C. 5 µL of this was transformed into *E. coli* and the plasmid was subsequently amplified and verified via Sanger sequencing.

3.2 Quantitative- PCR

3.2.1 RNA extraction

Cells were grown in 6 well plates until reaching 80% confluency. After washing with PBS, 1 mL of TRIzol® Reagent was added to each well. The wells were scraped and the lysate was transferred to 1.5 mL RNase-free Eppendorf tubes. 200 µL of chloroform was added to each sample and this was followed by vigorous shaking for 15 seconds. Tubes were then centrifuged at 10,000 g for 5 minutes. The upper aqueous phase, containing RNA, was transferred by pipetting to a new 1.5 mL Eppendorf tube. 500 µL of isopropanol was added to each tube to precipitate the RNA. Tubes incubated for 5 minutes at room temperature before centrifugation at 10,000 g for 5 minutes. After discarding the supernatant, the RNA pellet was washed with 1 mL 70% ethanol and centrifuged again at 10,000 g for 5 minutes. The ethanol was discarded, and pellets were allowed to air dry to ensure the removal of any residual ethanol before dissolving in 25 µL of DEPC-treated water. A ND-2000 spectrophotometer was subsequently used to measure RNA purity and concentration. RNase inhibitor (ThermoFisher) was added to the RNA, according to the manufacturer's instructions, to help prevent RNA degradation.

3.2.2 RNA treatment with DNase I

1 µg of each RNA sample was treated with a DNase I treatment kit (Life Technologies) as per manufacturer's instructions, to ensure no DNA contamination.

3.2.3 Complementary-DNA synthesis

SuperScript IV Reverse Transcriptase kit (Life Technologies) was used to synthesise complimentary DNA (cDNA). 10 µL of the mastermix was made according to the manufacturer's instructions and mixed with 10 µL of the DNase I-treated RNA samples. These were then incubated at 25°C for 10 minutes, 37°C for 120 minutes to synthesise the cDNA and 85°C for 5 minutes to denature the enzymes. Samples were then used immediately for quantitative PCR (qPCR) or stored at -20°C.

3.2.4 Data analysis

qPCR was performed in 96-well plates using a total volume of 20 µL per reaction containing of 5 µL of diluted cDNA (1 in 10 in nuclease-free water), 500 nM forward and reverse primers, 10 µL of 1X SYBR-Green PCR Mastermix and nuclease-free water. Each reaction was run in triplicates and cDNA was replaced with nuclease-free water as a negative control. Reactions were performed using the ABI StepONE sequence system with the following cycling conditions: 95°C for 15 minutes (initial denaturation), 40 cycles of 94°C for 15 seconds (denaturation) and 60°C for 30 seconds (annealing), and finally 72°C for 30 seconds (extension). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used for analysis of raw C_T values. Briefly, gene expression was normalised to the housekeeping gene β -actin, and values from treated cells were compared to the expression of untreated controls.

3.2.5 Primer optimisation

The primer sequences used were purchased as pre-designed from SigmaAldrich UK. Prior to use of primers in all samples, the concentrations to be used were optimised by varying the primer concentrations between 100 nM to 600 nM and also using different amounts of diluted cDNA – 1:5, 1:10, 1:15, and 1:20. The optimal primer concentration and cDNA amount was chosen according to the one that produced the maximum fluorescent signal and the smallest C_T value. The amplification efficiency of each of the primer pairs was calculated using serial dilutions of cDNA sample 1:1, 1:2, 1:10, 1:20, 1:100, 1:200, 1:1000, 1:2000. The C_T values at each were plotted against the logarithm of

the DNA copy number. With each serial dilution there should be a proportional shift in C_t value and the relationship between these data sets can be used to calculate the efficiency of the primer pair (Rodríguez et al., 2015); as shown for the β -actin primers in **Figure 3.1**.

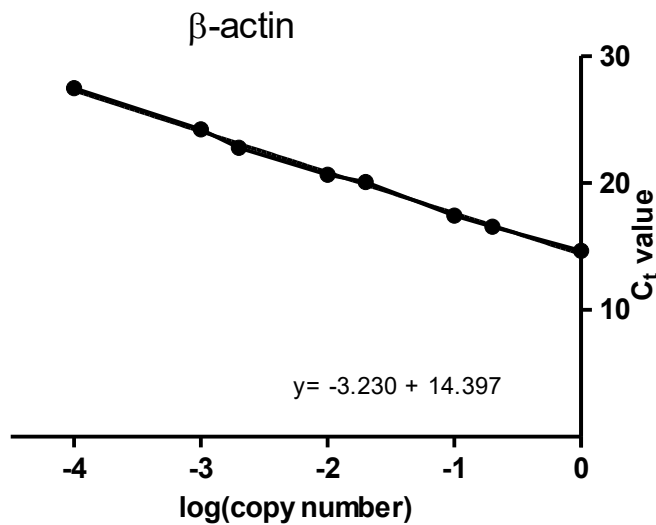


Figure 3.1 Example of primer efficiency calculation for β -actin A qPCR was performed on serial dilutions of cDNA. The log of the DNA copy number was plotted against the C_t value for β -actin. The regression equation is used to calculate the primer efficiency.

To calculate the primer efficiency:

$$\text{Primer efficiency} = 10^{\frac{-1}{-3.23}} * 100$$

$$\text{Primary efficiency} = 104\%$$

The efficiency of all primer sets was optimised to fall between 90-100%.

3.3 Cell culture

Cell lines were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5 g/L D-glucose, 4 mM L-Glutamine (Sigma, UK), supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/L streptomycin. Cells were incubated in a 95% O_2 and 5% CO_2 atmosphere at 37 °C. Cells were passaged in a 3:1 ratio via trypsinisation when reaching 70-80% confluency.

3.4 Transfection

3.4.1 Lipofectamine 2000

24 hours prior to transfection, 10^6 cells were plated into 6-well plates in 2 mL of DMEM supplemented with 10% (v/v) FBS. 4 μ g of plasmid DNA was combined with 250 μ L of Opti-MEM. In a separate tube, 10 μ L of Lipofectamine 2000 was combined with 250 μ L of Opti-MEM. These were incubated for 5 minutes at room temperature before mixing and incubation for a further 20 minutes at room temperature. The 500 μ L mix was then added to the 2 mL of media in each well of cells and incubated at 37 °C with 5% CO_2 overnight. The medium was then replaced with supplemented DMEM. Transiently expressing cells were used for experiments 3 days post-transfection.

3.4.2 Electroporation

An Amaxa® Cell Line Nucleofector® Kit L (Lonza) was used for electroporation of STC-1 cells. These were grown in 75 mm² flasks until 80% confluent. STC-1 cultures were trypsinised with 5 mL of trypsin for 5 minutes at 37 °C. 10 mL of DMEM was used to neutralise the trypsin and the solution was transferred into a 15 mL Falcon Tube (Corning™) and centrifuged at 1000 rpm for 4 minutes. Cells were then counted using a Neubauer haemocytometer and re-suspended in the appropriate electroporation buffer at a final concentration of 10⁶ cells/ 100 µL. This was mixed with the material to be electroporated to make a final volume of 100 µL/ per electroporation. The cell suspension was immediately transferred into electrocuvettes, which were subjected to shock in a Nucleofector II (Lonza). Immediately after shock, the electrocuvette was flushed with 500 µL of reduced Ca²⁺ media. Plastic pipettes were then used to transfer the electroporated cells into a well of a 12-well plate containing 1 mL of pre-warmed growth media. This was incubated at 37 °C until analysis of electroporation efficiency after 48 hours.

In order to produce edits at high efficiency, it was first essential to optimise the electroporation process for STC-1 cells. Electroporation of a GFP-expressing vector was used to calculate electroporation efficiency, as cells that were successfully transfected could be detected via flow cytometry analysis, giving information on the percentage of gated cells and the mean fluorescence intensity of each cell. Two different electroporation buffers, L and V, (Lonza) were used to re-suspend the cells prior to exposure to electroporation via 7 different Nucleofector II programmes of varying duration and intensity (**Figure 3.2**). The mortality caused by each condition was also analysed visually by microscopy. Buffer L with programme 7 (D023) was selected for on-going electroporation experiments, as it had the greatest percentage of cells gated, indicating the greatest percentage of cells expressing the vector but also showed the least indications of cell apoptosis.

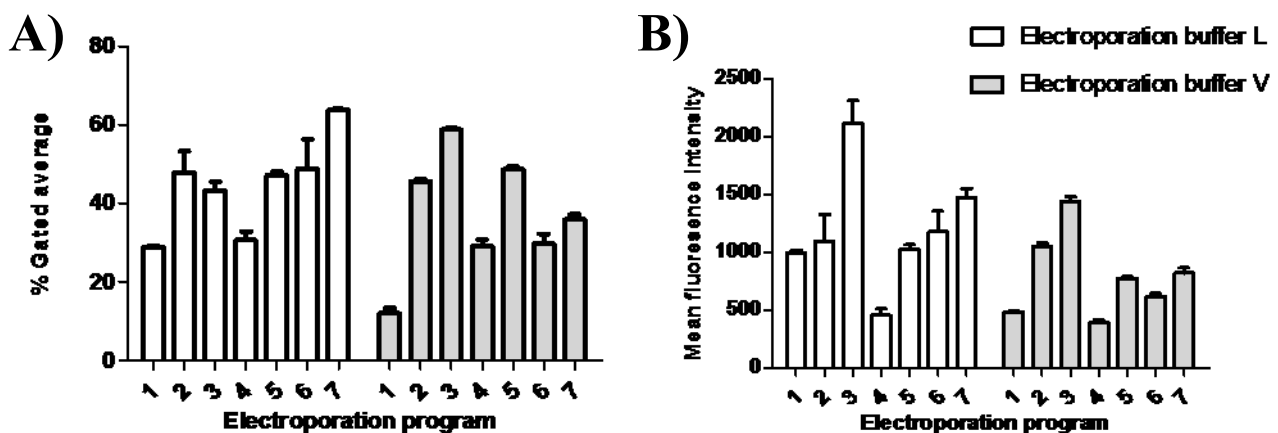


Figure 3.2 Optimisation of electroporation parameters for STC-1 cells A) A GFP-expressing vector was electroporated using 7 different optimisation Nucleofector II programmes into STC-1 cells re-suspended in Buffer L or Buffer V (Lonza). Flow cytometry was used to analyse the percentage of the cells that were gated, representing the percentage of those successfully expressing the vector (A) and the mean fluorescence intensity (B).

3.4.3 Stable cell lines

Stable cell lines were selected for after transfection by supplementing growth media with 0.5 mg/mL Geneticin (ThermoFisher), in 6mm² petri-dishes. Media was changed every 2/3 days. Separate colonies were selected with a sterile glass pipette and transferred to 96 well plates. Growth was

observed until screening analysis was performed to confirm stable expression of the transfected gene.

3.5 GPCR signalling assays

3.5.1 cAMP immunoassay

For measurement of cAMP accumulation, cells were plated in 96-or 48-well plates and grown to 80% confluency. Cells were pre-treated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 5 minutes prior to ligand addition (5 minutes) in the presence or absence of 3 μ M forskolin (FSK). Cells were then washed with ice-cold PBS. Cells were lysed by incubation with 50 μ L of Cisbio cAMP lysis buffer room temperature for 10 minutes on a shaking platform. Plates were then kept on ice, scraped and lysates were transferred to 1.5 mL Eppendorf tube and centrifuged at 14000 rpm for 10 minutes at 4 °C. The supernatants were transferred into a fresh tube. Levels of cAMP accumulation were measured using the Cisbio Homogenous Time Resolved Fluorescence (HTRF) cAMP dynamic 2 kit, a TR-FRET-based immunoassay kit. In a 384 white bottom well plate, 10 μ L of each sample, 5 μ L of dye d2 and 5 μ L of anti-cAMP cryptate conjugate was added and incubated in the dark for 1 hour at room temperature. The plate was read with PHERAstar FSX plate reader (BMG Labtech) equipped with HTRF 337 optic module, with excitation at 340 nm and measurements of emission at 615 nm and 665 nm. cAMP levels were interpolated from a cAMP standard curve (**Figure 3.3**) and normalised to protein concentration which was determined using a Bradford Assay. All experiments were conducted in triplicate and repeated at least 3 times.

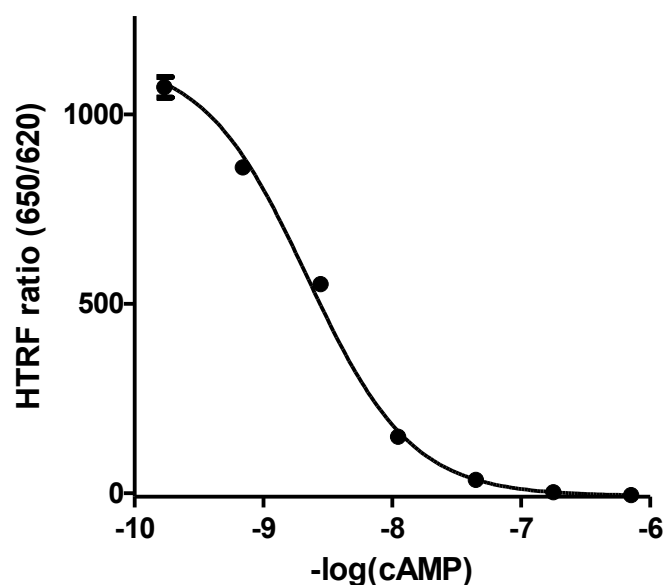


Figure 3.3 Standard curve for cAMP TR-FRET-based immunoassay Levels of cAMP accumulation were measured using the Cisbio Homogenous Time Resolved Fluorescence (HTRF) cAMP dynamic 2 kit, a TR-FRET-based immunoassay kit. The cAMP standard provided was serially diluted. In a 384 white bottom well plate, 10 μ L of each standard dilution sample, 5 μ L of dye d2 and 5 μ L of anti-cAMP cryptate conjugate was added and incubated in the dark for 1 hour at room temperature. The plate was read with PHERAstar FSX plate reader (BMG Labtech) equipped with HTRF 337 optic module, with excitation at 340 nm and measurements of emission at 620 nm and 665 nm. The HTRF ratio was then plotted against cAMP concentration to make a standard curve, from which cAMP concentrations can be extrapolated

3.5.2 IP₁ immunoassay

IP₁ assays were performed on cells plated at 80-90% confluency in 12 well plates. To measure the accumulation of IP₁, cells were incubated with ligands diluted in serum-free DMEM supplemented with 50 mM LiCl and 0.1% BSA fatty acid free for 30 minutes at 37°C. Cells were then washed with ice-cold PBS and lysed by incubating them with Cisbio IP₁ lysis buffer at room temperature for 10 minutes on a shaking platform. Samples were scraped and centrifuged as described for the cAMP accumulation assay. Levels of IP₁ accumulation were measured using the Cisbio HTRF IP-One kit, which functions using the same principles as the cAMP dynamic 2 kit. In a 384 white bottom well plate, 14 µL of each sample, 3 µL of anti-IP₁ dye d2 and 3 µL of anti-IP₁ cryptate conjugate was added and incubated in the dark for 1 hour at room temperature. The plate was read with PHERAstar FSX plate reader (BMG Labtech) equipped with HTRF 337 optic module, with excitation at 340 nm and measurements of emission at 620 nm and 665 nm. IP₁ levels were interpolated from a IP₁ standard curve (**Figure 3.4**) and normalised to protein concentration. All experiments were conducted in triplicate and repeated at least 3 times.

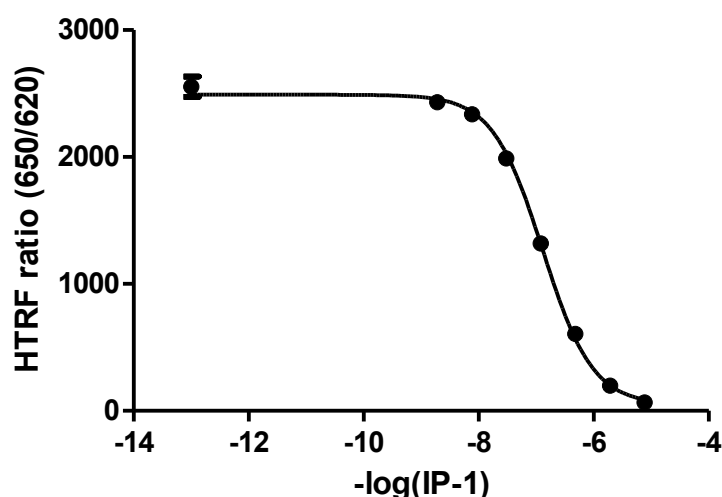


Figure 3.4 Standard curve for IP₁ TR-FRET-based immunoassay Levels of IP₁ accumulation were measured using the Cisbio Homogenous Time Resolved Fluorescence (HTRF) IP₁ kit, a TR-FRET-based immunoassay kit. The IP₁ standard provided was serially diluted. In a 384 white bottom well plate, 14 µL of each standard dilution sample, 3 µL of dye d2 and 3 µL of anti-IP₁ cryptate conjugate was added and incubated in the dark for 1 hour at room temperature. The plate was read with PHERAstar FSX plate reader (BMG Labtech) equipped with HTRF 337 optic module, with excitation at 340 nm and measurements of emission at 620 nm and 665 nm. The HTRF ratio was then plotted against IP₁ concentration to make a standard curve, from which IP₁ concentrations can be extrapolated

3.5.3 Calcium mobilisation

Calcium mobilisation was measured using the Fluo-4AM Direct Calcium Assay Kit (Invitrogen). Cells are plated into 35 mm² glass bottomed petri-dishes (Mattek). When at 60-70% confluency, cells were incubated with a 1:1 ratio of opti-MEM media (Sigma, UK) to calcium dye Fluo-4-AM Direct for 30 minutes at 37 °C and for a further 30 minutes at room temperature. Cells were imaged using a Leica Confocal Microscope using a 20 X dry objective and 488 nm excitation laser. Movies were recorded at 1 fps for 1 minute before ligand addition and then for a further 10-20 minutes until the

readout returned to basal levels. All experiments conditions were conducted in duplicate and repeated at least 3 times. The fluorescence intensity of each cell was quantified using the ImageJ plugin Time Series Analyser. The maximal intensity was obtained from subtracting the average background intensity (recorded before ligand addition) for each cell and averaged across 20 cells per condition.

3.6 Microscopy

3.6.1 Confocal live cell imaging

Antibody feeding assays were performed to visualise FLAG-tagged receptors in live cells. Cells were washed with anti-FLAG antibody produced in rabbit (1:500) for 20 minutes at 37°C and AlexaFluor 488 conjugated anti-rabbit secondary antibody (1:1000) for 20 minutes at 37°C in phenol red-free DMEM prior to ligand treatment. Cells were imaged using TCS-SP5 microscope (Leica) with a 63x oil-immersion objective and 1.4 numerical aperture (NA). Images were acquired using Leica LAS AF image acquisition software.

3.6.2 Flow cytometry

Cells were plated into 12 well plates. When at 80% confluency, cells were trypsinised and transferred to 1.5 mL Eppendorf Tubes. These were centrifuged at 10,000 rpm for 5 minutes. When using antibodies, the pellet was re-suspended in 200 μ L PBS + 2% FBS buffer, with anti-FLAG antibody produced in rabbit (1:500) and incubated on ice for 1 hour. Cells were pelleted, again, via centrifugation at 10,000 rpm for 5 minutes and then re-suspended in 200 μ L PBS + 2% FBS buffer with 488 nm conjugated anti-rabbit secondary antibody (1:1000) and incubated on ice for a further hour in the dark. Cells were washed 3 times with 500 μ L PBS + 2% FBS. The final resuspension was performed with 400 μ L of PBS + 2% FBS, after which the samples were transferred to round bottom polypropylene FACS tubes. These cells were then analysed on a FACS Calibur Flow cytometer.

3.7 Synthesis of genetically modified cells

Discovered some 20 years ago, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) are found in more than 40% of bacterial and 80% of archaeal genomes (Bhaya et al. 2011). In their native context, these are thought to act as an RNA-guided adaptive immune system against invading genetic elements (Wiedenheft et al. 2012). The CRISPR locus consists of alternating sequence of repeats and linkers, the latter of which consist of segments of foreign DNA, incorporated during an acquisition phase and inserted as protospacers within the CRISPR repeat arrays. When transcribed, the resulting CRISPR RNA (crRNA) guides nuclease proteins to specific sites identified via standard DNA-RNA base-pairing (Sander et al. 2014).

CRISPR systems are generally categorised into two main classes, with Class 1 dependent on multi-subunit protein aggregations and Class 2 utilising single effector proteins. The most commonly used in research, and the one to be employed in this project, is the Class 2 Type II CRISPR/Cas9 system adapted from *Streptococcus pyogenes*, which utilises the dsDNA endonuclease spCas9 (Nishimashu et al. 2014). Innately, spCas9 is guided, by the ribonucleotide complex formed from crRNA and transactivating CRISPR RNA (tracrRNA), to complementary regions of invading DNA. This is cleaved by spCas9 on the condition that it is flanked by a protolinker adjacent motif. The necessity of this site, of the form 5'-NGG-3' for spCas9, keeps the CRISPR array protospacers safe from cleavage and subsequent destruction as well as playing a role with spacer acquisition (Anders et al. 2014).

It was found that, by introducing unambiguous synthetic guide RNA (sgRNA); complementary to the 20 nucleotide region upstream of a target NGG site; alongside spCas9 nuclease, DNA cleavage could be targeted to specific locations in the mammalian genome. The resulting double stranded breaks (DSBs) are repaired by the cellular DNA repair machinery via two key mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). Without the presence of a homologous repair template, the latter of these repair mechanisms occurs most frequently. NHEJ is an error-prone process, which can often result in insertions or deletions (indels; Maruyama et al., 2015). When targeting DSBs to the coding regions of genes, the recurrent NHEJ process can introduce indels which act as frameshift mutations, disrupting the conserved triplet codon code and resulting in a non-functional protein product (Barrangou et al. 2016). Accordingly, this process can be utilised for precise genome engineering and the formation of targeted KOs.

3.7.1 Design and preparation of synthetic guide RNAs

Genomic DNA extractions from WT STC-1 cells were carried out using a Genomic DNA Extraction Kit (ThermoFisher) according to the manufacturer's instructions. Regions of interest were then amplified via HF-PCR, purified and the sequence was determined via Sanger Sequencing (Genewiz). The STC-1 genomic sequence was used to design sgRNAs, targeting the 20 nucleotide sequences upstream of a 5'-NGG-3' site in the genomic region of interest, the sequences of which can be found in the Appendix. These were ordered from Synthego. sgRNAs were diluted to a final concentration of 30 μ M in TE buffer.

3.7.2 CRISPR/Cas9 induced double stranded breaks

The *Streptococcus Pyogenes* Type II CRISPR/Cas9 system was utilised to generate genome edits, ribonucleoprotein (RNP) complexes of sgRNA with a recombinant spCas9 that had fused nuclear localisation sequences (NLS). 2 μ L of NLS-Cas9-NLS (NEB) (40 pmol) was incubated with 10 μ L sgRNA (300 pmol) with 3 μ L of 10 X Cas9 Reaction Buffer (NEB). TE buffer was used to make a final volume of 30 μ L. This was incubated at 10 minutes at room temperature to form RNP complexes. This 30 μ L reaction was then mixed with 1×10^6 STC-1 cells suspended in 70 μ L of Electroporation Buffer L (Lonza). Immediately, the mixture was transferred to electrocuvettes and electroporated as previously described. Cells were then recovered for 48 hours before re-plating and further screening.

3.8 Statistical analysis

Data is represented as the mean +/- the standard error. GraphPad Prism was used to determine significance ($p < 0.05$), using unpaired Student's *t* tests, One-way ANOVA with a Dunnett post-hoc, or Two-way ANOVA followed by a Bonferroni post-hoc test.

Chapter 4: Evaluation of the underlying mechanisms of FFAR2 crosstalk in enteroendocrine cells

4.1 Introduction

Since deorphanisation, both FFAR3 and FFAR2 have been studied extensively in many different heterologous and endogenous systems, with several reports indicating that FFAR2 can couple to $G\alpha_{q/11}$ and $G\alpha_{i/o}$ pathways, whereas FFAR3 couples exclusively with the latter (Milligan et al., 2016). In mammalian expression systems, SCFAs activate $G\alpha_{q/11}$ mediated calcium responses and induce $G\alpha_{i/o}$ inhibition of AC via both human and mouse FFAR2 (Brown et al., 2002; Le Poul et al., 2003). The dual-coupling ability of FFAR2 on activation by SCFAs has also been reported extensively in several different human and murine primary tissues (Nilsson et al., 2003). In EECs, it has been reported that the mechanism of SCFA-stimulated GLP-1 release is via FFAR2 $G\alpha_{q/11}$ mediated calcium influxes. Indeed, it has been observed that SCFAs can induce intracellular calcium responses in L-cell murine colonic crypts (Tolhurst et al., 2012) and that pharmacological blockade of the $G\alpha_{q/11}$ pathway attenuates gut hormone release induced from DREADD hFFAR2 activation (Bolognini et al., 2019). Contrary to the literature, previous work in this laboratory has suggested that SCFAs in the colon at 1 mM or 10 mM are unable to induce calcium or IP_1 responses in murine EECs, whereas FFAR2 synthetic ligands, such as 4-CMTB and Compound 1, can (Caengprasath, 2019). Although propionate robustly activates $G\alpha_{q/11}$ mediated pathways when rodent FFAR2 is expressed in heterologous systems, such as HEK-293 cells, this response is not observed in murine EEC models such as STC-1 cells, intestinal organoids and intestinal crypts. Furthermore, in these systems propionate was shown to solely activate $G\alpha_{i/o}$ pathways in a manner which was critical for the release of GLP-1.

It is likely that the cellular environment in which FFAR2 is being expressed is able to manipulate and alter its signalling patterns when activated by SCFAs, a phenomenon that has been described for other pleiotropically-coupled GPCRs such as the β -adrenoreceptor (β -AR; Daaka et al., 1997). FFAR2 is expressed in multiple tissues throughout the body, which display ligand-dependent selectivity in signalling pathways, each with differential functional outputs. For example, in pancreatic β -cells, acetate and 4-CMTB activate divergent FFAR2 downstream signalling cascades, with acetate inducing $G\alpha_{q/11}$ mediated PLC- β activation and calcium signals but 4-CMTB coupling to $G\alpha_{i/o}$. Interestingly, this was reflected in the functional output of the ligand, as they acted to activate and inhibit glucose-stimulated-insulin secretion (GSIS) respectively (Priyadarshini, 2015).

The current evidence suggests that the lack of $G\alpha_{q/11}$ signalling induced by SCFAs in murine EECs and in the colon may be due to system bias inducing a preference towards $G\alpha_{i/o}$ coupling. One hypothesis is that the co-expression of other GPCRs, such as FFAR3, in EECs, may attenuate the ability of FFAR2 to activate $G\alpha_{q/11}$ on SCFA stimulation. GPCR crosstalk can significantly influence $G\alpha_{q/11}$ and calcium signalling profiles and a similar phenomenon has been documented between the follicle stimulating hormone receptor (FSHR) and luteinising hormone receptor (LHR; Jonas et al., 2018).

FFAR2/FFAR3 crosstalk is a feasible explanation for diminished $G\alpha_{q/11}$ signals, as co-localisation of FFAR2 and FFAR3 has been noted in primary human tissues (Ang et al., 2018) and the receptors have shown to be co-expressed in the same subsets of murine EECs (Nohr et al., 2013). FFAR2 and FFAR3 are also co-expressed in ghrelin cells and primary murine gastric mucosal tissue, where SCFAs preferentially activate $G\alpha_{i/o}$ pathways through FFAR2, instead of $G\alpha_{q/11}$ (Engelstoft et al., 2013). This highlights the importance of the $G\alpha_{i/o}$ pathway in the regulation of ghrelin secretion. Furthermore, it is evident from FFAR2 and FFAR3 KO models that there is an intimate relationship between the two receptors (Tolhurst et al., 2012; Lin et al., 2012) and this potential cooperation and crosstalk is, thus, to be studied further in this project.

4.2 Objective and Aims

The objective was to determine whether crosstalk between FFAR2 and FFAR3 is able to influence the chosen G protein signalling pathway taken when the receptors were exposed to propionate and to see if there were any other candidate GPCRs potentially able to influence SCFA signalling cascades. To answer this, the key aims of the experiments were:

- To determine the SCFA signalling profiles in HEK-293 cells expressing FFAR2 and to confirm that these differ significantly from EEC-derived cell line STC-1 cells
- To determine the calcium mobilisation response to both endogenous and synthetic FFAR2 ligands in HEK-293 cells expressing FFAR2 or FFAR3 alone, and compare these with HEK-293 cells co-expressing both receptors in tandem
- To create STC-1 cells with a FFAR3 functional KO and to use this model to determine if the propionate mediated calcium response profiles in STC-1 cells is impacted via loss of FFAR3
- To analyse heterogeneity in G protein signalling and receptor expression profiles in STC-1 cells sourced from different suppliers and analyse whether any discrepancies in GPCR expression profiles can be correlated with discrepant calcium responses to ligands

4.3 Results

4.3.1 Propionate induces intracellular calcium responses in FLAG-FFAR2 HEK-293 cells but not in STC-1 cells

Firstly, it was necessary to confirm the observation that both propionate and synthetic ligands are able to induce calcium responses when FFAR2 is expressed heterogeneously in HEK-293 cells but that propionate-mediated calcium responses are absent in STC-1 cells (all FFAR2 receptors studied in this project were murine isoforms; hereby named FFAR2). In order to do so, calcium mobilisation was measured via Fluo4-AM calcium indicator dye and confocal imaging in either STC-1 cells or HEK-293 cells transiently transfected with FLAG-FFAR2, in order to quantify the intracellular calcium levels in response to sodium propionate (1 and 5 mM), the FFAR2 specific agonist 4-CMTB (10 μ M) or to NaCl (5 mM, control). In STC-1 cells, propionate was unable to elicit calcium mobilisation whereas 4-CMTB was (**Figure 4.1A**). In FLAG-FFAR2 HEK-293 cells, however, propionate at 1 mM or 5 mM was able to induce significant increases in intracellular calcium mobilisation, although levels were not as high as those induced by 4-CMTB (**Figure 4.1B**). The signalling profiles in response to the same ligands of STC-1 cells and FLAG-FFAR2 expressing HEK-293 cells were significantly different, with FLAG-FFAR2 HEK-293 cells showing much greater response to both 4-CMTB and propionate, but not to NaCl control (**Figure 4.1C**), perhaps representative of the much higher expression levels of FFAR2 reached when expressing from a plasmid in a heterologous system. The representative fluorescent intensity plots over time indicate that, on addition of 5 mM propionate, the intracellular calcium levels in FLAG-FFAR2 HEK-293 cells rise dramatically and remain high for a relatively sustained period of time (**Figure 4.1D**).

To investigate why FFAR2 is able to elicit a calcium response in HEK-293 cells but not in STC-1 cells when stimulated by propionate, two approaches were taken. Firstly to co-express a FLAG-FFAR3 plasmid with FFAR2 in HEK-293 cells and secondly, to use CRISPR/Cas9 in order to create FFAR3 KO STC-1 cells, to see if either of these interventions influenced the propionate-induced calcium responses.

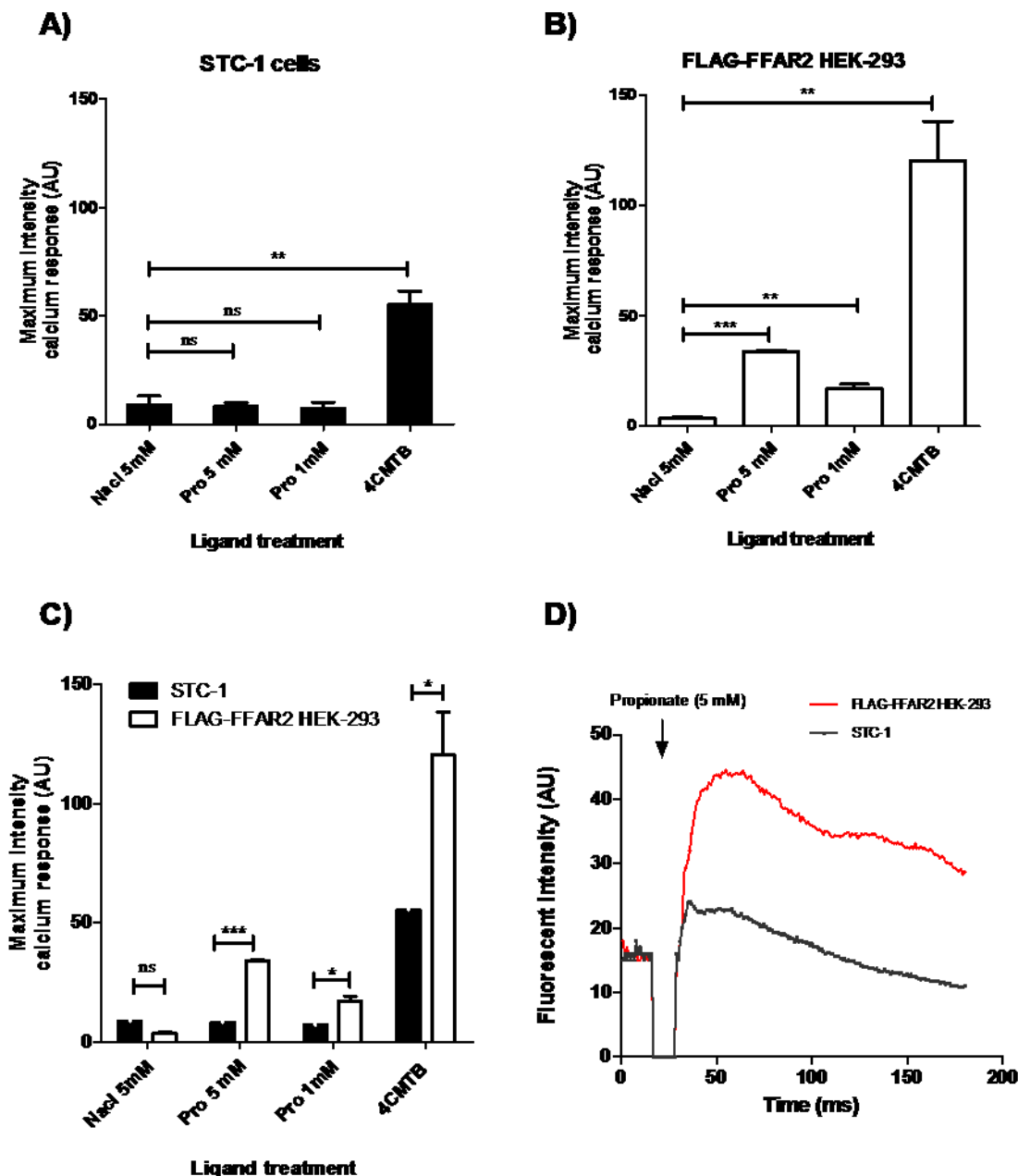


Figure 4.1 Propionate can induce intracellular calcium release in FLAG-FFAR2 HEK-293 cells but not in STC-1 cells. Intracellular calcium mobilisation measured in STC-1 cells (black bars) and FLAG-FFAR2 HEK-293 (white bars). Cells were incubated with fluorescence calcium indicator Fluo4-AM for 30 minutes at 37 °C and then for 20 minutes at RT and imaged live via confocal microscopy for 1 minute before NaCl/SCFA (1 or 5 mM) or 4-CMTB (10 μ M) addition. (A-B) Data is expressed as average maximal fluorescence intensities after ligand treatment $\pm$ SEM and is representative of duplicates of 20 separate cells across 3 distinct experiments in STC-1 cells (A) or FLAG-FFAR2 expressing HEK-293 cells (B). *t* test vs NaCl control *** p <0.001, ** p <0.01, * p <0.05 C) The average maximal fluorescence intensities of STC-1 cells and FLAG-FFAR2 HEK-293 in response to propionate (1 mM or 5 mM) and 4-CMTB are significantly different, two-way ANOVA with Bonferroni post hoc, *** p <0.001, ** p <0.01, * p <0.05 STC-1 response vs FLAG-FFAR2 HEK-293 response. D) Representative fluorescence intensity plots following propionate (5 mM) stimulation of STC-1 cells (black line) and FLAG-FFAR2 HEK-293 cells (red line), expressed in arbitrary units (AU)

4.3.2 FLAG-FFAR3 and HA-FFAR3 plasmids express in HEK-293 cells

For experiments to be performed using co-expression of FFAR2 and FFAR3 in a heterologous system, it was first necessary to synthesise a plasmid construct which expressed N-terminally tagged recombinant FFAR3 (all constructs used in this project were murine isoforms). These constructs were designed with a signal peptide for PM targeting with an N-terminal FLAG or HA tag for antibody detection. Before performing co-expression experiments, it was crucial to confirm that the FLAG-FFAR3 and HA-FFAR3 plasmids were able to be transfected and successfully expressed in HEK-293 cells. Incubation with anti-HA/ anti-FLAG primary antibodies and complementary fluorescent secondary antibodies was used to confirm the receptor presence via flow cytometry and live confocal microscopy. Flow cytometry analysis demonstrated that the mean gated percentage across three separate experiments was 88.2%, after subtracting the background fluorescence. The confocal imaging experiments suggested that the majority of receptor was expressed at the PM, but there was some evidence of constitutive internalisation into endosomal compartments (**Figure 4.2G**).

4.3.3 SCFAs activate $G\alpha_{i/o}$ pathways in FLAG-FFAR3 HEK-293 cells

To confirm the signalling properties of FLAG-FFAR3 it was stably transfected into HEK-293 cells. FFAR3 is known to activate $G\alpha_{i/o}$ signalling, and to verify this, FLAG-FFAR3 HEK-293s were subjected to 5-minute pre-treatment with IBMX (0.5mM) prior to exposure to 3 μ M forskolin (FSK), alone or with 10 mM SCFAs for 5 minutes. Acetate, propionate and butyrate all significantly reduced the FSK-induced rise in cAMP by an average of 52%, 51% and 51% respectively over 3 separate experiments. To confirm this was mediated via FFAR3, the experiment was repeated using 10 μ M of FFAR3 specific agonist, AR420626, which produced a more modest, but significant, cAMP decrease of 34% (**Figure 4.2A**).

4.3.4 The SCFAs elicit distinct pharmacological profiles at the FFAR3

The rodent FFAR3 is reported to have higher sensitivity to propionate over acetate and butyrate (Hudson et al., 2012) but it is not surprising that at a concentration of 10 mM (as shown in **Figure 4.2A**) the SCFAs displayed equipotent inhibition of cAMP accumulation, as 10 mM is reported to be the maximal response range for acetate, propionate and butyrate. To confirm the EC_{50} values for acetate, propionate and butyrate at the rodent FFAR3 receptor, SCFA-mediated inhibition of FSK stimulated cAMP accumulation was assessed when FLAG-FFAR3 HEK-293 cells were treated with FSK followed by either acetate, propionate or butyrate over a concentration range of 10 nM to 10 mM (**Figure 4.2 B-F**). From these dose-response curves, the EC_{50} values were calculated (**Table 4.1**). This indicated that EC_{50} values were similar to those previously reported and that acetate and butyrate have similar potencies, with pEC_{50} values of approximately 3.9. Propionate, however, is more potent at rodent FFAR3 with an pEC_{50} of 4.4.

	pEC ₅₀	Literature pEC ₅₀
Acetate	3.916 ± 0.25	3.73 ± 0.2
Propionate	4.44 ± 0.48	4.87 ± 0.12
Butyrate	3.905 ± 0.54	3.67 ± 0.17

Table 4.1 pEC₅₀ values for acetate, propionate and butyrate mediated FFAR3 Gα_{i/o} activation FLAG-FFAR3 HEK-293 cells were treated with IBMX (5 minutes, 0.5 mM) alone or with FSK (3 μM) and SCFAs over a concentration range of 1 μM to 10 mM. Data was used to plot a dose response curve using percentage of the maximal inhibition of cAMP accumulation by each of the SCFAs, collected across three distinct experiments (shown in Figure 4.2). These curves were used to extrapolate the EC₅₀ values. Data is expressed as mean pEC₅₀ values ± SEM. Literature pEC₅₀ values for FFAR3 were gathered from Hudson et al., 2012.

4.3.5 β-hydroxybutyrate exhibits Gα_{i/o} activation via FFAR3

β-OHB is a metabolite derived from fatty acids, produced by the liver as an alternative energy source to glucose in periods of fasting or intense exercise. β-OHB has been reported to both antagonise (Kimura, et al., 2011) and agonise (Won et al., 2013) FFAR3. To further investigate this, a cAMP assay was performed on IBMX (0.5 mM) pre-treated FLAG-FFAR3 HEK-293 cells with FSK (3 μM) and β-OHB. β-OHB was able to inhibit the FSK-induced cAMP response at concentrations from 0.1 mM to 10 mM (**Figure 4.2F**). The maximal reduction observed was at a 10 mM concentration, with a 32% reduction in cAMP. Interestingly, β-OHB at concentrations less than 0.01 mM or greater than 100 mM seemed to increase FSK-induced intracellular cAMP concentrations.

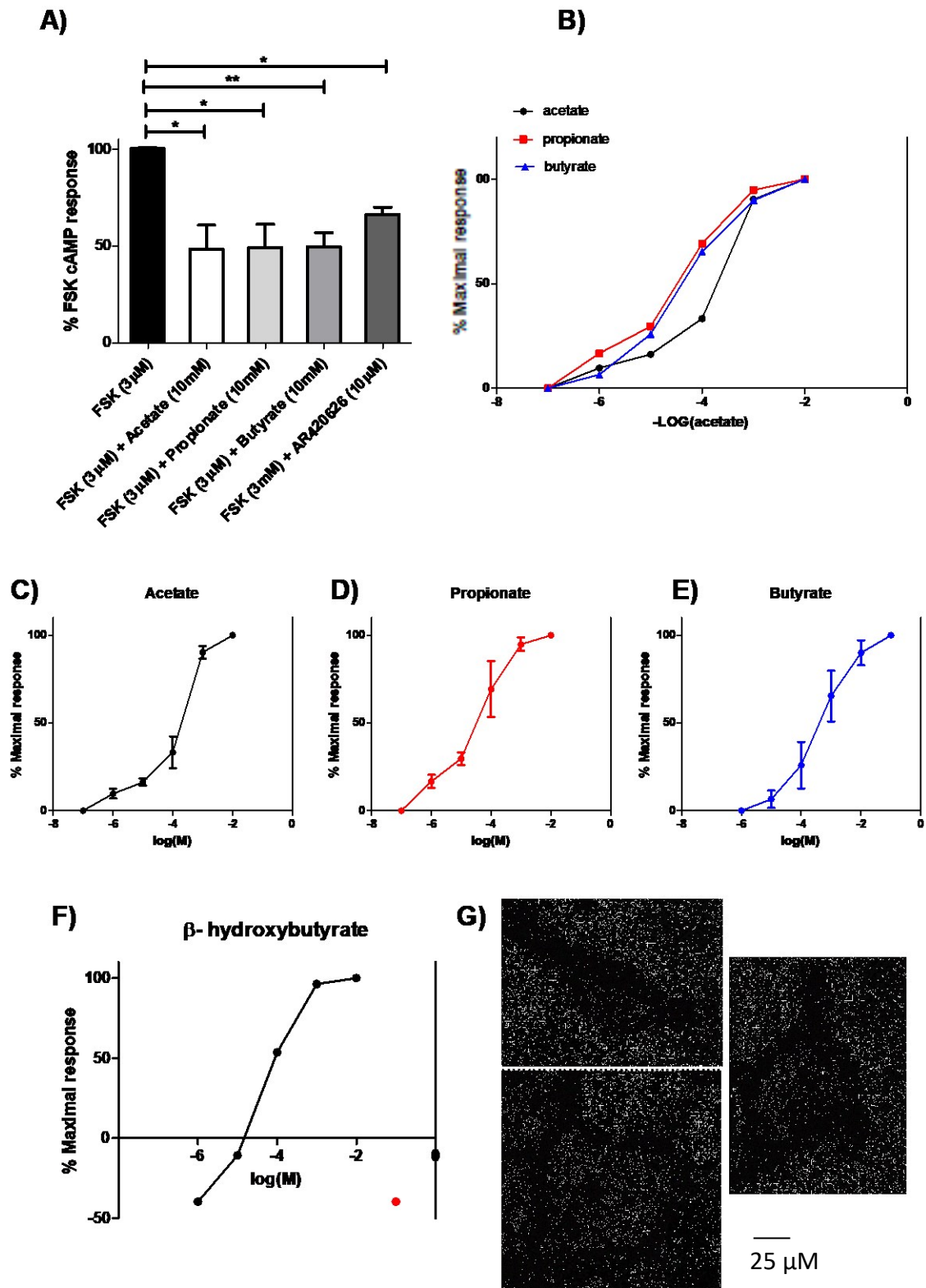


Figure caption overleaf

Figure 4.2 FLAG-FFAR3 expresses in HEK-293 cells and inhibits FSK-induced cAMP release A-F) Intracellular levels of cAMP measured in FLAG-FFAR3 transfected HEK-293 cells. A) Cells were treated with IBMX (5 minutes, 0.5 mM) and then with forskolin (FSK; 3 μ M) and short chain fatty acids (SCFAs; 10 mM) or FFAR3 agonist AR420626 (10 μ M) for 5 minutes. The maximal FSK-induced cAMP accumulation in the absence of ligand was defined as 100% and data is expressed as percentage change of FSK-stimulated positive control. All data represents mean $\pm$ SEM, collected over 3 distinct experiments. *t* test vs FSK-stimulated control **p*<0.05, ***p*<0.01. (B-F) cAMP inhibition dose-response curves. Cells were treated with IBMX (5 minutes, 0.5 mM) alone or with FSK (3 μ M) and SCFAs over a concentration range of 1 μ M to 10 mM. Data is expressed as a percentage of the maximal inhibition of cAMP accumulation for B) SCFAs C) acetate D) propionate E) butyrate F) β -hydroxybutyrate. Data represented as means $\pm$ SEMs taken over 3 distinct experiments. G) Live confocal images confirm expression of FLAG-FFAR3 in generated FLAG-FFAR3 stable HEK-293 cell line. Cells were incubated for 15 minutes with a 1 in 500 dilution of rabbit anti-FLAG antibody. After which they were washed and incubated for a further 15 minutes with a 1 in 1000 dilution of Alexa-488 anti-rabbit secondary antibody at 37 °C. Cells were imaged with confocal microscopy with a 495 nm laser (images representative of replicates imaged on at least three different occasions)

4.3.6 Co-expression of FFAR3 with FFAR2 in HEK-293 cells enhances the propionate induced calcium signals

To examine whether co-expression of FFAR3 with FFAR2 altered the calcium signalling of FFAR2 in HEK-293 cells, FLAG-FFAR3 and FLAG-FFAR2 were transiently co-transfected into untransfected HEK-293 cells. Cells were incubated with the calcium indicator dye, Fluo4-AM and a calcium mobilisation assay was performed to quantify the responses to propionate (5 mM) or NaCl (5 mM). Empty HEK-293 cells and FLAG-FFAR3 HEK-293 cells showed no response to NaCl or propionate. As before, propionate induced significant calcium mobilisation in FLAG-FFAR2 expressing HEK-293 cells compared to the NaCl control. The co-transfected FLAG-FFAR2 FLAG-FFAR3 HEK-293 cells also demonstrated sensitivity to propionate-mediated calcium signals. However, they responded significantly more robustly, with a near 2-fold increase in maximal fluorescent intensity compared to FLAG-FFAR2 HEK-293 cells (**Figure 4.3A**). Plotting the representative fluorescent intensity over time demonstrated the presence of FLAG-FFAR3 with FLAG-FFAR2 causes a more dramatic peak in fluorescent intensity in response to propionate addition (**Figure 4.3B**). Interestingly, both FLAG-FFAR2 and FLAG-FFAR2/FLAG-FFAR3 cells responded to the synthetic FFAR2 specific ligand, 4-CMTB, to a similar extent (**Figure 4.3C**). This suggests that crosstalk between FFAR3 and FFAR2 may enhance G α_q mediated signals specifically from SCFAs.

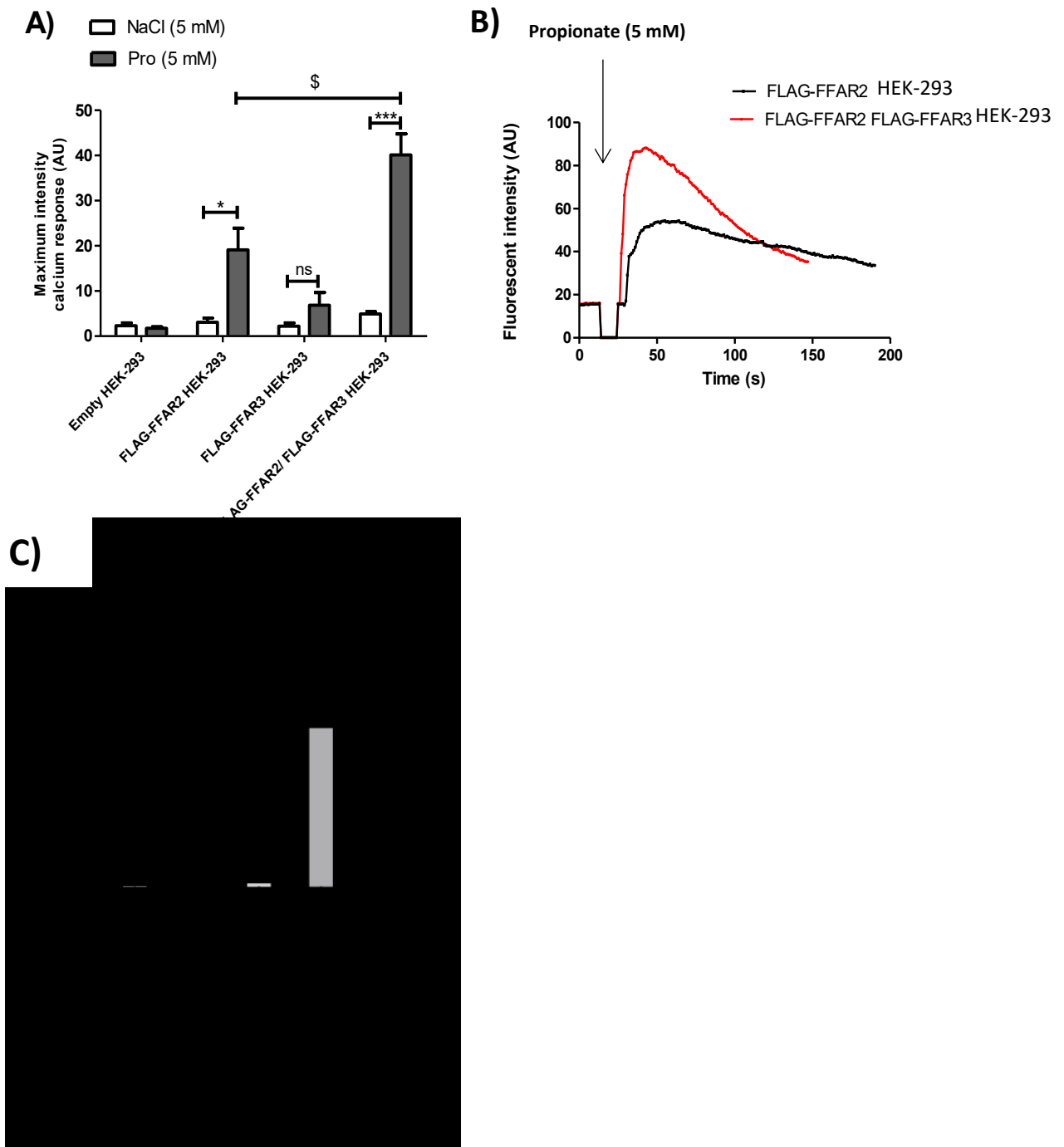


Figure 4.3 Co-expression of FLAG-FFAR3 with FLAG-FFAR2 potentiates the propionate induced intracellular calcium release in HEK-293 cells. Intracellular calcium mobilisation measured in untransfected (referred to as empty) HEK-293 cells, FLAG-FFAR2 HEK-293, FLAG-FFAR3 HEK-293 cells and FLAG-FFAR2/FLAG-FFAR3 HEK-293 cells. Cells were incubated with fluorescence calcium indicator Fluo4-AM for 30 minutes at 37 °C and then for 20 minutes at RT and imaged live via confocal microscopy for 1 minute before ligand addition (A) Data is expressed as average maximal fluorescence intensities after NaCl/Propionate treatment of cells. Data is expressed as average $\pm$ SEM collected from 20 distinct cells in duplicate from across 3 different experiments. Two-way ANOVA with Bonferroni post hoc, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ between group variance (propionate vs NaCl control for each cell type), \$ $p < 0.05$ within group variance (propionate vs propionate in different cell types) B) Representative fluorescence intensity plots following propionate (5 mM) stimulation of FLAG-FFAR2 HEK-293 cells and FLAG-FFAR2/FLAG-FFAR3 HEK-293 cells C) Data is expressed as average maximal fluorescent intensity after 4-CMTB (10 μ M) addition. Data is expressed as average $\pm$ SEM collected from 20 distinct cells in duplicate from across 3 different experiments. t test of FLAG-FFAR2 vs. FLAG-FFAR2/FLAG-FFAR3 is non-significant (ns). All data is expressed in arbitrary units (AU)

4.3.7 CRISPR/Cas9 mediated double strand breaks was able to cause a significant reduction in FFAR3 expression in STC-1 cells

To delete FFAR3 in STC-1 cells, CRISPR/Cas9 technology was utilised to make targeted DSBs in regions close to the transcription start site of the *ffar3* gene. The NHEJ repair pathway is an erroneous DNA repair pathway, often resulting in spontaneous indels. This process induces frameshift mutations that disrupt the conserved codon sequences, creating truncated and non-functional proteins that are targeted for degradation by the cell's quality control system, knocking out the gene function (Williams et al., 2016)

sgRNAs were designed to target the protein-coding region of *ffar3*, as near as possible to the transcription start site. Three sgRNAs were designed with the aim of forming DSBs, labelled gRNA_FFAR3KO_1, gRNA_FFAR3KO_2 and gRNA_FFAR3KO_3. For the first round of edits, gRNAs were electroporated in tandem; namely; gRNA1 and 2, gRNA1 and 3 and gRNA 2 and 3. The qPCR utilised to screen the three groups of edited cells, monitoring changes in FFAR3 expression, indicated that there was a 80%, 51% and 28% reduction in FFAR3 transcript, respectively (**Figure 4.4A**). The cells treated with gRNA1 and 2 had a significant reduction, and so these were selected to undergo a second round of CRISPR/Cas9 editing, with the same combinations of gRNAs (**Figure 4.4B**). Further significant reductions in FFAR3 transcript were detected in 2nd generation of edited cells treated gRNA1 and 2 ($p=0.04$), and gRNA2 and 3 ($p=0.003$; **Figure 4.4B**), the latter of which was selected for further experiments, and will hereby be termed STC-1 FFAR3 KO cells. The raw C_t values for this 2nd generation of edited cells were in the 32-34 range, suggesting a very low, barely detectable, level of expression.

4.3.8 CRISPR/Cas9 mediated FFAR3 KO did not significantly impact expression levels of FFAR2

It has been reported that the close proximity of the FFAR2 and FFAR3 genes, and their linkage at the chromosomal region 7A3, induces a tight coupling between them. Due to this interaction, manipulations of one of the genes can have downstream effects on the expression of the other, discrediting further observations (Zaibi et al., 2010). Therefore, it was necessary to ensure the expression levels of FFAR2 had not been impacted by the CRISPR/Cas9 machinery targeted at FFAR3. The RNA was isolated from STC-1 FFAR3 KO cells and STC-1 WT cells and a qPCR was performed to determine levels of FFAR2 transcript. There was no significant difference in the FFAR2 expression between WT STC-1 and the STC-1 FFAR3 KO cell line (**Figure 4.4C**), suggesting the KOs were specifically targeted to the FFAR3 gene.

4.3.9 CRISPR/Cas9 mediated FFAR3 KO significantly reduced the responsiveness of STC-1 cells to FFAR3 specific agonist, AR420626

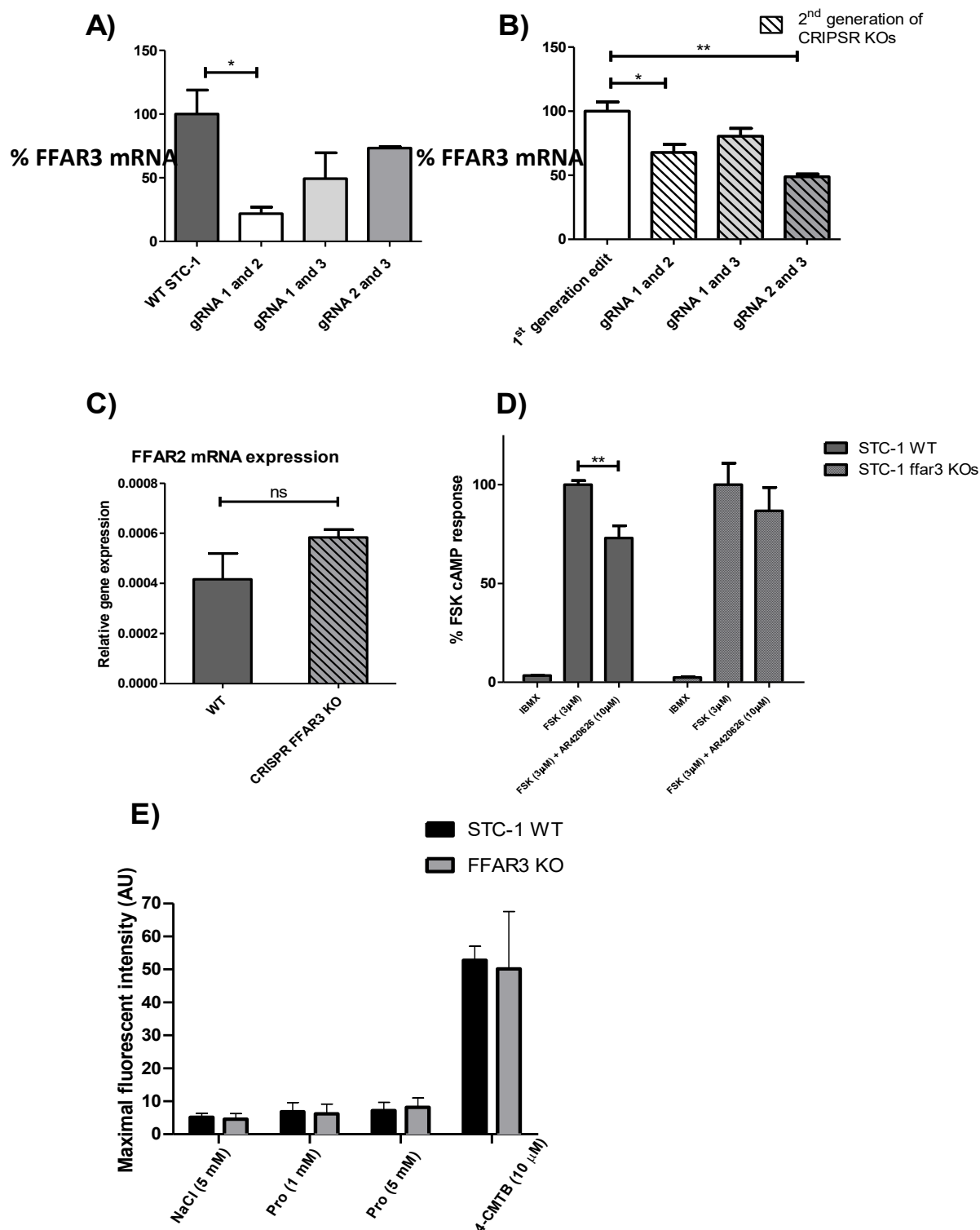


Figure 4.4 Generation of CRISPR/Cas9 mediated FFAR3 functional knockout STC-1 cells The Cas9 nuclease protein was co-electroporated into STC-1 cells with a combination of gRNAs, gRNA 1 and 2, gRNA 1 and 3 or gRNA 2 and 3. This was repeated on the 1st generation of knockouts (A) to synthesise a second generation of FFAR3 KO STC-1 cells (B). qPCR was used to evaluate FFAR3 transcripts in the 1st generation (A) and 2nd generation (B) of CRISPR-edited STC-1 cells. Data is expressed as a percentage of the FFAR3 transcripts compared to the un-electroporated control (STC-1 WT cells for A and the 1st generation of CRISPR KO STC-1 cells, shown in white on both A and B, for B). Data is represented as means $\pm$ SEM from 3 distinct experiments. t test against un-transfected control * p <0.05, ** p <0.01. C) qPCR was used to determine whether levels of FFAR2 transcript in the 2nd generation of edited STC-1 cells was disrupted by the CRISPR/Cas9 gene editing machinery, t test against STC-1 WT control is non-significant (ns). Data is expressed as mean $\pm$ SEM, taken from 3 distinct experiments. D) Intracellular levels of cAMP measured in WT and FFAR3 KO STC-1 cells. Cells were treated with IBMX (5 minutes, 0.5 mM) alone or with forskolin (FSK; 3 μ M) or AR42062 (10 μ M) for 5 minutes. The maximal FSK-induced cAMP accumulation in the absence of ligand was defined as 100% and data is expressed as percentage change of FSK stimulated control. Data is displayed as means $\pm$ SEMs collected over 3 different experiments. T test against FSK stimulated control, ** p <0.01. E) Intracellular calcium mobilisation measured in STC-1 WT cells and STC-1 FFAR3 KO cells, which were incubated with fluorescence calcium indicator Fluo4-AM for 30 minutes at 37 $^{\circ}$ C and then for 20 minutes at RT and imaged live via confocal microscopy for 1 minute before NaCl/Propionate (5 mM) or 4-CMTB (10 μ M) addition. Data is expressed as average maximal fluorescence intensities after ligand treatment of cells. Data is expressed as mean $\pm$ SEM from duplicates of 20 different cells for each treatment, collected in 3 different experiments. T test for each ligand, STC-1 WT vs STC-1 FFAR3 KO were non-significant.

The cAMP accumulation assay was performed with the FFAR3-specific agonist, AR420626, to compare the signalling profiles of WT STC-1 cells with the STC-1 FFAR3 KOs. Both cell lines were incubated with IBMX (0.5 mM) for 5 minutes and then subjected to exposure to FSK (3 μ M) with/without AR420626 (10 μ M). Whereas in WT STC-1 cells, AR420626 induced a significant ($p=0.005$) decrease in FSK-induced cAMP accumulation by approximately 30%, no significant reduction was observed in the FFAR3 KO STC-1 cells (**Figure 4.4D**), suggesting the functional activity of FFAR3 has been lost as a direct consequence of the genetic manipulation.

4.3.10 CRISPR/Cas9 mediated FFAR3 KO STC-1 cells remained unresponsive to propionate induced calcium mobilisation

To examine whether loss of FFAR3 in STC-1 cells altered the profiles of calcium mobilisation, as a marker of $G\alpha_q$ coupling, in response to FFAR2 ligands, a calcium mobilisation assay was performed on WT STC-1 cells and STC-1 FFAR3 KO cells. These were incubated with the calcium indicator dye, Fluo4-AM and fluorescent intensity was measured to quantify the responses to propionate (1 and 5 mM) or 4-CMTB (10 μ M), compared to a NaCl control. There was no significant difference between the reactivity of the two cell lines to NaCl (5 mM), propionate (1 mM), propionate (5 mM) or 4-CMTB (10 μ M). The calcium mobilisation signalling profiles in response to these ligands was very similar (**Figure 4.4E**), signifying that propionate is still unable to signal through FFAR2 $G\alpha_q$ pathway, even in the absence of FFAR3.

4.3.11 There is significant heterogeneity between STC-1 cell lines in their ability to respond to propionate via intracellular calcium mobilisation

The laboratory has received STC-1 cells from two distinct sources. Throughout the previous experiments described, the STC-1 cells utilised (now named STC-1a), were used at a passage number of 30-50 and were unable to elicit calcium signals in response to propionate. On the acquisition of a second batch of STC-1 stocks at earlier passages (now termed STC-1b), sourced from a different supplier, the evaluation of calcium mobilisation in response to propionate was repeated, as previous reports have suggested there is significant heterogeneity between STC-1 cell lines, able to manipulate phenotypic responses and secretory profiles (McCarthy et al., 2015). To examine whether STC-1b cells displayed distinct profiles of calcium mobilisation, as a marker of $G\alpha_q$ coupling, in response to FFAR2 ligands, a calcium mobilisation assay was performed to compare STC-1a and STC-1b. Both cell lines were incubated with the calcium indicator dye, Fluo4-AM and fluorescent intensity was measured to quantify the responses to propionate (1 and 5 mM) or 4-CMTB (10 μ M), compared to a NaCl control. There was no significant difference between the reactivity of the two cell lines to NaCl (5 mM), propionate (1 mM) or 4-CMTB (10 μ M). However, STC-1b cells showed a significant increase in maximal fluorescent intensity when exposed to 5 mM propionate (**Figure 4.5A**). The plot of fluorescence intensity (AU) with time, shows that there is a rapid increase in fluorescence following exposure to 5 mM propionate (**Figure 4.5B**), an effect which was not observed with the STC-1a cells.

4.3.12 STC-1 cells demonstrated significant heterogeneity between expression profiles of GPCRs

The observation that STC-1b cells responded with calcium influxes to propionate (5 mM), but STC-1a cells did not, prompted the theory that this may be a direct consequence of distinct intracellular environments that were manipulating the FFAR2 signalling profiles. It has been demonstrated that crosstalk with other GPCRs can alter signalling bias. It had already been concluded from the previous data that FFAR3 may not be inhibiting FFAR2 from coupling with $G\alpha_{q/11}$, but as FFAR1 and FFAR4 are also co-expressed in L-type EECs (Norton et al., 2017), they could also be feasible candidates to play

a role in receptor crosstalk. Additionally, altered signalling profiles could be due to discrepancies in FFAR2 expression levels, as FLAG-FFAR2 HEK-293 cells display calcium signals to propionate but express FFAR2 in much greater quantities. To analyse this further, RNA was extracted from both STC-1a and STC-1b cells and also CRISPR FFAR3 KO cells (which were derived from STC-1a cells). qPCRs were performed for the comparison of transcript levels of FFAR1, FFAR2 and FFAR4. Both FFAR1 and FFAR2 remained unchanged between the different cell lines (**Figure 4.5 C-D**). The levels of FFAR4 transcript, however, were approximately 3-fold lower in STC-1b cells than in STC-1a cells/ STC-1 FFAR3 KO cells (**Figure 4.5E**). As the STC-1b cells expressed FFAR4 significantly less, and were also able to elicit propionate mediated calcium influxes, it may be feasible that FFAR4 may be participating in GPCR crosstalk, thus, preventing FFAR2 $G\alpha_q$ coupling.

4.3.13 FFAR4 agonists reduce FSK-stimulated cAMP accumulation in STC-1 cells

FFAR4 has been reported to couple to both $G\alpha_q$ and $G\alpha_{i/o}$ pathways in EECs (Milligan et al., 2016). To confirm the latter, a cAMP accumulation assay was performed with FFAR4-specific agonist TUG-891. STC-1a cells were incubated with IBMX (0.5 mM) for 5 minutes and then subjected to exposure to FSK (3 μ M) with/ without TUG-891 (10 μ M). There was no significant increase in cAMP when using TUG-891 without FSK. However, there was a significant 40% reduction in FSK-stimulated cAMP accumulation in the presence of TUG-891, presenting evidence that FFAR4 can couple to $G\alpha_{i/o}$ pathways in STC-1 cells. This may provide a potential mechanism by which FFAR4 inhibits FFAR2 calcium signalling. There was no significant decrease in FSK-stimulated cAMP accumulation when the same experiment was performed using FFAR1 agonist, TAK-785 (10 μ M), which also did not increase intracellular cAMP when used without FSK (**Figure 4.5F**), suggesting there may not be functional FFAR1 in these cells.

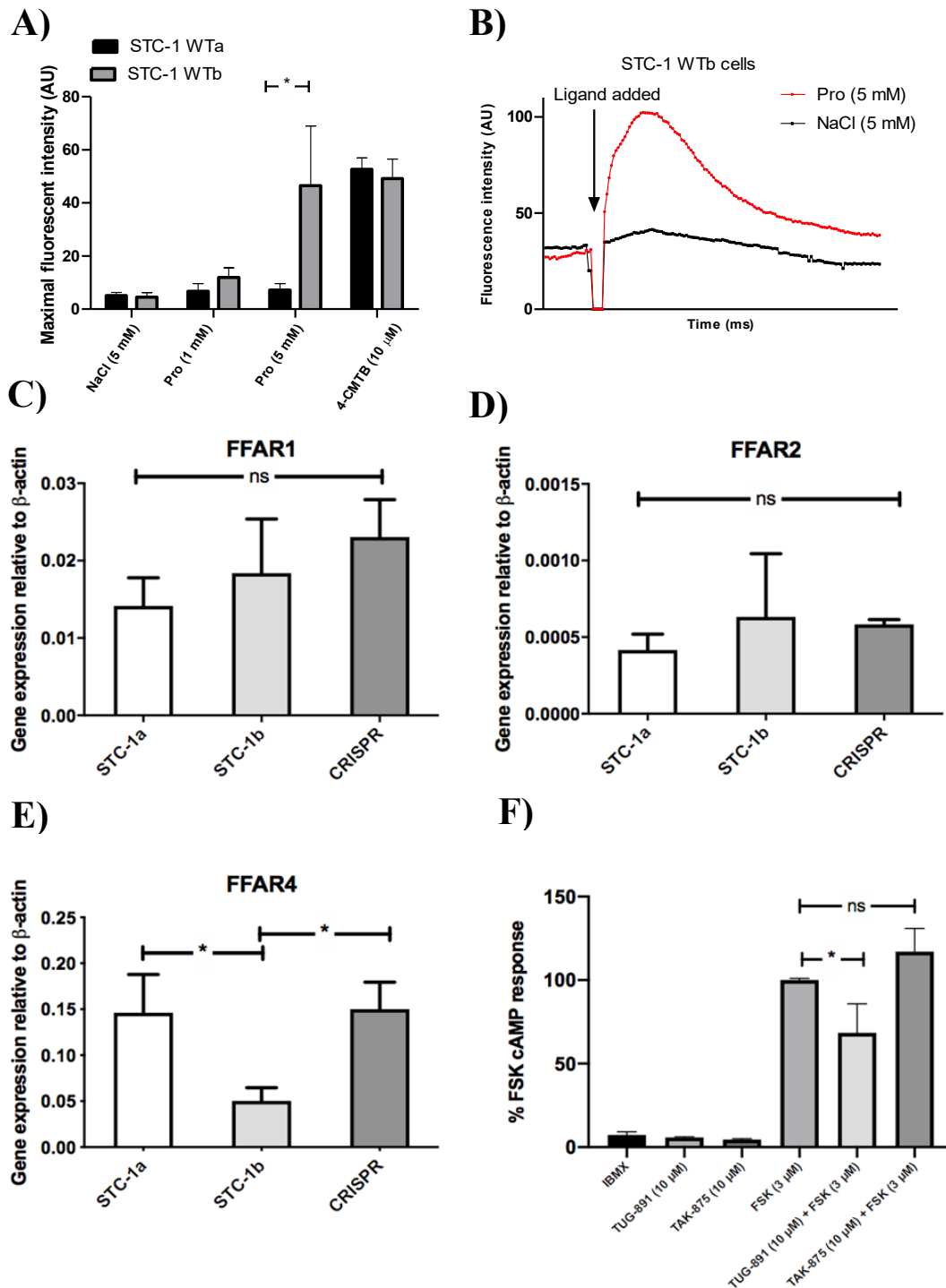


Figure 4.5 Heterogeneous calcium responses and expression profiles between STC-1 cells from different sources A and B) Intracellular calcium mobilisation measured in two strains of STC-1 WT cells (termed a and b), which were incubated with fluorescence calcium indicator Fluo4-AM for 30 minutes at 37 °C and then for 20 minutes at RT and imaged live via confocal microscopy for 1 minute before NaCl/Propionate (5 mM) or 4-CMTB (10 μM) addition. Data is expressed as average maximal fluorescence intensities after ligand treatment of cells. T-test STC-1a vs STC-1b * $p < 0.05$ B) Representative fluorescence intensity plots following propionate or NaCl (5 mM) stimulation of STC-1 WTb cells, expressed in arbitrary units (AU) (C-E) STC-1 WTa, STC-1 WTb and CRISPR FFAR3 KO cells were grown to confluency and were harvested, and qPCR was carried out to assess the levels of FFAR1 (C), FFAR2 (D) and FFAR4 (E). Expression levels were normalised to the levels of housekeeping gene β-actin. Data is shown as mean ± SEM, $n = 3$. One way ANOVA with Dunnett's post hoc * $p < 0.05$, non-significant (ns). F) Intracellular levels of cAMP measured in STC-1 cells. Cells were treated with IBMX (5 minutes, 0.5 mM) alone or with FSK (3 μM) and either no ligand, TUG-891 (10 μM) or TAK-875 (10 μM) for 5 minutes. The maximal forskolin-induced cAMP accumulation in the absence of ligand was defined as 100% and data is expressed as percentage change of FSK stimulated control. Data represented as means ± SEMs taken over 3 distinct experiments. T-test vs FSK stimulated control * $p < 0.05$, non-significant (ns)

4.4 Discussion

Although there is an extensive characterisation in the literature of both mouse and human FFAR2 and FFAR3 pharmacological and signalling properties, the majority of reports detail these when the GPCRs are expressed in heterologous systems, an environment distinct from endogenous systems. It has become evident that the signalling properties elicited by SCFAs and synthetic ligands in HEK-293 systems are not necessarily equivalent to those of EECs. Previous work in this laboratory has noted that SCFAs signal robustly and reliably via $G\alpha_{i/o}$ but not $G\alpha_q$ in EEC models, including STC-1 cells, intestinal crypts and organoids. However, when FFAR2 is expressed in HEK-293 cells, propionate can and does activate $G\alpha_q$ (Caengprasath, 2019). This project has further confirmed that propionate can induce calcium mobilisation via FFAR2 in HEK-293 cells, but has highlighted that in some, but not all, EEC intracellular environments, this is also possible. Some studies have observed SCFA-induced calcium mobilisation in EECs (Tolhurst et al., 2012) and chemogenetic manipulation to form DREADD receptors has demonstrated that DREADD-specific ligands can also induce similar effects (Bolognini et al., 2019), but the question remains as to why in some scenarios, FFAR2 demonstrates an apparent reluctance to couple to $G\alpha_{q/11}$ mediated signalling cascades. The traditional concept that each GPCR monomer acts independently to couple to its own array of G proteins and interact with an exclusive selection of ligands is now accepted to be much more complex. It is widely reported that homo- or hetero-dimerisation of GPCRs can have significant impacts on trafficking, signalling and ligand sensitivities. As it has been noted that the FFAR2 and FFAR3 are closely interlinked, co-localised and responsive to the expression levels of each other (Ang et al., 2018; Zaibi et al., 2010), it was consequently hypothesised that crosstalk between the two receptors was diminishing FFAR2 $G\alpha_{q/11}$ coupling.

To evaluate further the potential interaction of FFAR2 and FFAR3, two approaches were undertaken. The first of which was to induce co-expression of FFAR3 with FFAR2 in HEK-293 cells, to see if this undermined the propionate-induced calcium influxes. Plasmid constructs expressing FLAG-tagged and HA-tagged recombinant FFAR3 downstream of a CMV promoter were subsequently synthesised. Experiments have demonstrated that these can successfully be expressed in HEK-293 cells, as detected via live confocal images and flow cytometry. The observed inhibition of FSK-induced cAMP accumulation in FLAG-FFAR3 expressing HEK-293s in response to SCFAs and FFAR3 specific agonist, AR4260626, suggests that the transfection and expression of this receptor induced functional activity and that it couples to $G\alpha_{i/o}$ pathways as reported in the literature (Bolognini et al., 2016). Calculated EC_{50} values closely matched those reported elsewhere for rodent FFAR3, which indicate that propionate is more selective for and potent at FFAR3 than acetate and butyrate, which are equipotent at this receptor (Hudson et al., 2012). It was also interesting to note that physiologically high, but relevant concentrations of β -OHB, greater than 0.1 mM and less than 10 mM, were able to reduce intracellular cAMP levels, whereas physiologically low, but relevant concentrations enhanced cAMP accumulation. The complex pharmacology observed may be representative of β -OHB modulating the basal activity of FFAR3, which has been reported to be high (Priyadarshini et al., 2015). The contrasting effects that β -OHB has when used at different concentrations is a possible explanation for the conflicting reports as to whether β -OHB is an agonist or antagonist at FFAR3 (Kimura, et al., 2011; Won et al., 2013) and the observed properties may suggest partial agonism, where at low concentrations it acts to inhibit the high basal activity of FFAR3 (Hudson et al., 2012) and at higher concentrations it acts conversely to promote the activation. Additionally, there is some evidence of alternative non-receptor mediated signalling properties at supra-physiological concentrations.

After the confirmation of FFAR3 signalling properties, this study transiently co-expressed the FLAG-FFAR3 and FLAG-FFAR2 constructs in HEK-293 cells. The observation that transfection of FFAR3 could enhance calcium responses when expressed concurrently with FFAR2 suggests that it is not activity of FFAR3 preventing the activation of $G\alpha_q$ signalling from FFAR2. This suggests that rodent FFAR2 and

FFAR3 may interact to alter signalling in a similar fashion as reported previously for the human isoforms of the receptors, despite the significant species differences in structure, pharmacology and signalling profiles (Hudson et al., 2012). It has been demonstrated that expressing a fused human FFAR2-FFAR3 construct in HEK-293 cells significantly changes signalling in response to SCFAs, compared to when each monomer is expressed in isolation. When fused to FFAR3, FFAR2 seemed to more robustly couple to G_{α_q} calcium signalling, to activate p38 phosphorylation to a greater extent but was rendered unable to elicit $G_{\alpha_{i/o}}$ inhibition of AC (Ang et al., 2018). However, such artificial manipulation to generate a fused synthetic product may significantly disturb the sensitive three dimensional conformations of the two receptors, which is known to impact GPCR signalling and activity dramatically. Therefore, the question remains as to whether the observed changes in signalling are physiologically relevant.

Further work is needed to clarify whether FFAR3 is forming a heterocomplex with FFAR2 and how it can enhance calcium signalling. A way to approach this in a heterologous system, such as HEK-293 cells would be to co-express the receptors and to perform bioluminescence resonance energy transfer (BRET) analysis. BRET is a technique which has been utilised to study specific interfaces of receptor complexes, providing information not only about the receptor heteromer formation but also about the G protein-coupling properties characteristic for each heteromer or monomer composition. Complemented donor-acceptor resonance energy transfer (CODA-RET) is a methodology able to detect interactions between three or four separate entities at one time. The background of homomer interactions can be eliminated by designing the experiment so that a complementary signal is emitted only when GPCR subunits assemble into a heterocomplex. This acts as the donor signal, able to activate the acceptor molecules fused to the individual G proteins. This technique has been employed in live cells to describe how the heteromer formation of dopamine receptors (D1 and D2) can generate G-protein conformational rearrangements, inducing functional selectivity of signalling pathways in response to ligands (Urizar et al., 2011). A similar approach with FFAR2 and FFAR3 could provide evidence about whether it is a direct interaction between FFAR2 and FFAR3 that is inducing the enhanced calcium mobilisation in response to propionate. The synthetic FFAR2 ligand, 4-CMTB, has been demonstrated in this project to act in a manner distinct from endogenous ligands, as reported elsewhere (Caengprasath, 2019), as it is able to activate calcium mobilisation robustly in both EECs and heterologous systems in a way which is not enhanced by FFAR3 co-expression. CODA-RET may be utilised to shed light on the detailed molecular mechanisms underpinning the ligand-specific signalling profiles observed here and to characterise any ligand-induced conformational rearrangements of the downstream G proteins, which may be influencing the functional selectivity. Although BRET analysis holds several advantages over FRET methods, as utilised in identifying human FFAR2/3 dimers in HEK-293 cells (Ang et al., 2018), there are limitations with both BRET and FRET technologies. These include their inability to detect high-resolution localisation of molecules, their incapability to distinguish rapid dissociation/association patterns between molecules, their insensitivity to infrequent or transient events and the lack of information they give about the macro-complex stoichiometry (Calebiro and Sungkaworn, 2018). To overcome such restrictions, alternative high-resolution single molecule imaging techniques have been developed such as total internal reflection fluorescence (TIRF) illumination. Of particular utility here, would be photo-activatable dyes and localisation microscopy (PALM); a super-resolution approach to imaging heteromers (Jonas et al., 2015). As HA-tagged FFAR3 and FLAG-tagged FFAR2 constructs have been synthesised, HA-CAGE₅₅₂ and FLAG-CAGE₅₀₀ dyes could be utilised for PALM imaging. This could provide more information about, not only the potential interactions between FFAR2 and FFAR3 receptors, but the nature of the complexes formed. This could include how many monomers were in each complex, the relative ratios of each monomer subtype in each complex and time course of complex assembly and disassembly.

As well as the direct association of FFAR3 with FFAR2 causing shifts in conformation that may change the propensity to couple to certain G proteins, an alternative mechanism by which FFAR3 could be

increasing the SCFA-activated $G\alpha_{q/11}$ signals through FFAR2 in HEK-293 cells is via increasing the prevalence of FFAR2 expression on the PM. GPCR heteromerisation between α_{1B} -AR and α_{1D} -AR has been known to enhance the biosynthetic trafficking properties of these receptors from the golgi after translation (Uberty et al., 2003). Heteromer formation also alters the rate of internalisation and endocytosis. One example is vasopressin receptors V1a and V2, which both associate with β -arrestins and internalise when activated, but where V1a quickly dissociates from β -arrestins resulting in recycling back to the PM, V2 remains in complex with β -arrestin in intracellular compartments. However, when V1a and V2 form a heteromer, non-selective agonist stimulation leads to both receptors following the V2 pathway (Terrillion et al., 2004). As FFAR2 has been described to associate with β -arrestins, but FFAR3 has not (Milligan et al., 2016), it is possible they undergo distinct deactivation and internalisation processes, which may be subject to change on heterodimerisation.

The second approach to looking at the FFAR2/3 crosstalk was to make targeted CRISPR/Cas9 mediated FFAR3 KOs. Thus far, following two rounds of editing, approximately 90% of the FFAR3 transcript was been depleted from the STC-1 cells, suggesting that at least some cells of the polyclonal population were homozygous knockouts. Experiments demonstrated that propionate was still unable to elicit calcium mobilisation in these FFAR3-depleted cell line. Although we cannot rule out that there may be some remaining FFAR3 which may be able to contribute to FFAR2 signalling bias, it seems unlikely as the FFAR3 transcript was barely detectable. Unfortunately, attempts to synthesise a monoclonal homozygous FFAR3 KO population were hindered by the inability of EEC-derived cells to survive without surrounding growth factors (Goldspink et al., 2018), even in conditioned media. Therefore, all attempts to isolate a single clone via serial dilutions into 96 well plates and by picking STC-1 cell colonies failed and resulted in cell death.

Taking this two-pronged approach to evaluate FFAR2 and FFAR3 crosstalk and interplay has suggested that it is not FFAR3 responsible for the FFAR2 $G\alpha_{i/o}$ signal bias. Alternatively, it is feasible that there are other factors in the EEC cellular environment able to manipulate and bias the signalling patterns. There is a diverse array of GPCRs expressed in EECs, such as FFAR1, FFAR4, calcium-sensing receptor (CaSR), GPR119 and taste receptor subunits, all of which may be feasible candidates able to crosstalk with FFAR2. Furthermore, FFAR2 may be interacting with one or several of these, as part of a large communication network. The intricacy of this system is mirrored by the heterogeneous calcium responses to propionate observed from different supplies of STC-1 cells, and there remains some confusion about the underlying mechanism of these seemingly divergent reactions. It has been reported that the passage number and source of the STC-1 cells can have significant impacts on gene expression and signalling properties (Goldspink et al., 2018) and that within a single population there is significant heterogeneity (Wang et al., 2019). It was hypothesised that those STC-1 cells in which high concentrations of propionate was able to induce intracellular calcium mobilisation may be lacking, or have more of, a candidate GPCR for FFAR2 crosstalk. qPCR demonstrated that, indeed, there were significant differences in the expression level of FFAR4, and those cells which expressed FFAR4 to a lesser extent elicited more robust $G\alpha_{q/11}$ signals. Here, and elsewhere, it has been demonstrated that FFAR4 can couple to $G\alpha_{i/o}$ pathways in EECs (Milligan et al., 2016), so it could be feasible that FFAR4 may mediate a $G\alpha_{i/o}$ signal bias via crosstalk with FFAR2, especially as it is expressed at relatively high levels compared to the other GPCRs (**Figure 5.1**).

As well as L-type and K-type EECs, there are several other cell types that also co-express FFAR2 and FFAR4. For example, it has been demonstrated in mice and humans that FFAR4 is also expressed in gastric I cells (Milligan et al., 2016), in which SCFAs also elicit distinct $G\alpha_{i/o}$ signal bias through FFAR2 (Engelstroft et al., 2013). Here, FFAR4 is a chemosensor for dietary LCFAs, which, like SCFAs, potentially inhibit orexigenic ghrelin secretion *in vitro* gastric cells and in mouse models (Lu et al., 2012). Like FFAR2, FFAR4 can couple to both $G\alpha_{i/o}$ and $G\alpha_q$ pathways but both receptors inhibit ghrelin secretion via a PTX sensitive $G\alpha_{i/o}$ route in gastric cells and primary murine gastric tissue (Engelstroft et al.,

2013). FFAR2 is also expressed in mouse adipocytes and adipose tissue, where SCFAs induce $G\alpha_{i/o}$ pathway activation to elicit an anti-lipolytic effect (Ge et al., 2008) and to potentially modulate leptin secretion (Zaibi et al., 2008). Interestingly, adipose tissue also co-expresses FFAR4, which plays a role in regulation of glucose uptake in mature adipocytes (Milligan et al., 2016). The co-expression of FFAR4 with FFAR2 in cell types that display $G\alpha_{i/o}$ signal bias suggests a possible interaction between these receptors which is influencing G protein activation although experimental evidence is needed to understand whether this is the case, and if so, the fundamental mechanism underlying it. It is interesting to note that FFAR4 expression is considerably higher in obese humans (Little et al., 2014) and rodents (Paulsen et al., 2014). If it is found that FFAR4 does regulate FFAR2 mediated SCFA signaling pathways, it would be fascinating to discover if the higher levels of FFAR4 found in the obese phenotype has any impact on blunting SCFA signalling, and, subsequently, the downstream positive impacts FFAR2/3 agonism has on physiology, in these individuals.

A recent study has shown that, as well as differently sourced STC-1 cells having distinct properties (Goldspink et al., 2018) there is also significant heterogeneity within a population of STC-1 cells. They demonstrated that each cell has a unique, non-conserved expression pattern of gut hormones and L-AA sensing GPCRs, with immunostaining demonstrating the proportion of STC-1 cells that expressed each L-AA sensor ranged from 15% (TAS1R1) to 30% (CaSR). Although this paper did not analyse the proportions expressing members of the FFAR family, if they share similar expression profiles, the likelihood of co-expression of each FFAR isoform within the same cell decreases. This raises the question as to whether FFAR3 and FFAR2 are co-expressed in the same cells. As FFAR3 enhances propionate induced calcium mobilisation through FFAR2, if there was an absence of FFAR3 in EECs this may contribute to the increased $G\alpha_{i/o}$ signalling in EECs. The heterogeneity may also be indicative that it is unfeasible that it is GPCR crosstalk, alone, which is causing decreased $G\alpha_q$ coupling. Alternatively, it may not be a GPCR interaction causing the signal bias in the EEC cellular environment, as GPCRs are known to associate with proteins otherwise thought of as independent from canonical GPCR signalling cascades, such as tyrosine receptor kinases (TRKs; Pyne et al., 2007), integrins (Teoh et al., 2012) or syntrophins (Lyssand et al., 2011), forming a unique “signalosome” with the ability to alter receptor trafficking and expression at the PM as well as the signalling profiles and downstream physiological effects.

Each cellular environment is a context unique, adaptable and incredibly complex. The dynamics of the signalling environment, including the ratios of active to inactive receptor conformations, the G protein binding constants, the relative receptor expression levels, the lipid raft microdomain constitution, receptor trafficking and the level and constitution of the G protein monopoly expressed can all influence the ligand response behaviour drastically (Kinzer-Ursem et al., 2007; Allen., 2007). Between cell types there are distinct discrepancies in the expression, and, thus, availability of each G protein subunit. The profiles of GPCRs able to undergo multiplicity coupling is dependent upon the prevalence of each subunit and the ratios between them. There are a large range of mammalian G proteins, expressed differentially depending on the cell type, allowing stimuli to elicit cell-specific, appropriate responses depending on the environmental context (Wettschureck et al., 2005). Ghrelin cells are key examples of a cellular environment that seems remarkably susceptible to $G\alpha_{i/o}$ signal bias. Although these cells do express $G\alpha_{q/11}$ proteins, there is a suggestion that the more abundant presence of more obscure $G\alpha_{i/o}$ proteins is behind the signal bias. This suggestion came from the observation that ghrelin cells express G proteins $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$, G proteins which inhibit AC (Engelstroft et al., 2013). These G proteins are also expressed in other cell types such as pancreatic β -cells, where they mediate the inhibition of insulin release. As SCFAs have been reported to both activate (via $G\alpha_q$; McNelis et al., 2015) and inhibit (via $G\alpha_{i/o}$; Tang et al., 2015) insulin secretion via mediation of FFAR2 in pancreatic islets, the differential expression patterns of the $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$ proteins may be a possible explanation for the downstream coupling. It would be interesting to quantify the expression levels of $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$, or otherwise to evaluate the balanced ratio between $G\alpha_{i/o}$ subunits and $G\alpha_{q/11}$ subunits, in L-type EECs showing the propensity to signal via $G\alpha_{i/o}$.

instead of $G\alpha_q$. It is highly likely that these specialised cells express a significantly different G protein repertoire than the HEK-293 cells used as a model here. Not only are they isolated from different tissues of different species, but both immortalised cell lines have undergone artificial manipulation which has not only increased their replicability, but has also provided them with unique expression profiles.

In summary, it does not appear to be crosstalk with FFAR3 which is preventing $G\alpha_q$ coupling, but rather, there is evidence to suggest that FFAR3 enhances propionate induced calcium mobilisation via FFAR2. The apparent reluctance of FFAR2 to couple to $G\alpha_q$ pathways in EEC systems is likely to be a direct consequence of the unique signalosome, distinctive expression profile and specialised monopoly of proteins available to undergo dynamic interactions with the GPCR and GPCR signalling components and FFAR4 represents a realistic candidate. This is summarised in **Figure 4.6**.

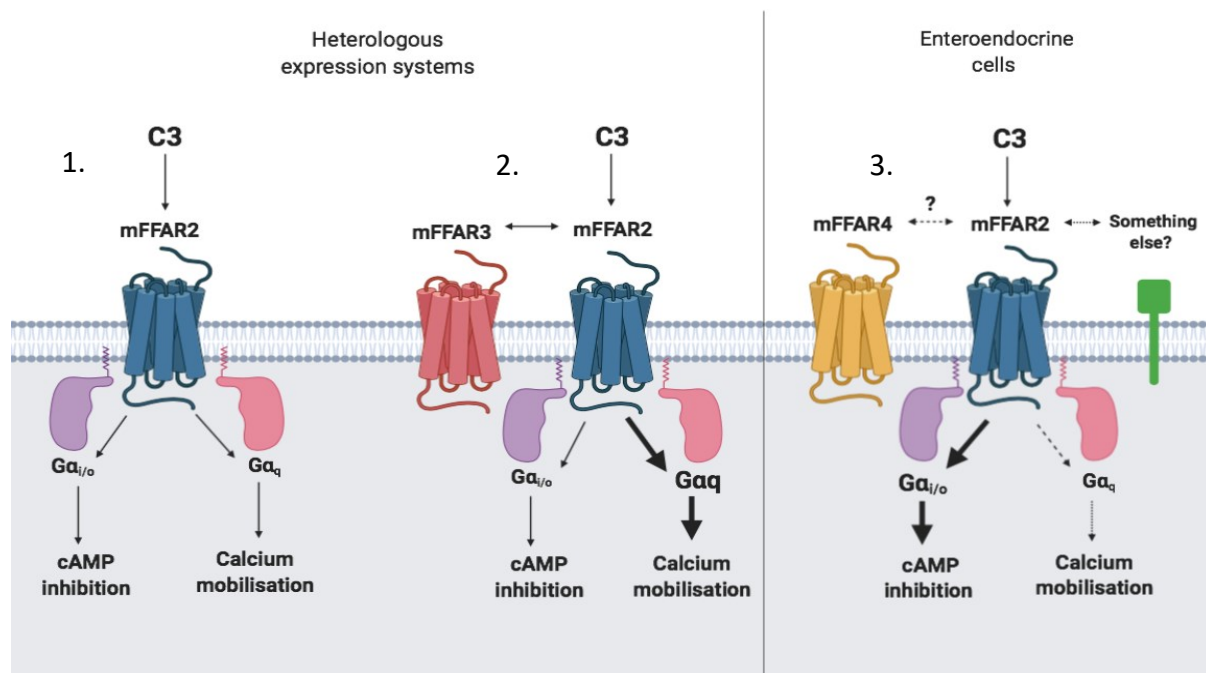


Figure 4.6 Schematic diagram of the proposed model of FFAR2 crosstalk 1. Mouse free fatty acid receptor 2 (FFAR2) expressed in isolation in heterologous systems can couple to $G\alpha_{i/o}$ and $G\alpha_q$ when stimulated with propionate (C3), inducing cAMP inhibition and calcium mobilisation respectively. 2. When mouse free fatty acid receptor 3 (FFAR3) is co-expressed with FFAR2 in heterologous systems, the calcium mobilisation in response to C3 is enhanced, suggesting a potential crosstalk between the two receptors. 3. In enteroendocrine cells (EECs), FFAR2 is seemingly unable to couple to $G\alpha_q$ in response to C3 in some intracellular environments. A key candidate receptor for crosstalk is mouse free fatty acid receptor 4 (FFAR4), which is expressed in EECs and is able to couple to both signaling pathways. Alternatively, the reluctance to couple to $G\alpha_q$ may be due to other interactions with proteins or combinations of proteins expressed uniquely in the EEC signalsome environment, or via a lack of $G\alpha_q$ G protein expression. **Image created with BioRender.**

Chapter 5: The role of SCFAs on nutrient-stimulated GPCR signal sensitivity in enteroendocrine cells

5.1 Introduction

Nutrients have been recognised as one of the most influential external factors governing epigenetic profiles, in a way that is conserved from single-celled organisms to virtually every organ of the body. The epithelial lining of the intestinal tract is particularly susceptible to diet-induced fluctuations in the extracellular and intracellular molecular array, due to the proximity to the gut lumen (Sanderson et al., 2004). Diet-induced changes in epigenetic profiles of the GI tract have been extensively reported and, amongst the many bioactive metabolites able to sculpt the epigenome, SCFAs have been suggested to play a fundamental role. In vivo studies have demonstrated that a high-fibre diet, yielding high concentrations of SCFAs in the intestine, can alter genetic profiles of the gut in genes involved in gut hormone release, such as preproglucagon; genes involved in nutrient trafficking such as GLUT-4 (Tappenden et al., 1998) and monocarboxylate transporter 1 (Haenen et al., 2013); genes involved in nutrient metabolism such as vitamin A processing enzymes (Goverse et al., 2017); and genes governing mucosal integrity maintenance such as mucin-3 (Paturi et al., 2012). There seem to be two fundamental mechanisms underlying SCFA-induced epigenetic fluctuations; firstly, FFAR2/3 mediated GPCR-signalling and, secondly, via the inhibition of histone deacetylases (HDACs).

SCFA-induced gene expression changes may occur as a direct result of the activation of FFAR2/FFAR3 signalling. GPCR signalling cascades have a large number of potential effector proteins, some of which have well-documented effects on gene transcription. For example, cAMP activates PKA, a kinase which can phosphorylate and regulate activity of cAMP response element binding protein (CREB), a transcriptional factor able to modulate the activity of several vital genes (Delghandi et al., 2005). Isoforms of PKC are also promiscuous effector kinases, activated by calcium signals and membrane DAG, with an array of downstream intracellular phosphorylation targets. The activation of some of these is able to modulate engagement of gene cis-regulatory elements (Tang et al., 2005) or suppress promoter activity (Yokoyama et al., 2013). There is some evidence that FFAR2 signalling contributes, albeit minorly, to moderating the expression of gut hormones genes in EECs in response to SCFAs. In human in vitro EEC cell lines, SCFAs stimulate *pyy* gene transcription in a manner which is partly dependent upon FFAR2 signalling. It seems as though the more modest increases in gene expression caused by acetate, as opposed to propionate or butyrate, are mediated via a FFAR2 PKC-dependent pathway, as this effect was abolished in FFAR2 KOs and mirrored by pharmacologically activating PKC. The small but significant increase in *pyy* expression caused by tiglic acid, an hFFAR2 agonist, was also curtailed by this genetic modification (Larraufie et al., 2018).

SCFAs are also known to inhibit HDACs. This influences the epigenome via altering the patterns of epigenetic marks; covalent tags that are added to the DNA structure or to the histone proteins. These reconstruct the three-dimensional architecture of the chromatin, manipulating the accessibility of the DNA and, thus, its susceptibility to undergo several processes, including transcription. Environmental factors such as nutrients, chemicals, toxins and pollutants can greatly alter the epigenome, which can impact physiology in ways that can contribute to disease pathogenesis (Zhang et al., 2018). The diversity of potential nutrient exposure induces a great complexity in the mechanisms by which dietary compounds alter epigenetic profiles, but the activation or inhibition of enzymes which catalyse the addition or removal of covalent tags is thought of as a primary mechanism. Acetyl or methyl groups are the predominant form of covalent tags. Where acetylation acts to enhance gene expression, methylation effectively switches the gene off. Acetylation occurs on lysine residues of histone proteins, masking the positive charges which would otherwise associate strongly with the negatively charged DNA backbone. Therefore, the chromatin becomes less tightly compacted. This manipulated architecture of the chromatin increases the accessibility of the DNA strands to multiple transcriptional factors and DNA binding

proteins key for the induction of transcription. Acetylation patterns fluctuate dynamically via the action of several enzymes, such as HDACs, which remove acetyl groups from histone residues (Delage et al., 2008). Butyrate is a strong inhibitor of HDACs, rebalancing the histone acetylation status and subsequently altering chromatin structure at concentrations ≤ 1 mM. Propionate has also been shown to inhibit HDACs at concentrations ≥ 10 mM but acetate does not have this ability (Waldecker et al., 2008). By doing so, butyrate can influence the expression of a magnitude of genes controlling diverse cellular processes, such as cell proliferation, cell differentiation, cell-to-cell adhesion, inflammation and chemokine release (Sanderson et al., 2004).

Gene expression differences are often observed in obesity (Ferriera et al., 2018) but whether this is a cause or consequence of obesity pathophysiology remains to be clarified due to the indiscriminate nature of the contributing factors such as metabolic status, gut microbiota composition and diet constitution. However, there does seem to be a fundamental link between SCFA-producing bacteria and the prevalence of epigenetic marks, including those in the promoter regions of genes encoding key nutrient sensing GPCRs. For example, as well as possessing significantly less diverse microbiomes and fewer SCFA-producing bacterial strains, the analysis of five FFAR3 promoter regions from blood samples demonstrated significant hypo-methylation in T2DM and obese patients compared to lean controls. Interestingly, with nutritional counselling and dietary changes, the methylation status was somewhat restored (Remely et al., 2014). The hypo-methylation observed leads to speculation that these individuals also have elevated expression of FFAR3 and altered SCFA signalling profiles. Although this study only looked at blood samples and methylation status, other studies have demonstrated links between GPCR RNA transcripts and proteins throughout the GI tract with BMI. For example, the qPCR and immunohistochemistry analysis of gastric tissue extracted from morbidly obese patients not only demonstrated significant gene expression changes in genes encoding GPCRs, such as TAS1R3 and FFAR4, but also of gustatory signalling elements and effectors, including G protein α -gustducin, effector enzyme PLC- β 2 and calcium channel TRPM5, compared to the profiles of tissue obtained from lean volunteers (Widmayer et al., 2012). The elevated level of FFAR4 observed in obese humans has also been mirrored by studies in lean and obese mice, in which, it was possible to identify the increased FFAR4 expression in EECs specifically (Widmayer et al., 2015). Further studies have demonstrated obese mice have altered colonic mRNA expression of FFAR1, FFAR2, FFAR3, FFAR4, and TAS1R3 but, each receptor subunit underwent divergent adaptive changes after gastric bypass surgery with some, such as FFAR2, being upregulated and others, such as TAS1R3, undergoing a downregulation (Peiris et al., 2018). Clearly, the expression of nutrient sensing pathway receptors and signalling components is incredibly sensitive and plastic, able to adapt dynamically to environmental factors in a diverse and complex manner.

5.2 Objective and Aims

The integration of two concepts, firstly, that the SCFAs are able to alter epigenetic profiles of a diverse range of genes and secondly, that the expression of nutrient sensing receptors and signalling proteins is not static but subject to manipulation by the environment, has led to the hypothesis that SCFAs can directly influence the expression profiles of nutrient sensing GPCRs in EECs. Thus, SCFAs may have a direct impact on the sensitivity of the EECs to nutrient-activated signalling pathways and subsequent effects on gut hormone secretion. I aimed to address this by:

- Verifying the nutrient sensing GPCR expression profile in STC-1 cells
- Analysing the impact of SCFAs on the expression of nutrient sensing GPCRs, including gustatory GPCRs and signaling components

- Dissecting the role of each SCFA; acetate, propionate and butyrate; in the expression profile of GPCRs and whether FFAR2/FFAR3 agonists can also induce the observed changes
- Pharmacologically inhibiting the FFAR2/3 $G\alpha_{i/o}$ signalling pathway to clarify its role on gene expression changes
- Evaluating whether SCFA induced upregulation of GPCRs induces a hypersensitivity to the ligands of that GPCR

5.3 Results: Treatment with SCFAs can change gene expression of specific GPCRs in STC-1 cells

5.3.1 Confirmation of the free fatty acid and taste GPCRs of STC-1 cells

The STC-1 cell line originates from a murine enteroendocrine tumour, retaining the ability to secrete several gut hormones in response to physiological stimuli such as nutrients and ligands, although different subpopulations of STC-1s have been reported to have significantly differing secretion profiles (McCarthy et al., 2015). All experiments with STC-1 cells described in Chapter 5 are STC-1a cells. To validate the expression of EEC markers in these STC-1 cells, the expression of PYY, CCK and GCG (preproglucagon) were confirmed via qPCR. Additional qPCRs were performed to elucidate which nutrient sensing GPCRs were expressed in STC-1s and to quantify the relative expression level, examining the FFAR1, FFAR2, FFAR3, FFAR4, TAS1R1, TAS1R2, TAS1R3 and TAS2Rs transcripts (**Figure 5.1**). The GPCR expression profile helped validate STC-1s as a model of EEC GPCR signalling as the results confirmed the presence of all the GPCR transcripts, albeit in differing amounts.

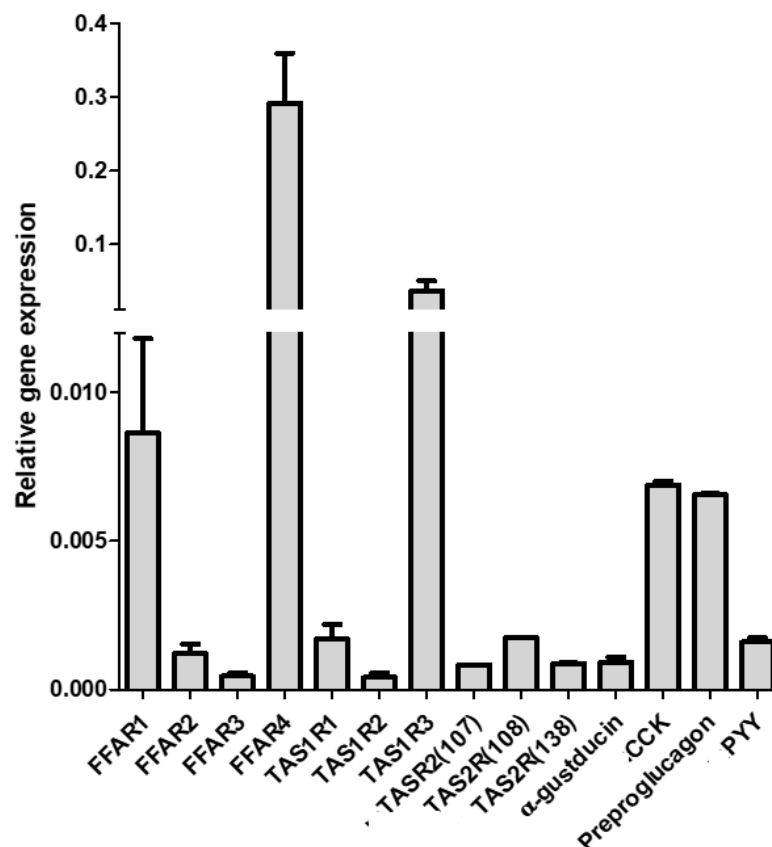


Figure 5.1 The expression profile of transcripts involved in GPCR-mediated nutrient sensing and gut peptide secretion in STC-1 cells STC-1 cells grown to confluency were harvested, and qPCR was carried out to assess the levels of EEC markers, FFAR1-4, TAS1Rs, TAS2Rs, and taste signalling proteins mRNA. Expression levels were normalised to the levels of housekeeping gene β -actin. Data is shown as mean $\pm$ SEM, $n=3$. Free fatty acid receptor (FFAR), Taste receptor family 1 (TAS1R), Taste receptor family 2 (TAS2R), cholecystikinin (CCK), peptide tyrosine tyrosine (PYY)

5.3.2 SCFAs can upregulate the taste receptor encoding gene transcripts

To test the hypothesis that SCFAs can influence the expression of nutrient sensing taste GPCRs, STC-1 cells were incubated for 2 hours with SCFAs in a 3:1:1 ratio of acetate: propionate: butyrate, as found endogenously in the proximal and distal colon (Hijova et al., 2007), at a concentration of either 5 mM or 10 mM. qPCR was used to analyse the relative changes in expression changes of the transcripts of TAS1R1, TAS1R2, TAS1R3, TAS2(108) and TAS2(138). Incubation with 10 mM SCFAs significantly upregulated all taste receptor mRNA transcripts ($p < 0.001$ compared to the NaCl control), whereas incubation at 5 mM only significantly upregulated TAS1R1 and TAS1R2 (**Figure 5.2**). TAS1Rs were more robustly upregulated by SCFAs than members of the bitter taste family. The maximal change in relative gene expression after SCFA (10 mM) incubation was observed for the TAS1R1 transcript, with a 6.2-fold increase in transcript levels compared to control, followed by TAS1R2 (3.8-fold change) and TAS1R3 (2.2-fold change). TAS2Rs were not significantly upregulated by SCFAs.

To further analyse the time scales of the upregulation of the TAS1R subunits, a time-point analysis was performed. In these experiments, STC-1 cells were incubated with 10 mM SCFAs or 10 mM NaCl for a duration of 1 to 5 hours, after which the RNA was then extracted, and qPCR was used to analyse relative gene expression changes (**Figure 5.3 A-C**). This experiment demonstrated that TAS1R1 was the only transcript significantly upregulated by SCFAs over the time course. Although

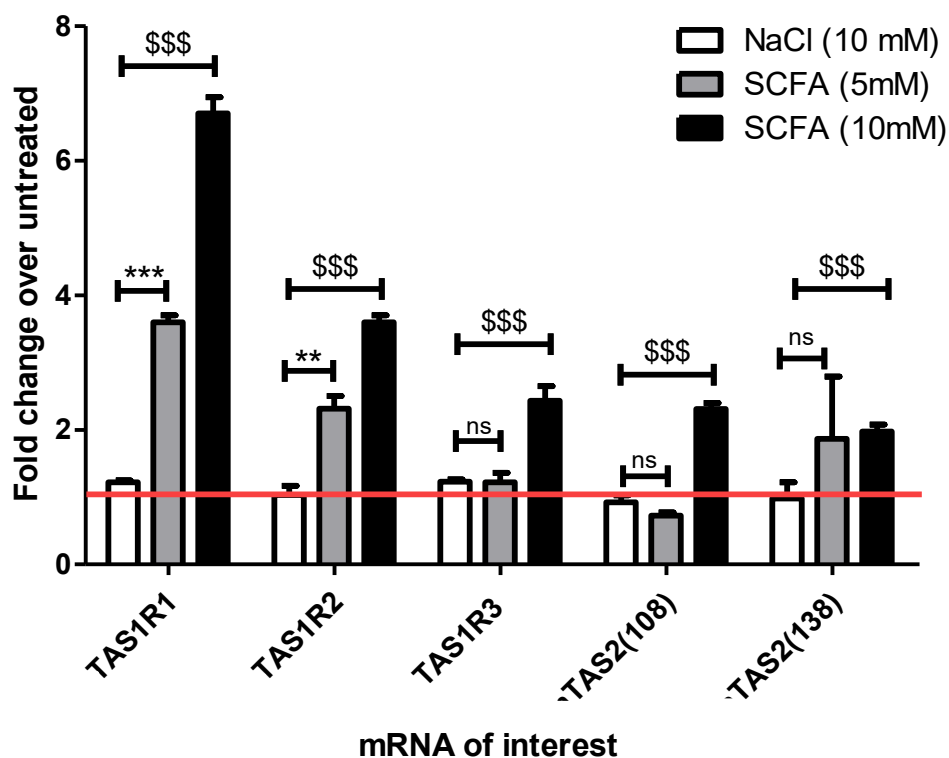


Figure 5.2 Exposure to SCFAs can influence the expression profile of taste receptor transcripts in STC-1 cells STC-1 cells were grown to 80% confluency and incubated with NaCl or SCFAs (5 or 10 mM) in DMEM -/- media for 2 hours at 37 °C. After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1, TAS1R3, TAS1R2, TAS2(108) and TAS2(138). Expression was normalised to the levels of housekeeping gene β -actin and normalised to the untreated control. Data is expressed as fold change in expression over the untreated control and represents the average $\pm$ SEM, $n=3$. The red line indicates a fold change of 1 where there has been no change in expression. Two-way ANOVA, with Bonferroni post hoc, \$\$\$ $p < 0.001$ SCFA (10 mM) vs NaCl control, ** $p < 0.01$, *** $p < 0.005$ SCFA (5 mM) vs NaCl control. Taste receptor family 1 (TAS1R), Taste receptor family 2 (TAS2R)

the trend in upregulation could be observed after a 1-hour incubation with SCFAs, this was potentiated with longer durations of incubation, becoming significant at 2-, 3- and 5-hour time points, where the upregulation reached a peak of 7-fold (**Figure 5.2A**). It was interesting to note that no significant upregulation of TAS1R2 was observed with these slightly different experimental parameters (**Figure 5.2B**), but as this transcript has very low expression levels (**Figure 5.1**), this could be an artefact of the normalisation process. The trend in receptor upregulation for TAS1R3, although not significant, could still be observed, but the variant nature of this upregulation minimised its significance (**Figure 5.2C**).

5.3.3 Incubation with 10 mM SCFAs did not significantly change the expression of FFARs at any time point

As performed previously, STC-1 cells were incubated with 10 mM SCFAs for a duration of 1-5 hours. qPCR to analyse the levels of mRNA transcripts of FFAR1, FFAR2, FFAR3 and FFAR4 over these time points was carried out. This demonstrated that SCFA treatment did not significantly up or downregulate FFAR family transcript levels compared to the NaCl control (**Figure 5.3 D-G**).

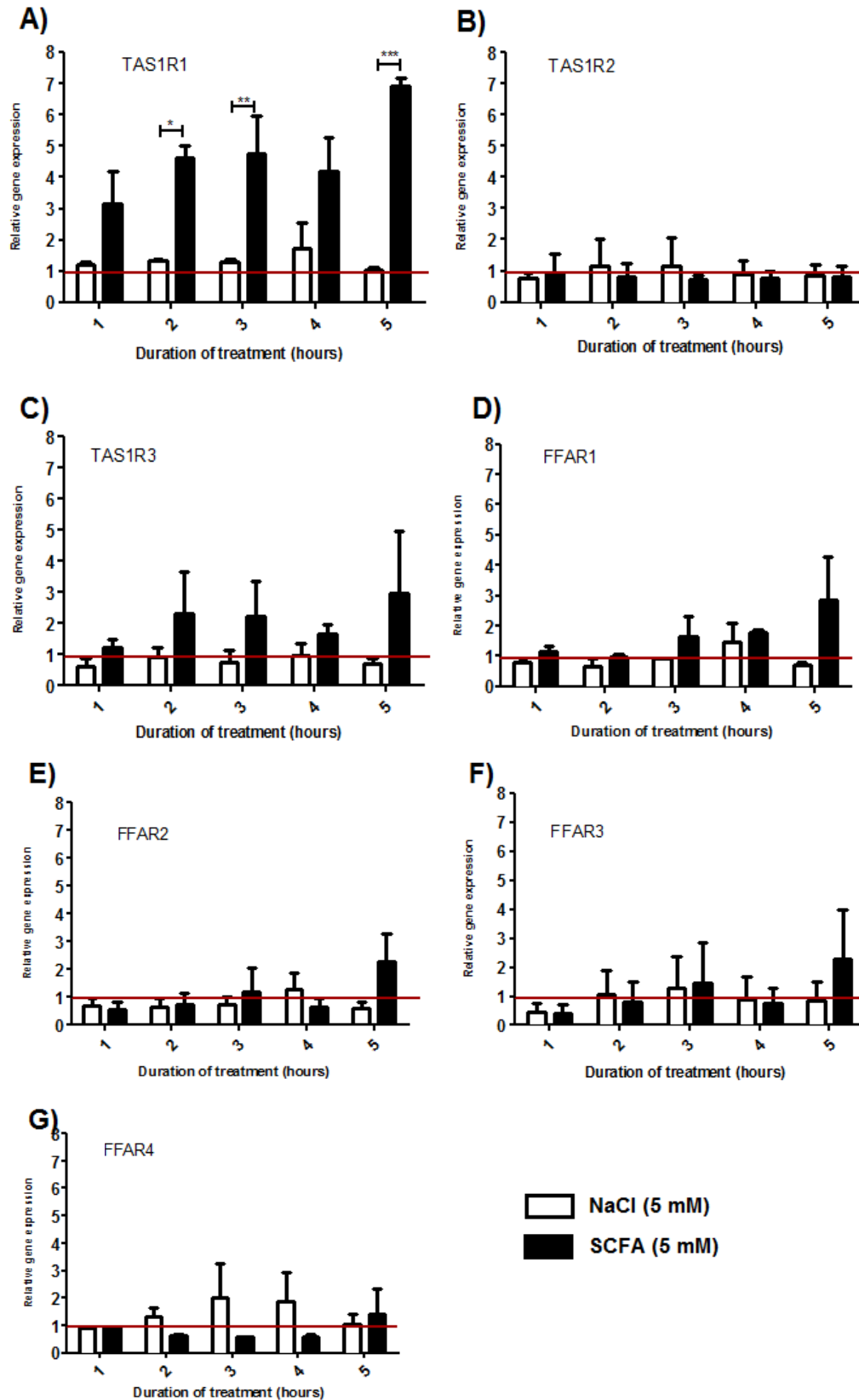


Figure 5.3 Time-course analysis of gene expression changes in nutrient sensing GPCRs after SCFA incubation
 STC-1 cells were grown to 80% confluency and incubated with 10 mM NaCl (white bars) or SCFAs (black bars) in DMEM $-/-$ media for 1-5 hours at 37 °C. After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1 (A), TAS1R2 (B), TAS1R3 (C), FFAR1 (D), FFAR2 (E), FFAR3 (F) and FFAR4 (G). Expression was normalised to the levels of housekeeping gene β -actin and normalised to the untreated control. Data is expressed as fold change in expression over the untreated control (the red line shows no fold change) and represents the mean $\pm$ SEM, from data collected in 3 distinct experiments. ANOVA with Bonferroni post-hoc, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Free fatty acid receptor (FFAR), Taste receptor family 1 (TAS1R)

5.3.4 Acetate, propionate and butyrate differ in their abilities to moderate umami taste receptor gene expression

As TAS1R1 and TAS1R3 were the most robustly upregulated and these subunits dimerise to form the functional umami taste receptor (Nelson et al., 2002), these were the transcripts which were chosen to be studied for the remainder of the chapter. Further experiments aimed to identify which SCFAs were responsible for the observed changes and the underlying mechanism. Results showed that

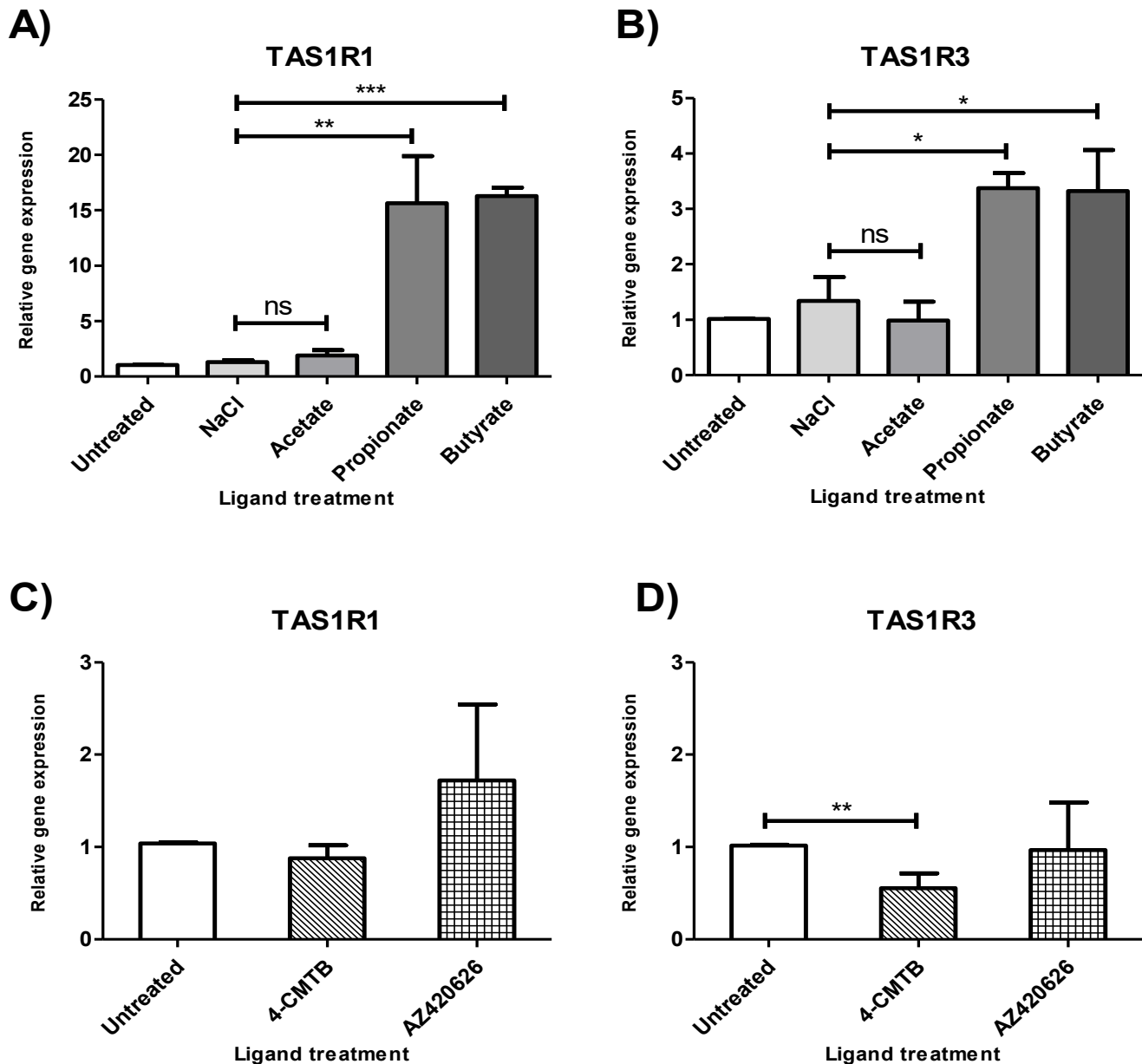


Figure 5.4 SCFAs and FFAR2/3 agonists influence gene expression of TAS1R1 and TAS1R3 differentially in STC-1 cells A and B) STC-1 cells were grown to 80% confluency and incubated with 10 mM NaCl or SCFAs in DMEM $\pm$ media for 5 hours at 37 °C. After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1 (A), TAS1R3 (B). Expression was normalised to the levels of housekeeping gene β -actin is expressed as fold change in expression over the untreated control C and D) STC-1 cells were grown to 80% confluency and incubated with 10 μ M of 4-CMTB or AZ420626 in DMEM $\pm$ media for 5 hours at 37 °C. After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1 (C), TAS1R3 (D). Expression was normalised to the levels of housekeeping gene β -actin is expressed as fold change in expression over the untreated control. All data represents the mean $\pm$ SEM from 3 distinct experiments. T-tests vs. control, * p <0.05, ** p <0.01, *** p <0.001. Taste receptor family 1 (TAS1R)

incubation with 10 mM propionate or butyrate for 5 hours was sufficient to induce the observed gene expression changes, showing that TAS1R1 was significantly upregulated by 18.4-fold ($p=0.0186$) and 16.5-fold ($p=0.0001$) for 10 mM of propionate and butyrate, respectively (**Figure 5.4A**). TAS1R3 was more modestly, but still significantly, upregulated 3.4-fold by 10 mM propionate ($p=0.001$) and 3.3-fold by butyrate ($p=0.04$; **Figure 5.4B**). Acetate, however, did not seem to be able to induce the gene expression changes. Although a modest 2-fold increase in TAS1R1 transcript was noted, it was non-significant (**Figure 5.4A**).

5.3.5 FFAR2- and FFAR3-specific agonists display distinct gene modulatory activities from SCFAs

To further examine whether direct activation of FFAR2 or FFAR3 could cause gene expression changes via GPCR signalling pathways, FFAR2 specific agonist, 4-CMTB, and FFAR3 specific agonist, AZ42062, were utilised. STC-1 cells were incubated in media supplemented with each agonist (10 μ M) for 5 hours, after which a qPCR was performed to quantify transcript levels of TAS1R1 and TAS1R3. Neither agonist was able to elicit any change in the expression of TAS1R1 (**Figure 5.4C**). 4-CMTB, however, effectively reduced TAS1R3 levels by approximately 50%. This demonstrates, again, that the synthetic ligands act in a significantly distinct manner from the endogenous ligands.

5.3.6 Pertussis toxin treatment of STC-1 cells is able to block the relative increase in umami taste receptor transcripts induced by SCFAs

SCFAs have been reported to alter gene expression both by HDAC inhibition and, more modestly, by FFAR2 signalling (Larraufie et al., 2018). We have observed that STC-1a cells exhibit SCFA-mediated $G\alpha_{i/o}$ signalling. To examine the contributions of $G\alpha_{i/o}$ signalling to gene expression changes, STC-1 cells were incubated with PTX, which, by inhibiting the $G\alpha_{i/o}$ subunit, would inhibit FFAR2 and FFAR3 mediated SCFA signalling pathways. After an 18 hour incubation with PTX, either NaCl (control), propionate or butyrate (10 mM) was applied to the STC-1 cells. A qPCR was performed to analyse the impacts of PTX pre-treatment on the SCFA-induced upregulation of umami taste receptor transcripts. Interestingly, treatment with PTX alone, without any SCFA addition, significantly upregulated TAS1R1 by 4.2-fold ($p=0.03$; **Figure 5.5A**), a trend also observed by a 2.6-fold in TAS1R3 upregulation (**Figure 5.5B**). However, when looking at the changes in umami taste receptor expression induced by propionate and butyrate relative to the effects of the NaCl control, the PTX pre-treated STC-1 cells demonstrated significant reductions in the relative upregulation of both TAS1R1 and TAS1R3 transcripts. For TAS1R1, propionate-induced upregulation fell significantly from 18.4-fold to 4.6-fold. Similarly, the butyrate induced upregulation reduced significantly from 16.5-fold to 6.3-fold (**Figure 5.5C**). The TAS1R3 transcript demonstrated no upregulation at all when STC-1 cells had been PTX pre-treated, with a significant reduction in the 3.4-fold propionate mediated upregulation to 1-fold (no change over control). Butyrate, similarly, did not induce any gene expression changes when cells were treated with PTX, with a 1.2-fold increase over control (**Figure 5.5D**). It remains to be determined whether the effects of PTX on gene expression changes are due to direct blockade of the contribution of FFAR2/3 mediated $G\alpha_{i/o}$ signalling or due to a more general inhibition of the $G\alpha_{i/o}$ downstream of other receptors.

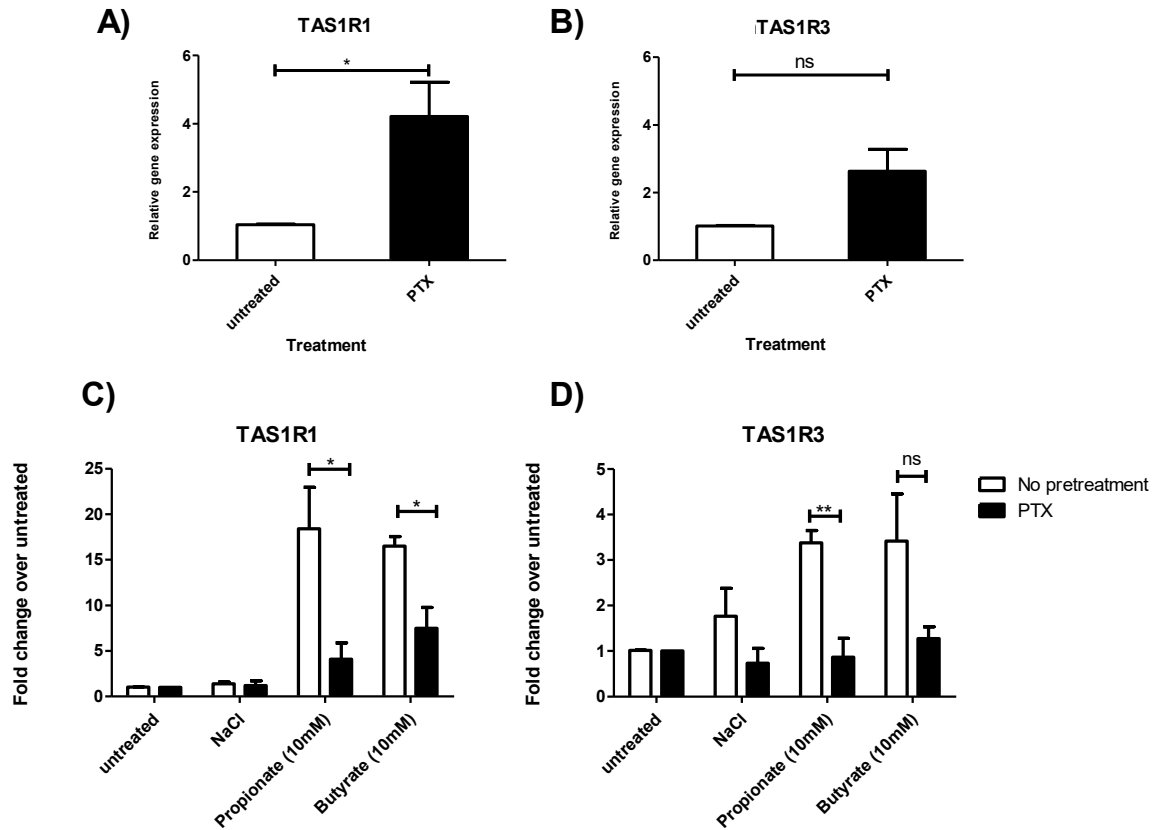


Figure 5.5 The use of pertussis toxin to block $G\alpha_{i/o}$ signalling has significant effects on SCFA-mediated changes in taste receptor gene expression in STC-1 cells A and B) STC-1 cells underwent a PTX pre-treatment (200 ng/ μ L, 18 hours). After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1 (A), TAS1R3 (B) transcripts. Expression was normalised to the levels of housekeeping gene β -actin is expressed as fold change in expression over the untreated control. All data represents the mean $\pm$ SEM from 3 distinct experiments. T-tests vs. control, * p <0.05, ** p <0.01, *** p <0.001. C and D) After a PTX pre-treatment (200 ng/ μ L, 18 hours) or no pre-treatment, STC-1 cells were grown to 80% confluency and incubated with 10 mM of NaCl, propionate or butyrate in DMEM $-/-$ media for 5 hours at 37 °C. After incubation, cells were washed with ice-cold PBS and an RNA extraction was performed to be used for qPCR analysis of taste receptors TAS1R1 (C), TAS1R3 (D). Expression was normalised to the levels of housekeeping gene β -actin is expressed as fold change in expression over its own NaCl-treated control. Two-way ANOVA, Bonferroni post hoc of no pre-treatment vs. PTX treatment for each ligand * p <0.05, ** p <0.01, *** p <0.001, non-significant (ns)

5.4 Results: STC-1 cells respond to umami taste ligands

Despite the evidence that SCFAs can upregulate the expression of the umami taste receptor genes, increased mRNA concentrations do not necessarily correlate in a linear fashion with protein translation. Therefore, the aim was to quantify whether there was a link between upregulation of gene expression and an enhanced functional umami taste response. The lack of commercially available selective umami receptor ligands necessitated that L-MSG, the classical umami tastant, was utilised. It has been reported that the human, but not rodent, umami receptor is much more selective for L-MSG over other L-AAs. Therefore, L-Ala was also selected, as this had been reported to elicit the strongest calcium responses from the mouse umami receptor (Toda et al., 2013).

5.4.1 STC-1 cells express taste receptor specific G protein, α -gustducin

The literature concerning the expression patterns of signalling components throughout the GI tract suggests that it is possible the umami taste receptor couples to α -gustducin in EECs (Sternini et al., 2010). qPCR results confirmed that α -gustducin was expressed in STC-1 cells, with transcript concentrations averaging 0.001-fold of the β -actin house-keeping gene, roughly equating to the basal TAS1R1 mRNA concentrations (**Figure 5.1**). In gustatory cells the umami taste receptor induces calcium signalling via its $G\beta_3\gamma_{13}$ subunit interacting with PLC- β_2 , whereas the α -subunit reduces intracellular cAMP levels via activating phosphodiesterases (Kinnamon et al., 2009). However, it is possible that taste receptors couple to α -gustducin independent pathways in STC-1 cells, including $G\alpha_q$, $G\alpha_s$ and $G\alpha_{i/o}$. Thus, the aim was to analyse the effects of stimulating STC-1 cells with umami ligands on intracellular concentrations of second messengers, calcium and cAMP.

5.4.2 IMP synergises the IP_1 accumulation caused by L-MSG and L-Alanine in STC-1 cells

The literature suggests that the human and mouse umami taste receptor couples to calcium signalling in heterologous systems (Nelson et al., 2002; Toda et al., 2013) but there have been no reports as to whether this remains the case when expressed at endogenous levels in STC-1 cells. To test this, an IP_1 assay was performed in STC-1 cells in response to a 30-minute exposure to L-MSG or L-Ala (10 mM) in the presence of LiCl (50 mM). There was no significant difference in the concentration of IP_1 when STC-1 cells were treated with the L-AAs alone. However, the addition of IMP (2 mM), although not affecting the basal levels of IP_1 when used in isolation, significantly increased the IP_1 accumulation in STC-1 cells when used concurrently with either umami taste ligand. IMP with L-Ala or L-MSG induced a 4.8% or 10.8% increase respectively over the untreated basal IP_1 levels (**Figure 5.6A**).

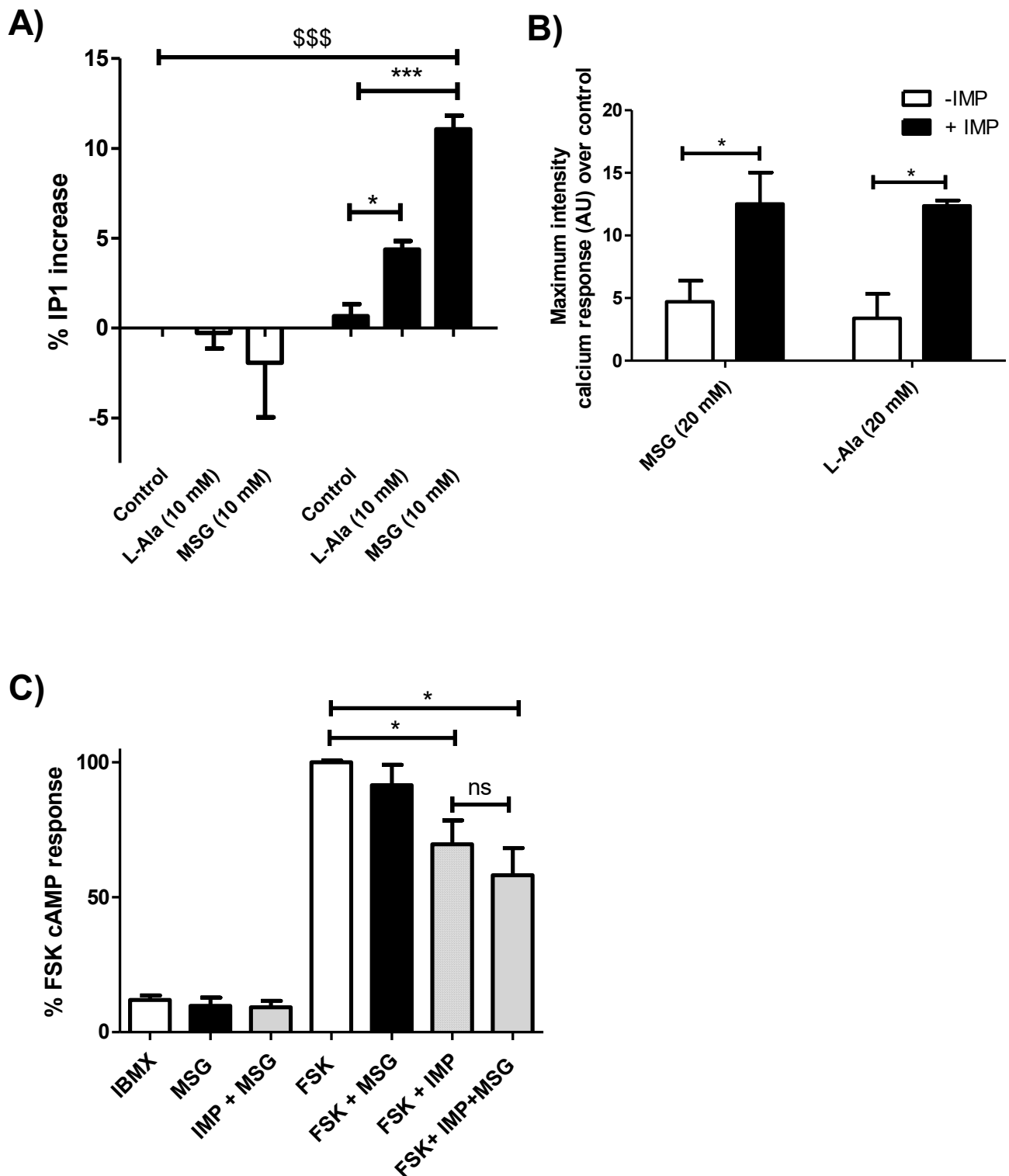


Figure 5.6 Umami taste ligand GPCR effector signalling assays in STC-1 cells A) Intracellular accumulation of IP₁ measured in STC-1 cells pre-treated with IMP (black bars; 2 mM) or NaCl (white bars; 2 mM) for 5 minutes prior to treatment with either L-Ala (10 mM), L-MSG (10 mM) or NaCl (10 mM; control) for 30 minutes. Data is expressed as mean $\pm$ SEM from 3 distinct experiments. Two-way ANOVA, Bonferroni post hoc, \$\$\$ p <0.001 IMP vs no IMP, * p <0.05, ** p <0.01, *** p <0.001 control vs. L-Ala/ L-MSG treatment. B) Intracellular calcium mobilisation measured in STC-1 cells, incubated with fluorescence calcium indicator Fluo4-AM for 1 hour and imaged live via confocal microscopy for 1 minute before the addition of IMP or NaCl (2 mM) and then for a further minute before the addition of L-Ala (20 mM) or L-MSG (20 mM). Data is expressed as mean $\pm$ SEM maximal fluorescence intensities over the control and is taken from 20 cells run in duplicate in 3 separate experiments. Two-way ANOVA, Bonferroni post hoc * p <0.05, IMP vs no IMP C) STC-1 cells were treated with IBMX (5 minutes, 0.5mM) and then treated with L-MSG (20 mM) and/or IMP (2 mM) with/without forskolin (FSK, 3 μ M), for 5 minutes. The maximal forskolin-induced cAMP accumulation in the absence of ligand was defined as 100% and data is expressed as percentage change of FSK stimulated control. All data represents mean $\pm$ SEM, collected over 3 distinct experiments. One-way ANOVA with Dunnett's post hoc, * p <0.05

5.4.3 IMP synergises the calcium mobilisation caused by L-MSG and L-Alanine in STC-1 cells

To visualise the calcium responses to umami ligands, STC-1 cells were incubated with Fluo-4AM calcium dye. On addition of umami ligands (20 mM), waves of calcium could be detected which seemingly occurred with homogeneity throughout the entire STC-1 cell population. Statistical analysis of the maximal fluorescence intensity demonstrated the addition of L-MSG and L-Ala to STC-1 cells induced an approximate 5-fold increase in maximal fluorescent intensity (AU) over the NaCl control. Addition of IMP (2 mM) did not induce calcium mobilisation alone (**Figure 5.7C**), but significantly enhanced the calcium response to both L-MSG (increasing from 5- to 12-fold over NaCl control) and L-Ala (increasing from 4- to 11- fold over NaCl control; **Figure 5.6B**). The enhancement of the umami receptor mediated signalling by IMP provides evidence that the calcium signals are being mediated by modulation of taste receptor subunits, as opposed to other L-AA sensitive receptors expressed in STC-1 cells.

5.4.4 L-MSG signalling via the umami taste receptor has no significant impact on cAMP accumulation in STC-1 cells

It is also possible umami taste receptors can couple to $G\alpha_{i/o}$ or $G\alpha_s$ proteins, or otherwise act to modulate intracellular cAMP levels. To evaluate this in STC-1 cells, intracellular levels of cAMP was measured following 5-minute exposure to L-MSG (20 mM) and/or IMP (2 mM). Neither L-MSG alone, or in combination with IMP, was able to significantly increase intracellular cAMP concentrations, suggesting that $G\alpha_s$ pathways are not involved in umami taste receptor signalling (**Figure 5.6C**). This experiment was then performed in the presence of FSK (3 μ M), to assess $G\alpha_{i/o}$ signalling. Interestingly, a significant (approximately 40%) decrease in FSK-stimulated cAMP was observed when using L-MSG with IMP, but not when using L-MSG alone. Further analysis, however, demonstrated that IMP could significantly inhibit cAMP accumulation independently of L-MSG (**Figure 5.6C**). IMP can degrade spontaneously to inosine, a compound with the ability to mediate other GPCR isoforms, such as the ubiquitously expressed adenosine receptor family (Welihinda et al., 2016), so it is possible the observed inhibition of cAMP accumulation may be due to activation of adenosine receptor dependent pathways instead.

5.4.5 Butyrate pre-treatment enhances the calcium mobilisation in response to L-Ala

As butyrate had been shown to significantly upregulate transcript levels of both the umami receptor subunits (**Figure 5.4**), it was hypothesised that pre-treatment with butyrate may enhance the signalling from the umami receptor if the increases in transcript correlated with an increased surface expression of the protein. Therefore, STC-1 cells were incubated overnight in media supplemented with either 5 mM butyrate or 5 mM NaCl (control) and a Fluo4-AM calcium mobilisation assay was performed to compare the calcium responses to L-MSG and L-Ala (20 mM), in the presence of IMP (2 mM). Results indicated that overnight butyrate exposure did slightly enhance the calcium response to umami ligands. There was a significant increase in the maximal fluorescent intensity of the L-Ala induced calcium response from 11- to 26-fold over the control. Plotting the fluorescent intensity over time demonstrated that butyrate pre-treated cells elicited a steeper gradient and higher peak of fluorescence on addition of L-Ala (**Figure 5.7C**). This trend was also observed for L-MSG, in which the calcium response increased non-significantly from 11- to 23-fold (**Figure 5.7A**). The representative fluorescence plot over time shows a slight enhancement in fluorescence in butyrate pre-treated cells, although the difference is somewhat less apparent than that observed for L-Ala (**Figure 5.7D**).

5.4.6 Butyrate pre-treatment enhances increases intracellular levels of IP₁

To analyse if the observed enhancement in calcium signals was also reflected in intracellular IP₁ concentrations, STC-1 cells were incubated overnight with 5 mM butyrate or NaCl (control). They were then exposed to IMP (2 mM) with L-MSG or L-Ala and an IP₁ accumulation assay was

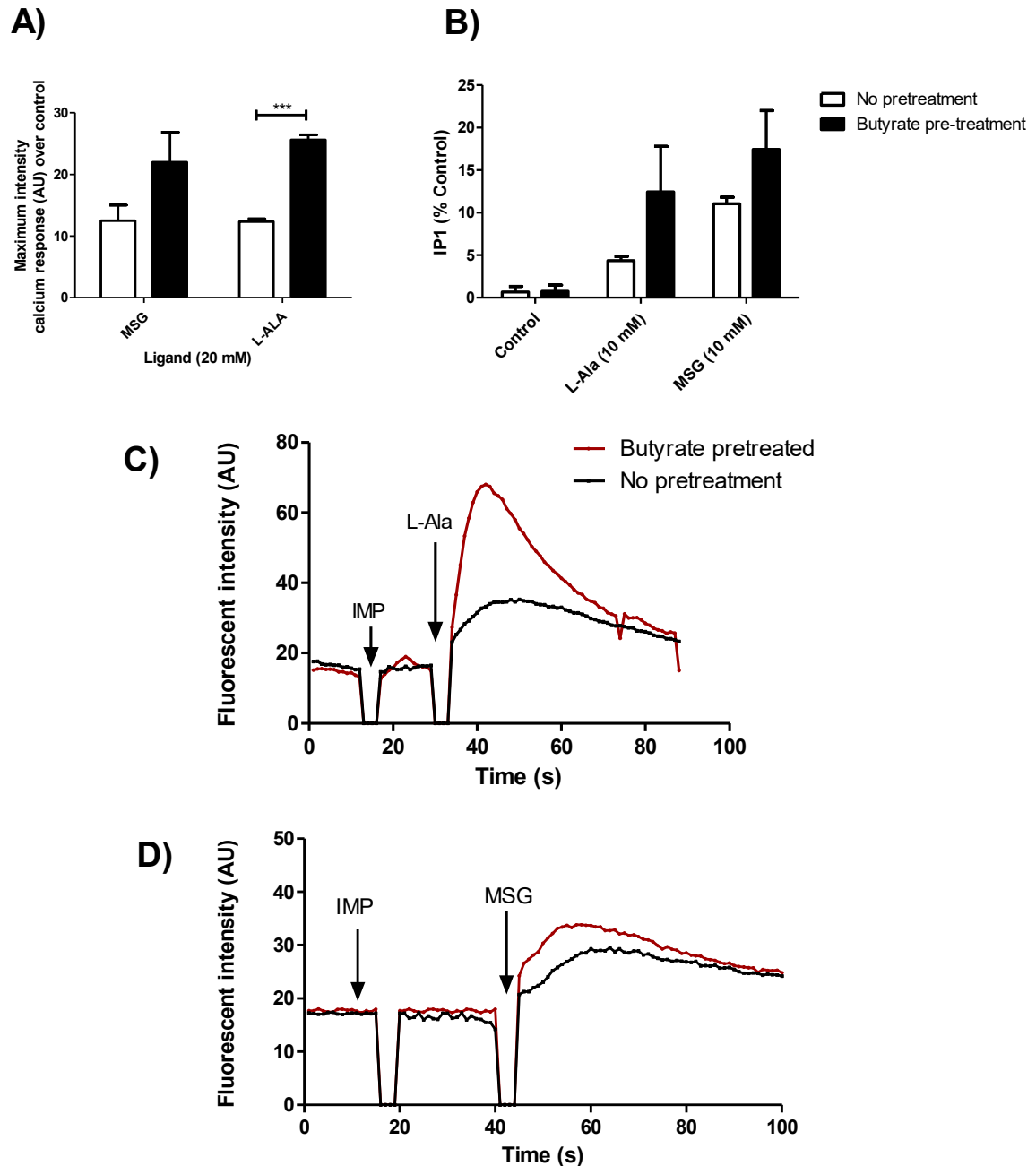


Figure 5.7 Butyrate pre-treatment enhances STC-1 calcium response to L-Alanine but not L-MSG STC-1 cells were pre-treated with 2 mM butyrate (black bars) or NaCl (white bars) for 12 hours. A) Intracellular calcium mobilisation measured was then measured in butyrate pre-treated/ not pre-treated STC-1 cells which were incubated with fluorescence calcium indicator Fluo4-AM for 1 hour and imaged live via confocal microscopy for 1 minute before the addition of IMP (2 mM) and then for a further minute before the addition of L-Ala (20 mM) or L-MSG (20 mM). Data is expressed as mean $\pm$ SEM maximal fluorescence intensities over the control and is taken from 20 cells run in duplicate in 3 separate experiments. Two-way ANOVA, Bonferroni post hoc, ***p < 0.001 butyrate vs. NaCl pre-treatment. B) Intracellular accumulation of IP-1 was measured in butyrate/NaCl pre-treated STC-1 cells after incubation with IMP (2 mM) and either L-Ala (10 mM), L-MSG (10 mM) or NaCl (10 mM; control) for 30 minutes. Two-way ANOVA showed no significant difference between butyrate and NaCl-pre-treated groups. C and D) show representative fluorescence intensity plots following IMP (2 mM) and L-Ala (C; 20 mM) or L-MSG (D; 20 mM) stimulation in butyrate pre-treated (red lines) and NaCl pre-treated (black lines), expressed in arbitrary units (AU)

performed. Interestingly, results demonstrated that the basal concentration of IP₁ was 50% higher, on average over the three experiments, in butyrate pre-treated cells than in control. Furthermore, butyrate pre-treated cells tended to have elevated IP₁ accumulation when exposed to the umami ligands and IMP, when compared to NaCl pre-treated cells, although these increases were not significant (Figure 5.7B).

5.5 Discussion

There is extensive literature documenting the ability of SCFAs to modulate gene expression in various cells, tissues and species (Sanderson et al., 2004; Chambers et al., 2018). There are also multiple observations that diet constitution and adiposity both correlate with transcripts of GPCR signalling elements. Combining these reports led me to question whether SCFA could directly modulate GPCR signalling gene expression in EECs. To address this, the mRNA expression profile of untreated STC-1 cells was first confirmed, which suggested that a variety of nutrient sensing receptors and taste receptor signalling elements as well as gut hormone transcripts are present. This agrees with published results of GPCR expression profiles throughout the mouse GI tract. After verification of which GPCR signalling components are present in STC-1 cells they were incubated with SCFAs to examine whether direct application could induce gene expression changes in gustatory GPCR signalling elements. This is the first report that SCFAs, particularly propionate and butyrate, can directly, independently and robustly upregulate the transcripts encoding taste receptors in EECs. A 3:1:1 ratio of acetate: propionate: butyrate at a physiologically relevant concentration of 10 mM was able to induce significant upregulation of taste receptors from both families; TAS1Rs and TAS2Rs. Considering downregulation and subsequent desensitisation of GPCRs upon repeated continuous or intermittent ligand exposure is a common phenomenon and something which is greatly limiting when considering GPCRs as potential drug targets (Calebiro and Godbole, 2018), a mechanism which may promote upregulation may be an extremely useful tool in preventing the phenotypical blunting of signals observed after tachyphylaxis.

The observation that SCFAs can directly modulate GPCR gene expression provides a potential link between the disrupted microbiome, SCFA production and fluctuations in GPCR expression patterns reported in obese humans (Remley et al., 2014) and mice (Widmayer et al., 2015; Peisis et al., 2018). The discrepancies in the relative levels of the components of the gustatory GPCR signalling networks in EECs in obese individuals could be an explanation for the curtailed post-prandial secretion of anorexigenic gut hormones, such as GLP-1 (Ranganath et al., 1996). Here, in STC-1 cells, SCFAs did not have any impact on the expression levels of FFAR transcripts. These have also been reported to be expressed differentially in the colon of obese mice and humans when compared to lean counterparts (Remley et al., 2014; Widmayer et al., 2015; Peisis et al., 2018). This may suggest the observed *in vivo* changes in FFAR expression is unlikely to be mediated solely via SCFA action but may be dependent upon a great number of factors. This is unsurprising as many dietary and endocrine signals are able to influence gene expression and the pathophysiological changes that occur during the onset of an obese phenotype hold great complexity (Clement et al., 2003).

There were two mechanisms hypothesised to be responsible for the effects of SCFAs on the expression of umami receptor subunit transcripts; indirectly via signalling through FFAR2 and/or FFAR3 and directly via inhibition of intracellular HDACs. However, it is not unreasonable that each SCFA may be mediating changes in gene expression via different contributions of several signalling mechanisms as the SCFAs do differ in their signal bias, potency and affinity at rodent FFAR2 and FFAR3 (Hudson et al., 2012) and also in their ability to inhibit HDACs (Waldecker et al., 2008).

Acetate, which shows very little potency as a HDAC inhibitor (Waldecker et al., 2008) did not elicit significant upregulations. However, both propionate and butyrate, which inhibit HDACs to a greater

extent than acetate, robustly enhanced gene expression of both TAS1R1 and TAS1R3. This may suggest that the SCFA mediated upregulation occurs predominantly via HDAC inhibition, as reported elsewhere (Jansen et al., 2004; Sanderson et al., 2004; Larraufie et al., 2018), a process which regulates approximately 2% of mammalian genes (Davie et al., 2003). This is further supported by the lack of gene upregulation observed with the use of FFAR synthetic agonists. 4-CMTB displays characteristic signalling properties, distinct from endogenous ligands, but can activate $G_{q/11}$ and $G_{i/o}$ pathways in EECs (Bolognini et al., 2016). Although we cannot rule out receptor-independent effects of this ligand, the observation that 4-CMTB significantly reduced TAS1R3 by approximately 50% may suggest a role of FFAR2 in moderating transcription or translation of TAS1R3, although, if so, this pathway seemingly opposes the HDAC inhibition pathway. This project confirms previous reports that synthetic ligands of FFAR2/3 possess signalling properties and signal bias unique from endogenous ligands (Wacker et al., 2017) and highlights that the characteristic signalling properties may have significant impacts on the downstream gene expression changes.

One recent study, which looked at the effects of the SCFAs on regulation of *pyy* transcription in human EECs, suggests that although the primary mechanism of gene regulation does seemingly appear to be HDAC inhibition, there was a secondary minor contribution through an FFAR2-dependent route. In this study, acetate acted through FFAR2 to induce a 2-fold increase in the levels of the *pyy* transcript (Larraufie et al., 2018). In this project, acetate did not significantly upregulate taste receptor transcripts, although it showed a similar trend by causing a 2.2-fold increase in TAS1R1 transcript levels. However, where we exposed STC-1 cells to SCFAs for 5 hours, the paper described incubated the EECs for a longer duration of 24 hours. It could be that the FFAR2-dependent signalling routes mediated gene expression changes take longer to occur than the more-immediate HDAC inhibition. We cannot discount that regulation of unrelated genes, each located within a unique DNA microenvironment, occurs by different mechanisms. However, acetate selectively activates human FFAR2 over FFAR3, whereas the rodent orthologues do not show this selectivity (Hudson et al., 2012) so it may be fundamental differences in the signalling properties of acetate between species orthologues which may explain why acetate does not significantly impact gene expression in this study. Despite this, there is evidence to suggest that FFAR2 signalling can alter gene expression in mouse, as well as humans, as mice fed a diet rich in fermentable carbohydrates increased PYY-expressing cells by 87% in a manner dependent upon FFAR2 (Brooks et al., 2016).

The experiments performed with an 18-hour PTX pre-treatment aimed to clarify whether FFAR2/3 $G_{i/o}$ signalling was involved in the observed changes in gene expression of the umami taste receptor. The use of PTX provided evidence to suggest that both propionate and butyrate induced upregulation is occurring via mechanism at least partly independent of FFAR2/3 $G_{i/o}$ signalling, as PTX did not completely abolish gene upregulation. It was interesting to observe that the 18-hour treatment with PTX in isolation also significantly upregulated both umami taste receptor transcripts. PTX has been reported elsewhere to have significant impacts on the gene expression of its target; $G_{i/o}$ proteins (Eschenhagen et al., 1992). PTX has also been shown to reduce the enhancement of HDAC activity that is caused by certain anti-apoptotic growth factors such as lysophosphatidic acid (Ishdorj et al., 2008). This is perhaps an explanation for its ability to modulate genes, such as the umami taste receptor genes, which are independent from PTX pharmacological targets. However, it is also possible PTX acts directly to alter gene expression by inhibiting $G_{i/o}$ signalling and increasing basal intracellular cAMP concentrations.

Despite the somewhat confusing results obtained from the utilisation of PTX, the pre-treatment did curtail the relative changes in gene expression of the umami taste receptor mediated by SCFAs. PTX seemingly had more impact on the impacts of propionate over butyrate, perhaps highlighting discrepancies in their signal transduction. Propionate is only able to modestly inhibit HDACs at high

concentrations of > 10 mM (Waldecker et al., 2008). If this was the sole method by which propionate was mediated fold change in mRNA transcript would be significantly less than the fold change caused by butyrate, but this was not the case. Therefore, it may be more feasible that propionate is acting via a secondary FFAR2/3 signalling mechanism, further supported by the observation that its action was seemingly more susceptible to $G\alpha_{i/o}$ inhibition with PTX. If propionate is acting via FFAR2 or FFAR3 to modulate gene expression, it may be hypothesised that FFAR3 is the more likely candidate, as propionate is nearly 10 times more selective for rodent FFAR3 than FFAR2 (Hudson et al., 2012). Furthermore, FFAR3 has been reported elsewhere to influence gene expression in other cellular models. Primary culture of pancreatic islets isolated from FFAR3 KO mice demonstrated significantly different transcriptomes than WT, with several genes associated with insulin secretion and glucose regulation disturbed (Priyadarshini et al., 2015). To test this further, the CRISPR/Cas9 mediated FFAR3 KO STC-1 cells could be utilised. It is reported that butyrate, however, is a very potent HDAC inhibitor (< 1 mM; Waldecker et al., 2008), well below the concentration range utilised in this project (1-10 mM), maybe suggesting that the predominant mechanism for butyrate mediated upregulation is more likely to be via its modulatory action on HDACs.

A recent paper analysing the heterogeneity of STC-1 cells has highlighted that each cell has a unique, non-conserved expression pattern of L-AA sensing GPCRs. Immunostaining for the TAS1R1-TAS1R3 receptor only stained positive in 15% of cells, the least abundantly expressed of all the peptide sensors, despite the fact that mRNA transcripts of TAS1R1 were relatively high (Wang et al., 2019). It is well documented that mRNA transcript levels do not necessarily correlate with protein expression in mammalian cells (Greenbaum et al., 2003), and GPCRs must go through complex endosomal trafficking networks to reach the PM, in order to be receptive to external stimuli (Duvernay et al., 2005). This prompted experiments to determine whether the umami taste receptor was playing a functional signalling role in STC-1 cells. As other GPCRs able to sense L-AAAs and peptides are expressed in EECs (Symonds et al., 2014), the lack of selective umami taste agonists or antagonists makes it difficult to decipher the precise contributions of each L-AA sensitive GPCR. This is further complicated as the mouse umami taste receptor responds to a broader set of ligands than its human analogue, which is highly specific to L-Glu (Toda et al., 2013). However, the synergistic effects of IMP offer a mechanism to detect umami-specific responses (Nelson et al., 2002), although it should be noted that IMP can easily be converted to inosine, shown to elicit pharmacological activity at the family of ubiquitously expressed adenosine receptors (Welihinda et al., 2016), a possible explanation for its ability to reduce FSK-mediated cAMP accumulation. The observation that umami ligands, L-MSG and L-Ala, only caused calcium mobilisation and intracellular accumulation of IP_1 in the presence of IMP, suggests that TAS1R1-TAS1R3 is functional and coupled to calcium signalling in STC-1 cells, similarly to other heterologous expression systems (Nelson et al., 2002). In the taste buds the umami receptor activates PLC- β via the $\beta\gamma$ subunit (Kinnamon et al., 2009), but whether this is the case in STC-1 cells, or whether the $G\alpha_q$ is mediating IP_3 production remains to be determined. Umami taste ligands had no significant impact on the cAMP levels of STC-1 cells compared to those treated with IMP alone. This suggests it does not couple to $G\alpha_{i/o}$ to inhibit AC. Here, we showed that the gustatory-specific α -gustducin is expressed in STC-1 cells. In gustatory cells, activation of the umami taste receptor induces α -gustducin to activate phosphodiesterases, decreasing intracellular cAMP concentrations through hydrolytic enzymatic reactions (Kinnamon et al., 2009). However, in the cAMP assays performed in this project IBMX, a phosphodiesterase inhibitor, was utilised to increase the detectability to cAMP. However, this may have interfered with the interaction of α -gustducin with phosphodiesterases, thus, preventing their subsequent activation. To investigate whether umami taste receptor promotes phosphodiesterase activity in STC-1 cells via α -gustducin, an assay excluding IBMX, and thus, the possible interference, needs to be designed. A PDE-subtype selective inhibitor could be utilised in its place.

After determining that umami taste ligands in the presence of IMP were able to elicit calcium mobilisation in STC-1 cells, the question remained as to whether pre-treatment with SCFAs could induce upregulation of the umami taste receptor to an extent which was obvious from a functional perspective. It was hypothesised that SCFA pre-treatment may induce a hypersensitivity to umami taste ligands. To test this hypothesis, STC-1 cells were incubated overnight with 1 mM butyrate, a concentration known to be potent enough to inhibit HDACs and activate FFAR2/3 signalling (Waldecker et al., 2008; Hudson et al., 2012). The overnight incubation with butyrate significantly increased basal IP₁ levels by 100% which is intriguing as, unlike acetate and propionate, there is no evidence that butyrate induces G α_q calcium mobilisation via FFAR2 in EECs (Tolhurst et al., 2012). Despite this increase in basal IP₁ concentrations, there remained a trend for a larger percentage increase in IP₁ after butyrate pre-treatment. It was interesting to note that the L-Ala- but not the L-MSG-induced calcium mobilisation significantly increased when STC-1 cells had been exposed to butyrate. It cannot be discounted that this is due to the effect of butyrate on the expression of other genes able to modulate nutrient sensing mechanisms, as HDAC activity modulates 2% of the mammalian genome (Davie et al., 2003). One such candidate would be the CaSR, a receptor with promiscuous pharmacology, with many aromatic and short-chain aliphatic L-AAAs acting as PAMs to enhance the calcium-induced activation (Conigrave et al., 2008). The CaSR is not activated at sub-threshold concentrations of calcium (< 1 mM) but at normal media extracellular calcium concentration (1.5 mM), many L-AAAs can activate G α_q pathways and, thus, intracellular calcium mobilisation via the CaSR (Hira et al., 2008). As L-Ala is more potent than L-Glu at the human isoform of the CaSR (Conigrave et al., 2008), the observation that L-Ala calcium responses are enhanced more by butyrate pre-treatment may lead one to speculate about whether SCFAs also upregulate CaSR expression. However, other L-AA sensitive GPCRs are known to be expressed on EECs, such as GPCR6A and GPR119 (Norton et al., 2017). Despite the lack of clarity about the fundamental underlying mechanism, butyrate pre-treatment does seemingly enhance L-AA induced calcium mobilisation somewhat. Therefore, it would be interesting to clarify whether this may also augment gut hormone secretion in response to L-AAAs. If so, this synergistic interaction could perhaps be used in the design of satiating dietary constitutions.

Due to the reported heterogeneity of STC-1 cells, with reports indicating that only 15% of the unstimulated STC-1 cell population express the umami taste receptor (Wang et al., 2019), it would be interesting to investigate further whether the SCFA induced upregulation of this receptor, robustly observed in this project, is conserved only to the subset of cells previously expressing it or if SCFAs can act to induce the expression in those cells from which it was absent beforehand. Both are feasible explanations for the enhanced calcium mobilisation. Elsewhere, with other genes, SCFAs have been demonstrated to induce novel gene expression in cells. Using 3D organoid models to monitor cell differentiation and development in real time, SCFAs have been noted to induce the differentiation of progenitor cells into enteroendocrine cells via promoting the expression of transcriptional factor *neurogenin-3* (Petersen et al., 2014). This ability of SCFAs to increase the prevalence of L-type cells by double in mouse and human organoid cultures is also mirrored by *in vivo* studies in mice. In these, a diet rich in fermentable carbohydrates increased PYY-expressing cells by 87% via a FFAR2 dependent mechanism (Brooks et al., 2016), suggesting it could be feasible that SCFAs induce umami taste receptor expression in cells previously lacking it. In addition to the gustatory system and the digestive system, the umami taste receptor is found in the respiratory system, brain, testis and spermatozoa (Li et al., 2013). Therefore, it would also be interesting to see if taste receptor upregulation by SCFAs is a conserved phenomenon throughout divergent umami-expressing cell types. If so, the expression in the testis and spermatozoa could provide a link between high fibre diets and male fertility.

When considering extending these findings to *in vivo* models, it is important to consider that the extent to which the TAS1R1-TAS1R3 has a functional role in gut physiology remains to be fully

determined. Nevertheless, it is co-localised with CCK in the mouse proximal intestine, it induces CCK secretion from STC-1 cells (Daly et al., 2012) and has been shown to enhance the peristaltic reflex and activate sensory afferents *in vivo* (Kendig et al., 2014). Certain L-AAs have also been shown to signal in a lactisone-dependent manner to elicit CCK secretion from porcine jejunum tissue (Tian et al., 2019). However, attempts to dissect the specific functional roles of each L-AA sensing receptor often elicit conflicting results. For example, the CaSR was reported to be solely responsible for L-Phe induced CCK secretion from STC-1 cells (Hira et al., 2008), contradicting reports that this was partly umami mediated (Daly et al., 2012). Methods to segregate the functional roles, aside from observing the synergistic effects of IMP, have included genetic KD and KO models, although care should be taken as compensatory behaviour is a common phenomenon (El-Brolosy et al., 2017). The unique pharmacological profiles of each receptor have also allowed some distinction between the properties of the umami taste receptor and the CaSR. For example, by comparing the phenological effects of L-cysteine versus L-tryptophan as the latter has no functional activity at the umami taste receptor (Kendig et al., 2014). However, much of the evidence pointing towards the umami taste receptor as a functional nutrient sensor able to elicit gut hormone secretion has been performed in rodent models. As the human umami receptor is seemingly much more selective for L-MSG, whereas the rodent isoform responds to some extent to most of the 20 L-AAs (Nelson et al., 2002), there are likely to be fundamental differences in the role the umami taste receptor has between species in gastrointestinal nutrient sensing. The rodent TAS1R1-TAS1R3 receptor, with its ability to respond to the majority of the 20 essential amino acids in the millimolar range (Nelson et al., 2002), has been suggested to act as a more general amino acid sensor in endocrine cells, thought to contribute to the role that mammalian target of rapamycin complex 1 (mTORC1) has in integrating signals of amino acid availability to control cell growth and translation of new proteins. TAS1R3-deficient rodent pancreatic β -cell lines had reduced 4EBP-1-dependent translational activity, approximately half the insulin protein content but equal transcript levels and demonstrated reduced amino acid-induced insulin secretion (Wauson et al., 2012). This suggests a link between umami taste receptor and endocrine functions and raises the question of whether a similar mechanism is at play in murine EECs.

It is also important to note that although SCFAs do robustly upregulate umami transcripts in STC-1 cells, not only does the anatomy and morphology of the GI tract differ enormously from this immortalised cell line, but the biochemistry and metabolic signalling environment is much more complex. Additionally, STC-1 cells are derived from mice, not humans, and there are differences between species in where the umami taste genes are located within the DNA architecture. Whether the same upregulation is observed in different species, which encode the umami taste genes on different chromosomes, is questionable. Nevertheless, studies have demonstrated that altering the macronutrient contents of diets can directly regulate transcript levels of TAS1R1 and TAS1R3 in other animal models. For example, supplementing the diet with certain L-AAs increased these transcripts in porcine jejunum tissue, via a mechanism dependent on umami taste receptor signalling (Tian et al., 2019). This may suggest that the expression of umami taste receptor is susceptible to environmental and nutritional factors which signal through GPCRs in various species and models.

In summary, we can conclude that SCFA signalling can remodel epigenetic profiles of gustatory taste receptor signalling elements in EECs, particularly the TAS1R1 subunit of the umami taste receptor. This receptor is expressed in STC-1 cells and seemingly induces calcium mobilisation when exposed to L-AAs in the presence of IMP. This could be one potential route for the L-AA induced CCK secretion from STC-1 cells (Daly et al., 2012). Although further clarification of the precise mechanism is needed, butyrate seemingly enhances the L-Ala induced calcium mobilisation. Overall, using the evidence gathered here, it is highly plausible that SCFAs could act to hypersensitise EECs to the presence of other bioactive nutrients, enhancing the prototypical EEC signalling response. This effect could be highly beneficial when considering the positive physiological and metabolic impacts of the

EEC-derived gut hormones and may be a secondary reason as to why a diet high in NDCs is satiating (Byrne et al., 2015).

Chapter 6: Final conclusion

The work performed in this project has investigated the molecular mechanisms underlying the versatile bioactivity of SCFAs in EECs. A key finding was that SCFA are not only able to act as signalling molecules in their own right, by directly modulating FFARs, but are also able to influence the functional signalling capacity of other dietary ligands in EECs. This observation that SCFAs are able to mould the gustatory taste element signalosome shows that SCFAs dynamically reprogram elements of GPCR signalling pathways that are responsive to other, seemingly unrelated, metabolites and nutrients. This further confirms the versatility of the SCFAs as bioactive signalling molecules in EECs. As EECs are essential to physiology and metabolism due to their propensity to secrete anorexigenic gut hormones, characterisation of the signalling pathways leading to this functional response may have important therapeutic value.

This project has provided evidence to suggest that GPCR signalling networks within EECs are highly adaptable and able to mould dynamically in response to intracellular and/or extracellular environmental factors. Throughout the various experiments, significant heterogeneity in both the expression profile of GPCRs and the functional signalling capacity of differentially sourced STC-1s has been identified. This suggests that the observed discontinuity in the selected G protein pathway FFAR2 activates, in response to SCFA stimulation, is a result of promiscuous coupling that is highly sensitive to the slight environmental discrepancies between cells. The essential components of the cellular environment able to significantly modulate G protein-coupling profiles and, thus, influence the predominant signalling cascade activated in response to SCFAs, remain to be characterised fully. However, the preferential activation of $G\alpha_{i/o}$ pathways in EECs is plausibly a consequence of the direct interaction of FFAR2 and FFAR3 with other nutrient sensing GPCRs, or the crosstalk between coinciding nutrient sensing pathways, a plausible explanation due to the heterogeneity in the gene expression profiles of GPCRs between STC-1 cell types. Throughout this project, it has also been shown that the mechanisms underlying endogenous SCFA signalling at FFAR2 and FFAR3 display distinct pharmacology from the synthetic ligands. These discrepancies have been observed immediately, with differences in the preferences of G protein-coupling, but also in the longer-term, perhaps representative of the distinct downstream signalling cascades activated by the G proteins, such as the induced changes in gene expression. The work in this project could be extended to further encompass how the observed changes in signalling and expression are tied with gut hormone secretion in response to nutrient stimulation.

This project has gone somewhat into demonstrating the promiscuous and complex pharmacology of FFAR2 and FFAR3, but obtaining a crystal structure will provide valuable insights into the intricate structural and conformational interactions underlying this. Currently, the closest resolved structure is of FFAR1 (Tikhovnova et al., 2017) but, as this only shares 30% homology, the clues it provides to SCFA receptor pharmacology is somewhat limited. However, utilisation of the FFAR1-homology models has led to the suggestion that the SCFAs share similar, but not identical, orthosteric binding sites to FFAR1; an inter-helical region between helices 4 and 5. Similar analysis of this model has been used to propose a potential mechanism for allosteric modulation, implying it is possibly being mediated via a positively charged arginine residue on helix 2 (Tikhovnova et al., 2015). The crystal structure of FFAR1 has also offered clues as to the reasons underlying the distinct pharmacological profiles observed when activating FFAR2 with endogenous as opposed to synthetic ligands. For example, FFAR2 specific synthetic agonist, 4-CMTB, has been demonstrated to influence FFAR2 activity in two distinct mechanistic phases, consisting of an initial transient orthosteric activation followed by a prolonged modulation of the allosteric site (Tikhovnova et al., 2017). The distinct ability of 4-CMTB to interact with both sites, and thus, induce a ligand-specific conformational change in FFAR2, may explain why 4-CMTB activates FFAR2 to elicit $G\alpha_{q/11}$ mediated calcium mobilisation in EECs, whereas SCFAs do not. Despite this knowledge, to fully dissect whether the three predominate SCFAs; acetate, propionate and butyrate; are able to manipulate the conformational and structural properties of FFAR2 and FFAR3 in unique and divergent ways, crystal

structures of these receptors would be fundamental. A crystal structure would also help model the pharmacodynamics of FFAR2/3 ligands, allowing greater characterisation of the association and dissociation rates and, thus, ligand occupancy times, a property which is fundamental when considering utilisation for therapeutic benefit (Guo et al., 2012).

Chemogenetic technology is rapidly progressing and there have been developments detailing the use of photoactivatable GPCRs to discern receptor specific effects (Acosta-Ruiz et al., 2019). For receptors, such as FFAR2 and FFAR3, whose co-expression and similar pharmacology make the individual contributions to the observed signalling profile notoriously difficult to decipher, these techniques show some promise. One such example is the specific activation of tagged GPCRs by photoswitchable ligands. The tags, such as HALO- or SNAP-tags, are genetically fused onto the N-terminal of the GPCR monomer, facilitating localisation. These enable the receptor to be specifically activated when there is an external light source of the correct wavelength, by ligands that consist of three regions essential for their function. The first region specifically covalently binds the HALO- or SNAP- tag of the receptor, bringing the ligand in close proximity to the receptor at a specific orientation. This is fused to a photoactivatable domain, sensitive to a certain wavelength of light, which induces a reversible conformational change, acting as a switch. On activation, the induced change in this light sensitive domain brings the third domain, a domain with similar morphology to the endogenous ligand, to interact with the orthosteric binding site, and, thus, activate the receptor. The bulky ligand is unable to activate the receptor unless the light source is applied, allowing detailed analysis of receptor-specific effects and allowing the signal to be terminated effectively (Tian et al., 2016). By facilitating the ability to return the receptor to an inactive state by removing the light source, this method offers the advantage over traditional DREADD technology, utilised elsewhere with FFAR2 (Bolognini et al., 2019). In addition, as the light-sensitive molecule is not intrinsic to the target receptor, as is the commonly the case with traditional opsin-incorporating GPCR techniques (Morri et al., 2018), it is more plausible that the endogenous receptor pharmacology will be preserved intact, with the pattern of G protein-coupling being conserved. Therefore, designing photoactivatable ligands that specifically target tagged FFAR2/FFAR3 and using these concurrently in genetically modified models, expressing the tagged isoforms of these receptors, could help paint a more comprehensive picture of the intricate signalling dynamics and interactions of the SCFA receptors.

In conclusion, this project has offered valuable information about the mechanisms underlying EEC sensitivity and functional response to bioactive nutrient stimulation. The summation and integration of these various signalling events leads to a single physiological response in EECs, one of the most integral being the secretion of anorectic peptide hormones. Uncovering the underlying mechanisms that determine the response of EECs to the dynamic array of nutrients to which they are exposed may aid in the design of novel therapies that act to selectively augment the signalling pathways that give rise to anorexergic gut hormone secretion. As gut hormones induce countless positive physiological and metabolic adaptations, as well as inducing satiety, targeted therapeutic augmentation of their secretion could be a viable approach to prevent weight gain and adiposity.

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