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The pharmacology and function of receptors for short chain fatty acids

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Running title page

Running title: Pharmacology of Short Chain Fatty Acid Receptors

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Text pages: 44
Number of figures: 3
Number of references: 114
Number of words in Abstract: 207
Number of words in text: 7371

ABBREVIATIONS

BHB, ² -hydroxybutyrate; ECL, extracellular loop, fMLP, N-formylmethionyl-leucyl-phenylalanine; GPCR, G protein-coupled receptor; GSIS, glucose-stimulated insulin secretion; HDAC, histone deacetylase; HTS, high throughput screen, LE, ligand efficiency; NAM, negative allosteric modular; PAM, positive allosteric modulator; PBMC, peripheral blood mononuclear cell; PMN, polymorphic nucleocyte; PTX, pertussis toxin; ROS, reactive oxygen species; SAR, structure activity relationship, SCA, small, non-fatty acid, carboxylic acids; SCFA, short chain fatty acids; TM transmembrane; TPN, total parenteral nutrition

Chemical Names

4-CMTB, [(S)-2-(4-chlorophenyl)-3- methyl-N-(thiazol-2-yl)butanamide]; CATPB, (S)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid; compound 1, 3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic

acid; GLPG0974, -[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid; AR420626, N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide; compound 6, 2-methyl-5-oxo-4-(3-phenoxyphenyl)-N-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide; sorbic acid, 2,4-hexadienoic acid.

ABSTRACT

Despite some block-buster G protein-coupled receptor (GPCR) drugs only a small fraction (~15%) of the more than 390 non-odorant GPCRs have been successfully targeted by the pharmaceutical industry. One way that this issue might be addressed is via translation of recent de-ophanization programmes that have opened the prospect of extending the reach of new medicine design to novel receptor types with potential therapeutic value. Prominent among these receptors are those that respond to short chain free fatty acids of carbon chain length 2-6. These receptors, FFA2 (GPR43) and FFA3 (GPR41), are each activated by the predominant short chin fatty acids acetate, propionate and butyrate, ligands that originate largely as fermentation by-products of anaerobic bacteria in the gut. However, the presence of FFA2 and FFA3 on pancreatic ^{2}-cells, FFA3 on neurons and FFA2 on leukocytes and adipocytes means that the biological role of these receptors likely extends beyond that of the widely accepted role of regulating peptide hormone release from enteroendocrine cells in the gut. Here we review the physiological roles of FFA2 and FFA3, the recent development and use of receptor selective pharmacological tool compounds and genetic models available to study these receptors and present evidence of the potential therapeutic value of targeting this emerging receptor pair.

INTRODUCTION

Short Chain Fatty Acids (SCFAs) are saturated aliphatic organic acids containing 2 to 6 carbon atoms. Within the body they are predominantly the by-product of the fermentation of non-digestible carbohydrates (fibers) through the action of intestinal anaerobic bacteria (den Besten *et al.*, 2013). The amount of SCFAs released in the intestine is influenced by several factors, among which are the strain and quantity of microbiota in the colon, substrate source and intestinal transit time (Wong *et al.*, 2006). In general, SCFAs reach an intestinal concentration in the millimolar region, in which acetate (C2), propionate (C3) and butyrate (C4) represent the most abundant metabolite species (e95%) (Topping and Clifton, 2001). In 2003, the previously orphan seven transmembrane (TM) domain polypeptides GPR41 and GPR43 were identified as G protein-coupled receptors (GPCRs) that are activated by SCFAs (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). Following this discovery, interest surrounding SCFAs and their receptors has risen dramatically, both in relation to the discovery of selective ligands and the physiological role of these receptors. GPR41 and GPR43 were subsequently renamed FFA3 and FFA2, respectively (Stoddart *et al.*, 2008a) based on their responsiveness to SCFAs.

FFA2/FFA3 receptor structure and signal transduction

The genes encoding FFA2 and FFA3, together with the medium/long chain fatty acid receptor GPR40 (FFA1, Stoddart *et al.*, 2008a), cluster as a group of intronless sequences located in human at chromosome 19q13.1. They were first identified during a search for novel human galanin receptor subtypes (Sawzdargo *et al.*, 1997). A further gene that shares 98% identity with FFA3, was also identified within this region, and is designated GPR42. This latter potential receptor is now classified as a functional polymorph of

FFA3, although its physiological role and expression in humans is yet to be fully clarified (Liaw and Connolly, 2009; Puhl *et al.*, 2015). FFA2 and FFA3 are closely related, with 43% amino acid identity (Stoddart *et al.*, 2008a). This translates into poor ligand selectivity between the two receptors. Nonetheless, there is a rank order of potency for SCFAs in activating human FFA2 and FFA3 receptors, where FFA2 is activated more potently by shorter chain fatty acids whilst, in general, the opposite is the case for FFA3. Specifically, the rank order of potency for human FFA2 is reported as C2=C3>C4>C5=C1, whereas at human FFA3 it is C3=C4=C5>C2>C1 (Milligan *et al.*, 2009) (**Figure 1**). Although this results in acetate being significantly more potent at human FFA2 than at human FFA3 (Schmidt *et al.*, 2011) and is sometimes, therefore, employed as a selective activator of FFA2, there is great need to identify more selective synthetic ligands that would allow improved discrimination between the biological functions of FFA2 versus FFA3 both *in vitro* and *in vivo*.

The de-orphanization of GPR41 and GPR43 led to the observation that the carboxylic acid group of SCFAs is the key element in the activity of these endogenous molecules at both receptors. Previous studies had shown that positively charged amino acids within the TM regions are essential for the binding and function of other GPCRs whose ligands contain a carboxylic acid group (Stitham *et al.*, 2003; He *et al.*, 2004; Tunaru *et al.*, 2005; Sabirsh *et al.*, 2006). This, together with the observation that uncharged ester derivatives of SCFAs are inactive at FFA2 and FFA3 (Le Poul *et al.*, 2003), led Milligan's group to hypothesize that basic residues might also play a crucial role in the binding of SCFAs to their receptors (Stoddart *et al.*, 2008b). Sequence alignment of FFA2 and FFA3 with FFA1 revealed that five positively charged amino

acids were conserved across these fatty acid receptors. Generation of homology models, linked to a mutagenic strategy, was then employed to identify the key polar amino acids for ligand recognition contained in the water-filled cavity within the TM domains of FFA2 and FFA3 (Stoddart *et al.*, 2008b). From this, four positively charged amino acid residues were identified: Histidine (His) in TM IV (residue position 4.56); Arginine (Arg) in TM V (5.39); His in TM VI (6.55); Arg in TM VII (7.35) (Stoddart *et al.*, 2008b) (for a more detailed consideration of the position and significance of these amino acids in FFA2 and FFA3 see Ulven, 2012). Studies in cells expressing alanine-substituted forms of these residues in FFA2 and FFA3 led to the conclusion that both Arg 5.39 and 7.35, as well as His 6.55, were essential for co-ordinating the recognition and functionality of SCFAs at both FFA2 and FFA3. Indeed, in each case, these alterations completely abrogated response to SCFAs (Stoddart *et al.*, 2008b). By contrast, mutation of His 4.56 displayed a more diverse outcome between the two receptors, suggesting that this amino acid may not be directly involved in the binding of the carboxylate group but, rather, plays a role in fatty acid chain length selectivity (Stoddart *et al.*, 2008b). The identification of these key residues involved in the orthosteric binding site paved the way for the study of other, and potentially selective, small molecule ligands. Schimdt and colleagues established structure-activity relationships (SAR) of a group of small, non-fatty acid, carboxylic acids (SCAs) at FFA2 (Schimdt *et al.*, 2011). Although this work identified some molecules that were relatively selective for FFA2 over FFA3, for example 2,2-dimethylacrylic acid has approximately 800 times higher potency at FFA2 compared to FFA3, the potency of such molecules was still very modest and too low to be useful as pharmacological tools for *in vitro* and, particularly, *in vivo* studies. Given the small size

of these molecules, the low potency of both SCFAs and SCAs is hardly surprising. However, they did display high ligand efficiency (LE). LE is a measure of ligand binding free energy per heavy atom count and is mathematically expressed by delta g (Hopkins *et al.*, 2014). This concept has been widely used for the selection and optimization of fragments or small ligands at specific pharmacological targets. Schmidt *et al.* (2011) indicated that the ligand efficiency for C2, C3 and SCAs was approaching the maximal possible delta g value, indicating that it would be unlikely that potency at FFA2 and FFA3 could be improved without increasing ligand size substantially.

An additional binding pocket in FFA2, distinct from that for the endogenously produced SCFAs, was first hypothesized by researchers at Amgen. This was based on outcomes from a high throughput screen (HTS) campaign to identify FFA2 activators (Lee et al., 2008). This resulted in the characterization of the first moderately potent FFA2 selective, synthetic ligand, [(S)-2-(4-chlorophenyl)-3- methyl-N-(thiazol-2-yl)butanamide] (4-CMTB) (**Figure 1**). This ligand was shown to cause activation of both $G\pm_{i/o}$ and $G\pm_{q/11}$-mediated pathways via FFA2 with no effect at FFA3 (Lee *et al.*, 2008). Despite this, some subsequent studies have indicated very limited ability of this ligand to produce elevation of Ca^{2+} levels in transfected cells (see later). Moreover, further analyses of the pharmacological properties of this ligand revealed an ability to exert positive cooperativity with both C3 and C2, indicating that 4-CMTB behaves as an allosteric agonist at FFA2 (Lee *et al.*, 2008; Wang *et al.*, 2010; Smith *et al.*, 2011). Mutagenic studies have revealed that extracellular loop 2 (ECL2) plays an important role in the allosteric effect of 4-CMTB. Replacement of ECL2 in FFA2 with the equivalent region from FFA3 completely abolished the observed positive cooperativity between 4-

CMTB and C3 (Smith *et al.*, 2011). Despite this, the details of the 'allosteric' binding pocket for 4-CMTB remain to be fully defined.

As noted earlier, in terms of signal transduction FFA2 is a promiscuous receptor that has the ability to couple to pathways transduced by both $G\pm_{i/o}$ and $G\pm_{q/11}$ proteins (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). Moreover, screens performed in strains of the yeast *S. cerevisiae* containing different yeast/mammalian G± subunit chimeras, also indicated an ability of FFA2 to interact with $G\pm_{12}$, $G\pm_{13}$ and $G\pm_{14}$ (Brown *et al.*, 2003). However, interaction of FFA2 with these G proteins has yet to be validated in cells that express native, full length mammalian G proteins. By contrast, the activation of FFA3 appears to induce only $G\pm_{i/o}$-mediated signaling as receptor effects are generally attenuated by pertussis toxin (PTX) treatment of cells (Brown *et al.*, 2003; Le Poul *et al.*, 2003). In addition, SCFA occupancy of FFA2 has been reported to recruit both ² -arrestin-1 and, in particular, ² -arrestin-2 to the receptor and these are responsible for FFA2 internalization from the cell surface and the initiation of G protein-independent signal transduction (Hudson *et al.*, 2012b and 2013b; Lee *et al.*, 2013). To date there are no published data regarding the ability of FFA3 to recruit arrestin isoforms.

Physiological roles of FFA2/FFA3

Roles in immune cells

When the tissue expression of FFA2 was first described, its most notable presence was in cells of the innate immune system, both polymorphic nucleocytes (PMNs) and peripheral blood mononuclear cells (PBMCs) (Le Poul *et al.*, 2003) (**Figure 2**). Recent studies have confirmed presence of FFA2 in neutrophils, eosinophils (Maslowski *et al.*,

2009), and in leukocytes of the lamina propia (Nøhr *et al.*, 2013). The presence of FFA3 remains contentious (Brown *et al.*, 2003; Maslowski *et al.*, 2009). Naturally, given that SCFAs are a by-product of anaerobic fermentation by gut bacteria, it is to be expected that immune cells should be able to respond to such ligands.

One area of particular interest is inflammatory conditions of the lower gut, such as ulcerative colitis and Crohn's disease (Xavier and Podolsky, 2007). Butyrate enemas have been used to attempt to treat colitis, with mixed results (Bocker *et al.*, 2003), and a high fiber diet can result in improvement in ulcer and colitis scores, with matching reduction in neutrophil infiltration (Kataoka *et al.*, 2008). Despite these promising results, two different studies examining the role of FFA2 in rodent models of colitis have generated conflicting effects. Maslowski et al. (2009) found that colitis could be rescued in germ-free mice by FFA2 agonism with acetate, whereas Sina et al. (2009) found that SCFA recruit PMNs via FFA2 to worsen tissue damage. Moreover, a`first in man` clinical trial with the FFA2 antagonist, GLPG0974 (see later), for the treatment of ulcerative colitis did not provide any immediate beneficial effects, resulting in this study being terminated. Clearly, FFA2 signaling in leukocytes is a complicated scenario, which deserves further dissection and analysis.

Most reports suggest that SCFAs mediate a shift from pro-inflammatory to anti-inflammatory cytokine release from leukocytes. C2 and C4 can inhibit TNF-± release (Säemann *et al.*, 2000; Maslowski *et al.*, 2009; Ohira *et al.*, 2013), and C4 in particular can mediate a switch from a Th1 (which is exaggerated in Crohn's disease) to a Th2 profile of cytokine production (Säemann *et al.*, 2000; Cavaglieri *et al.*, 2003). Both FFA2 and FFA3 knock-out mice do not recruit Th1 cells in a rectal inflammation model (Kim

et al., 2013). Finally, C3 can trigger the release of the anti-inflammatory IL-10 from regulatory T cells, and this happens in a FFA2-specific manner (Smith *et al.*, 2013).

In terms of cell signaling, C2 and C3 (Le Poul *et al.*, 2003; Maslowski *et al.*, 2009) but not C4 (Nakao *et al.*, 1992) increase intracellular Ca^{2+} in PMNs, in a manner that has been described as partially (Nakao *et al.*, 1992) or fully PTX insensitive, and intracellular store-dependent (Le Poul *et al.*, 2003), raising the possibility that this effect may be mediated via FFA2 and $G\pm_{q/11}$. PMNs require calcium for chemotaxis towards N-formylmethionyl-leucyl-phenylalanine (fMLP), a peptide which is often used as a model of bacteria-stimulated chemotaxis (Chen and Jan, 2001). PMNs migrate along a C3 or C4 gradient, in a FFA2, p38, ERK1/2, and pAkt specific manner; and this appears to be PTX-sensitive, implicating $G\pm_{i/o,}$ in addition to $G\pm_{q/11}$ (Sina *et al.*, 2009; Vinolo *et al.*, 2011). Neutrophils use reactive oxygen species (ROS) in phagosomes to kill internalized bacteria (Nordenfelt and Tapper, 2011), but ROS also play a role as signaling molecules involved in chemotaxis (Hattori *et al.*, 2010). C4 inhibits ROS production in neutrophils stimulated with fMLP via cAMP, and a PTX-sensitive release of G protein G_{23} complex. Conversely, C2 increases ROS production in a PTX-insensitive manner (Vinolo *et al.*, 2009), and macrophages lacking FFA2 do not produce ROS in a model of gouty arthritis (Vieira *et al.*, 2015). Potentially this may reflect opposing signaling via FFA2 and $G\pm_{q/11}$ and FFA3 and $G\pm_{i/o}$. ROS also contribute to apoptosis in neutrophils - a process which helps limit the extent of inflammation (Nordenfelt and Tapper, 2011) – and C2 increases apoptosis (Maslowski *et al.*, 2009), perhaps again via FFA2.

Broadly speaking, it would appear that C2 and C3 stimulate, whilst C4 inhibits, the various functions of innate immune cells. This may explain conflicting results using

mixes of SCFAs, although non-receptor mediated effects cannot be ruled out. In terms of cytokine release, all SCFAs appear to have anti-inflammatory effects. There are not yet many data demonstrating the signaling pathways upstream of cytokine release, or indeed whether both FFA2 and FFA3 are involved. As with analysis of function of FFA2 and FFA3 in other tissues (see later), the low potency and pleiotropic effects of the SCFAs, means that without access to new, well characterized and highly selective synthetic ligands, unravelling the specific roles of FFA2 and FFA3 in immune cells and in immune cell-mediated disease processes will remain challenging.

Roles in the intestine

C4 is an important energy source for enterocytes, and SCFAs have a multitude of positive effects in the colon, such as helping maintain the intestinal barrier, and decreasing the risk of cancer (Canani *et al.*, 2011) (**Figure 2**). Indeed, model animals on total parenteral nutrition fare better when supplemented with SCFAs, showing less mucosal atrophy, an improvement that correlates with increased expression of proglucagon (Gee *et al.*, 1996; Pratt *et al.*, 1996; Tappenden and McBurney, 1998). Studies using fluorescently labelled L-cells show FFA2 to be highly expressed in colonic L-cells, and FFA3 in small intestinal L-cells (Tolhurst *et al.*, 2012). FFA3 partially co-localizes with gastrin and ghrelin in the stomach, completely co-localizes with CCK, GIP and secretin in the proximal small intestine and with PYY, NT, and GLP-1 in the distal small intestine. A gradient of expression of FFA3 increases distally in D-cells and enterochromaffin cells. Conversely, FFA2 is only observed sparsely in a subpopulation of enteroendocrine cells of the small intestine, although the lack of fluorescence in the model used cannot be taken to be conclusive proof of lack of expression (Nøhr *et al.*,

2013). In accordance with the expression profile of FFA2 and FFA3, stimulation of enteroendocrine cells with SCFAs can trigger GLP-1 (Reimer and McBurney, 1996; Lin *et al.*, 2012; Tolhurst *et al.*, 2012; Nøhr *et al.*, 2013; Psichas *et al.*, 2015), GLP-2 (Akiba *et al.*, 2015) PYY (Lin *et al.*, 2012; Psichas *et al.*, 2015) and GIP (Lin *et al.*, 2012) release. However, reports as to whether oral SCFA can increase enteroendocrine hormones *in vivo* are mixed. One study found oral SCFAs had no effect on GLP-1, but decreased the plasma GIP response to glucose challenge (Tang *et al.*, 2015). FFA2 agonists may also act on EC cells to trigger 5-HT release, which together with GLP-2 would help prevent mucosal injury (Akiba *et al.*, 2015), suggesting a mechanism for the benefits to adding SCFAs to TPN. Finally, there is a link between SCFAs and decreased intestinal motility, which is FFA3 and neuroendocrine independent (Dass *et al.*, 2007). Overall, results show that FFA2 and FFA3 are involved in enteroendocrine hormone production and intestinal functions, but further studies with selective synthetic ligands and new animal models are needed to better define the roles of these receptors in the intestine.

Roles in the pancreas

Early reports showed that C2 improves glucose clearance in rats (Shah *et al.*, 1977), and improves glucose-stimulated insulin secretion (GSIS) in isolated rat islets (Patel and Singh, 1979). More recently it has been demonstrated that islets express both FFA2 and FFA3 (Brown *et al.*, 2003; Leonard *et al.*, 2006; Regard *et al.*, 2007) (**Figure 2**), and crucially they are found in beta cells of the islet (Tang *et al.*, 2011). Interestingly, FFA2 is upregulated in pregnancy in the mouse, suggesting that it may aid in this insulin-

resistant state (Layden *et al.*, 2010). This suggests a simple relationship of FFA2/3 agonism leading to insulin secretion, but the reality is likely to be more complicated. One study has reported that while C2 increases insulin secretion via FFA2 (Priyadarshini *et al.*, 2015), C3 inhibits insulin secretion via FFA3 (Priyadarshini and Layden, 2015). Other results indicate that C2 has an autocrine role, suggesting it to inhibit insulin secretion via $G\pm_{i/o}$ coupled to both FFA2 and FFA3 in beta cells (Tang *et al.*, 2015). The latter appears more logical, given the importance of cAMP in boosting GSIS (Yajima *et al.*, 1999). Interpretation must remain cautious at this point, however, because it is possible that different SCFAs display ligand bias (see later) at FFA2/3. For example, FFA2 agonist-mediated enhancement of GSIS activation is reported to be via PLCβ (Priyadarshini *et al.*, 2015). Perhaps FFA2-dependant effects of C2 in the islet are biased to $G\pm_{q}$ over $G\pm_{i/o}$. Studies in FFA2 knock-out mice showed a depressed level of plasma insulin in response to an oral glucose load, but it is difficult to draw firm conclusions from these data given that FFA2 also plays a role in GLP-1 secretion (Tolhurst *et al.*, 2012) and insulin sensitivity (Bjursell *et al.*, 2011), both of which would be anticipated to have a knock-on effect on insulin levels. To describe separate, potentially opposing roles of FFA2 and FFA3 in the islet, selective ligands for each receptor are sorely needed.

Roles in adipose tissue

There is strong evidence for the presence of FFA2 in adipocytes (**Figure 2**). Two of the original de-orphanization studies noted this (Brown *et al.*, 2003; Le Poul *et al.*, 2003), and subsequent studies have agreed (Ge *et al.*, 2008; Al-Lahham *et al.*, 2010; Lemor *et al.*, 2010; Kimura *et al.*, 2013). The presence of FFA3, however, remains a subject of debate. FFAR3 mRNA is reported to be in adipose tissue and adipocyte cell

lines (Le Poul *et al.*, 2003; Xiong *et al.*, 2004; Mielenz *et al.*, 2008; Al-Lahham *et al.*, 2010) but, equally, many groups report that adipocytes themselves do not possess the receptor (Brown *et al.*, 2003; Hong *et al.*, 2005; Zaibi *et al.*, 2010; Bellahcene *et al.*, 2013). Knocking out FFA2 expression has not provided clarity as to its possible function in adipocytes. FFA2 receptor knock out lines have been reported to show both increase adiposity (Kimura *et al.*, 2013), and protect against diet-induced obesity (Bjursell *et al.*, 2011). In addition, lean and obese humans have similar expression levels of FFA2 (Dewulf *et al.*, 2013). Still, it is clear that SCFAs act on adipocytes. C2 derived from alcohol metabolism reduces circulating FFAs (Crouse *et al.*, 1968), and resistant starch supplementation inhibits lipolysis and hormone-sensitive lipase to the same effect (Robertson *et al.*, 2005; Ge *et al.*, 2008). Exogenous C2, C3 and C4 all inhibit lipolysis (Hong *et al.*, 2005; Ge *et al.*, 2008; Zaibi *et al.*, 2010). Importantly this effect is PTX-sensitive (Ohira *et al.*, 2013), and absent in FFA2 knock-out mice (Ge *et al.*, 2008). Reports suggest that FFA2 activation reduces insulin sensitivity in the adipocyte, by $G_{i/o}$-mediated inhibition of Akt phosphorylation downstream of the insulin receptor (Kimura *et al.*, 2013).

SCFA can drive adipocyte differentiation from stem cells (adipogenesis). C2 and C3 trigger the pre-adipocyte cell line 3T3-L1 to differentiate, and C3 increases expression of the adipogenesis markers PPAR-³2 and C/EBP±. When these cells are treated with siRNA against FFA2, expression of PPAR-³2 and aP2 (another adipocyte marker) decrease (Hong *et al.*, 2005). FFA2 knock-out mice have less adipocytes (Bjursell *et al.*, 2011) but, curiously, these findings from rodent models may not translate to humans. Adipocytes cultured from the omentum did not show any increase in aP2 after

treatment with FFA2 agonists (Dewulf *et al.*, 2013). A lack of difference in white adipose tissue between FFA2 knock-out and wild type pups in the embryonic stage have even led one group to conclude that the effect of FFA2 in adipogenesis may be an *in vitro* artefact (Kimura *et al.*, 2013).

Finally, the adipocyte does not function as merely an inert energy store; it is also an endocrine cell, secreting metabolic hormones including adiponectin and leptin (Sethi and Vidal-Puig, 2007). Higher levels of leptin have been reported in both FFA2 (Bjursell *et al.*, 2011) and FFA3 knock-out mice fed a high fat diet (Bellahcene *et al.*, 2013) although a different FFA2 knock-out line had plasma leptin levels comparable to wild type (Kimura *et al.*, 2013). *In vitro*, SCFAs also increase leptin mRNA in bovine adipocytes (Soliman *et al.*, 2007) and increase leptin secretion from primary murine adipocytes (Xiong *et al.*, 2004), both in a PTX-sensitive manner. As all these somewhat contradictory studies demonstrate, the true role of the FFA2 in the adipocyte is far from fully defined.

Roles in neurons

The autonomic nervous system regulates energy output, and in particular the sympathetic nervous system acts to decrease energy use during periods of starvation (Kimura *et al.*, 2011). It has been proposed that SCFAs can act at FFA3 as a switch to modulate sympathetic regulation of energy. In times of plenty, plasma levels of C4 will increase (especially if diets are high in fiber) (Nilsson *et al.*, 2010), whereas during starvation, levels of ketone bodies such as ² -hydroxybutyrate (BHB) – a putative FFA3 antagonist (Inoue *et al.*, 2012) (or possibly agonist) (Won *et al.*, 2013) will increase.

FFA3 is expressed by both prevertebral and paravertebral ganglia (Kimura *et al.*, 2011; Won *et al.*, 2013; Nøhr *et al.*, 2015) (**Figure 2**). FFA3 knock-out mice have a lower resting heart rate, and less sympathetic innervation of the heart, whereas C3 given to wild type mice can raise heart rate. Co-culturing cardiomyocytes and neurons together reveals a FFA3-dependent relationship where C3 can increase beat rate. This effect is PTX-sensitive, and involves $G_{i/o}$, PLC^{β}, and ERK/12 MAP kinases. In this system, BHB can antagonize the effect of C3 (Kimura *et al.*, 2011). C3 causes the release of noradrenaline from the superior cervical ganglion via the same signaling cascade and synapsin 2. Again, this is inhibited by BHB (Inoue *et al.*, 2012). However, there is a conflicting report that describes a different mechanism of FFA3 action. Won *et al.*, (2013) showed inhibition of N-type Ca^{2+} channels by $G_{i/o}$ complex generated by activation of FFA3, an effect that was elicited by each of C2, C3 and BHB, and would presumably decrease catecholamine release from neurons (Won *et al.*, 2013). How these data fit within the sympathetic modulation theory remains to be defined.

FFA3 is also expressed in ganglia of the enteric and sensory nervous systems, i.e. submucosal ganglia, myenteric ganglia, nodose ganglion, dorsal root ganglia, and trigeminal ganglia (Nøhr *et al.*, 2013, 2015). The physiological function of FFA3 in these ganglia has yet to be described. Finally, FFA3 co-localizes with a neuronal marker in the portal vein wall, where it is proposed that C3 can act to form a gut-brain axis regulating intestinal gluconeogenesis (De Vadder *et al.*, 2014).

Experimental challenges and current perspectives for the validation of FFA2/FFA3 as therapeutic target(s)

Target validation is an essential step in drug-development studies and assists in defining the physiological role(s) of a GPCR and its importance in patho-physiological conditions, with the aim of developing a pipeline of potential therapeutic medicines (Smith, 2003). Among several approaches, validation of a GPCR can be achieved by employing ligands that selectively perturb the target of interest *in vitro* and/or *in vivo*.

Synthetic ligands for SCFA receptors

There is a general paucity of selective ligands for FFA2 and FFA3 and the co-expression of these two GPCRs in several tissues (see earlier) presents major obstacles in understanding the patho-physiological role of each receptor. This is further complicated by differences between the pharmacology of species orthologs of FFA2 and FFA3 (**Figure 1**). For example, although C2 has been used in a number of *in vivo* studies both because it is the most abundant SCFA in the body (McOrist *et al.*, 2008) and because of its reported selectivity for FFA2 over FFA3 (Schmidt *et al.*, 2011), this selectivity of C2 is most pronounced at the human SCFA receptors. By contrast, (Hudson *et al.*, 2012b) demonstrated that this is not the case for the murine orthologs of FFA2 and FFA3 (**Figure 1**). Indeed, for the mouse receptors C2 is equipotent in activating FFA2 and FFA3. Moreover, no endogenous SCFA is sufficiently selective to define a role for FFA2 over FFA3, or *vice versa*, in murine cells and tissues (Hudson *et al.*, 2012b). This highlights the need for more potent and markedly more selective ligands for the two receptors.

Orthosteric agonist ligands

Pleiotropic non-receptor-mediated effects, as well as the low potency and lack of selectivity of the SCFAs between FFA2 and FFA3, has certainly become a major obstacle to the study of the function of these receptors, both *in vitro* and *in vivo*. The work of Schmidt et al. (2011) highlighted that although small molecules containing a carboxylic acid group can achieve close to optimal LE and a reasonable degree of selectivity at FFA2 vs FFA3, their modest potency, and a lack of knowledge of potential 'off-target' effects, means they remain unsuitable for *ex-vivo* and/or *in vivo* study of these receptors, although they have been used in a limited number of cases (e.g. (Priyadarshini *et al.*, 2015)).

The first class of more potent synthetic ligands of FFA2 were initially described and patented by Euroscreen (Hoveyda *et al.*, 2010). From this series of ligands, Hudson et al., (2013b) synthesized and characterized 'compound 1' (3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid) as a selective orthosteric agonist at human FFA2 (**Figure 1**). In a range of assays, including those reflecting FFA2-mediated $G\pm_{i/o}$, $G\pm_{q/11}$ and ² -arrestin pathways, this ligand is able to activate human FFA2 with potency in the high nanomolar range. The mode of binding of this ligand has not yet been completely elucidated. 'Compound 1' and related molecules contain a carboxylic acid pharmacophore, and this is required for function because replacement by a methyl or *tert*-butyl ester eliminates activity (Hudson *et al.*, 2013b). Moreover, it is clearly orthosteric in action as it also lacks function at mutants of the key positively charged residues of the orthosteric binding site of human FFA2 (Hudson *et al.*, 2013b). Moreover, function of 'compound 1' is inhibited by the orthosteric antagonist, CATPB (Hudson *et al.*, 2013b) in a competitive and surmountable fashion. However, 'compound

1' contains also the N-thiazolyamide pharmacophore, as found in the allosteric agonist 4-CMTB. Despite this, 'compound 1' does not appear to act as a 'bitopic' ligand (Lane *et al.*, 2013) because in ^{2}-arrestin-2 recruitment assays 'compound 1' did not also display competitive interactions with 4-CMTB (Hudson *et al.*, 2013b). Studies with related compounds, designated 9, 14, 101 and 105, containing both carboxylate and N-thiazolyamide moieties (Brown *et al.*, 2015) indicated these ligands were also able to interact with the orthosteric binding site of FFA2, but not with the allosteric binding site.

In cells endogenously expressing FFA2 'compound 1' has been reported to reduce lipolysis in both the human and mouse immortalized adipocyte cell lines, SW872 and 3T3-L1 respectively, with a mechanism that has been shown to be $G\pm_{i/o}$ dependent. Moreover, 'compound 1' has also been reported to induce GLP-1 release from the murine STC-1 enteroendocrine cell line (Hudson *et al.*, 2013b). Importantly, although 'compound 1' displays reasonable potency at rodent orthologs of FFA2 (Hudson *et al.*, 2013b), certain other compounds from this chemical series show markedly lower potency at the rodent forms compared to human (Hudson *et al.*, 2013b). Although potentially a good deal might be learned from a systematic SAR analysis of this compound series, such studies have not been reported to date. Docking studies of 'compound 1' to a homology model of human FFA2 revealed that the phenyl substituent in this ligand may interact with residue position 3.29 of the receptor (Hudson *et al.*, 2013b). Interestingly, this residue differs between human (serine) and rodent (glycine) orthologs. More recently, a further homology model of this receptor, based on the x-ray structure of the related receptor FFA1, has indicated that tyrosine 90 (residue position 3.33) is also in close proximity to this phenyl ring (Sergeev *et al.*, 2016) and mutation of this residue has

also previously been shown to markedly reduce the potency of 'compound 1' (Hudson *et al.*, 2013b).

Orthosteric antagonist ligands

Receptor antagonists, by blocking the action of either endogenous or synthetic agonists, routinely provide powerful tools to help define biological roles of a receptor. Interest in the biological actions and potential therapeutic application of FFA2 antagonists emerged with the discovery that FFA2 is expressed by neutrophils and can dictate their migration in inflammatory states, including those of the lower intestine (Maslowski *et al.*, 2009; Sina *et al.*, 2009). Moreover, recent studies have showed that FFA2 and FFA3 activation in beta cells of the pancreas can block insulin secretion (Tang *et al.*, 2015), suggesting that antagonists at either (or both) of these receptors could be beneficial for the treatment of type II diabetes.

In recent years, two series of FFA2 antagonists have been described. The first series of antagonists was reported by Euroscreen (Brantis *et al.*, 2011). Among those compounds, (S)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid (CATPB) (**Figure 1**) inhibited effects of C3 in cells expressing human FFA2 in both [^{35}S]GTPγS binding and calcium-based assays, with reported pIC_{50} of 7.70 and 8.00, respectively (Brantis *et al.*, 2011). Lack of effect in cells expressing FFA3 indicated CATPB to be specific for FFA2. Experiments using [^{3}H]CATPB showed that C3 was able to fully displace the radiolabeled ligand, consistent with, although not defining, that CATPB binds to the orthosteric site of human FFA2 (Brantis *et al.*, 2011). Subsequent experiments employing [^{35}S]GTPγS binding revealed that CATPB was also able to

decrease the constitutive activity of human FFA2 expressed in HEK293 cells, indicating that CATPB acts as an inverse agonist at this receptor (Hudson *et al.*, 2012b).

The second series of FFA2 receptor antagonists contains an azetidine pharmacophore and was reported by Galapagos NV as potentially of interest for their effects in metabolic and inflammatory disorders, based on an ability to block SCFA-induced neutrophil migration (Sanière *et al.*, 2012). The most studied compound from this series is 4-[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid (GLPG0974) (**Figure 1**). This ligand showed high potency to antagonize acetate-mediated Ca^{2+}-elevation, with a reported pIC_{50} of 8.04 (Pizzonero *et al.*, 2014). Using human neutrophils this compound blocked both acetate-induced migration and expression of the neutrophil activation marker, CD11b[AE], consistent with a potential capacity of GLPG0974 to decrease inflammatory processes (Pizzonero *et al.*, 2014). Good pharmacodynamic properties and the pharmacokinetic profile of this ligand resulted in 'first in man' trials of GLPG0974 in a phase 2, randomized, double blind, and placebo-controlled clinical trial in 2013. During this study, the safety and efficacy of the compound were tested in patients affected by mild to moderate ulcerative colitis. However, no improvement in the clinical profile of patients over a short-term treatment resulted in termination of this programme.

FFA2 antagonists could represent an extremely useful tool for pre-clinical drug development and proof of concept studies. Unfortunately neither of the FFA2 antagonists that have been described to date in primary peer-reviewed publications (CATPB and GLPG0974) appear able to interact with rodent orthologs of FFA2 (**Figure 1**). Recently, Sergeev and colleagues analyzed the binding interaction of [^{3}H]GLPG0974 at hFFA2.

From this study it emerged that the orthosteric antagonists, GLPG0974 and CATPB, do not require interaction with both Arginine residues, Arg 5.39 and 7.35, in the orthosteric binding pocket in order to engage with the receptor (Sergeev *et al.*, 2016). In addition, it was found that these different classes of antagonists displayed preferential interaction with different arginine residues (Sergeev *et al.*, 2016). The characterization of ligand-receptor interactions is likely to be important for the design of ligands that also display antagonism at rodent orthologs of the receptor.

Allosteric ligands

Allosteric modulators are defined as ligands that interact at a site of a receptor which is distinct from and does not overlap with the orthosteric binding site. The interaction of a ligand with an allosteric binding site can generate a conformational change in the receptor that is transduced to the orthosteric site and/or directly to the intracellular effector (Kenakin and Miller, 2010; Wootten *et al.*, 2013). Allosteric ligands can regulate the affinity and/or the potency of orthosteric ligands in a positive (positive allosteric modulators, PAMs) or negative (negative allosteric modulators, NAMs) manner (Kenakin and Miller, 2010; Wootten *et al.*, 2013. Molecules may also bind to the allosteric site but have no effect on receptor activity in which case they are called neutral allosteric ligands (NALs). In addition to potentially modulating the activity of orthosteric ligands allosteric modulators may also possess agonist activity, or intrinsic activity, in their own right (Kenakin and Miller, 2010; Wootten *et al.*, 2013). This is often only evident at high levels of occupancy of the allosteric site and in highly sensitive signal transduction assays.

Therapeutically, allosteric ligands potentially offer certain advantages over orthosteric ligands. This includes improved selectivity due to the fact that allosteric sites are often in non-conserved regions of the receptor and that the co-operativity of allosteric ligands can be receptor subtype specific. Furthermore, the effects of allosteric modulators are saturable, and this can limit possible side-effects including overdose. Moreover, PAMs and NAMs have the advantage of maintaining both temporal and spatial properties of endogenous ligand function, without altering or disrupting the physiological system (Kenakin and Miller, 2010; Wootten *et al.*, 2013).

Currently phenylacetamides, of which 4-CMTB (**Figure 1**) is by far the most studied example, represent the only described class of allosteric modulators at FFA2. 4-CMTB is an allosteric agonist at FFA2 in that it has the ability to activate $G\pm_{i/o}$, $G\pm_{q/11}$ and ^{2}-arrestin-mediated FFA2 pathways directly as well as behaving as a PAM of the potency of SCFAs (Lee *et al.*, 2008; Wang *et al.*, 2010; Smith *et al.*, 2011). However, some studies suggest that 4-CMTB might affect various FFA2-mediated signaling responses somewhat differently than SCFAs (Smith *et al.*, 2011). Whether 4-CMTB displays 'functional selectivity' (Hudson *et al.*, 2013a) at FFA2 thus deserves further investigation.

In vitro, 4-CMTB has been found to produce inhibition of lipolysis in both mouse and human adipocytes (Lee *et al.*, 2008; Wang *et al.*, 2010; Brown *et al.*, 2015), to induce GLP-1 release from enteroendocrine cells (Brown *et al.*, 2015) and to promote chemotaxis of neutrophils (Vinolo *et al.*, 2011). Unfortunately, 4-CMTB has poor pharmacokinetic properties (Wang *et al.*, 2010) and consequently it is not suitable for *in vivo* validation of FFA2.

There is an even greater paucity of available FFA3-selective ligands. The only currently described class of synthetic ligands was identified by Arena Pharmaceuticals (Leonard *et al.*, 2006). This series of ligands was later shown to contain each of allosteric agonists, PAMs, NAMs and PAM-antagonists (Hudson *et al.*, 2014), although the basis for the SAR of such effects remains uncharted. One compound derived from this series, AR420626 (**Figure 1**), has been used to demonstrate the involvement of SCFAs in both GLP-1 secretion from colonic crypts (Nøhr *et al.*, 2013) and ghrelin secretion from gastric mucosa cells (Engelstoft *et al.*, 2013). Although this class of compounds needs to be further developed and characterized, the diverse and rather complex pharmacology of this series of ligands could provide new insight into the biological functions of FFA3. Interestingly, FFA2 and FFA3 allosteric modulators show similar function at human and rodent orthologs of those receptors, where they retain both their potency and allosteric properties (Lee *et al.*, 2008; Hudson *et al.*, 2012b and 2014). This is in contrast to the behavior of the synthetic orthosteric ligands discussed above. Although it has been reasoned that allosteric binding sites should be under less evolutionary pressure to be maintained than orthosteric sites because endogenously produced regulators to not bind to these regions (May et al., 2007, Hudson et al., 2013a) it has been helpful that the allosteric ligands described above do display function at rodent orthologues of FFA2 and FFA3. Considering that different species ingest different amounts of fiber and they are consequently exposed to varying concentrations of SCFAs (Dranse *et al.*, 2013; Milligan *et al.*, 2014), it is reasonable to imagine that this may have driven alterations in the orthosteric binding site between species (Hudson *et al.*, 2014). This could be extremely important in terms of drug development programs. As already introduced, as well as

showing activity at the human receptor, ligands preferably should show activity in different species as pre-clinical studies are performed in animal models. In this regard, allosteric modulators at FFA2 could be useful approach to validate FFA2/FFA3 *in vitro* and *in vivo.*

Biased ligands

In the last 20 years it has become clear that some ligands have the ability to preferentially activate specific receptor-mediated intracellular signaling pathways over others. This phenomenon is defined as “biased signaling” or “functional selectivity” and is believed to reflect the capacity of a receptor to adopt multiple activated states and/or the ability of a ligand to preferentially induce specific receptor active states (Kenakin, 2013; Kenakin and Christopoulos, 2013). It has been suggested that such ‘biased’ ligands may have clinical benefit if they can facilitate beneficial physiological processes without simultaneously driving signals that may be contra-indicated. This has generated many ideas in ligand design and, although still to be shown directly to result in clinical benefit, highlights the need to pharmacologically characterize ligands in an array of functional assays (Kenakin and Christopoulos, 2013; Kenakin, 2015). FFA2 in particular has been reported to be a promiscuous receptor due to its ability to interact with various G proteins and also to recruit arrestins (Brown *et al.*, 2003; Stoddart *et al.*, 2008b). Signal bias is certainly not restricted to synthetic ligands. For example, endogenous ligands for chemokine receptors clearly exert bias (Zweemer *et al.*, 2014) and for receptors, such as FFA2, that respond to multiple endogenously generated ligands, there may be potential for these to display variation in signal flux.

At present it is unclear whether different SCFAs display bias or indeed, whether such bias at SCFA receptors could have a therapeutic application. Certainly, however, biased agonists at FFA2 or FFA3 would be invaluable tools to understand the physiological implications of distinct signaling pathways to the biological effect of these receptors. For example, the role of FFA2 in pancreatic beta cells remains controversial (Priyadarshini *et al.*, 2015; Tang *et al.*, 2015). Nonetheless, $G\pm_{q/11}$ and $G\pm_{i/o}$-dependent pathways in beta cells seem to exert opposite effects relative to insulin secretion, with activation of $G\pm_{q/11}$ signaling leading to increased levels of this hormone, and of $G\pm_{i/o}$ to diminished secretion (Winzell and Ahrén, 2007; Priyadarshini *et al.*, 2015). Whether FFA2 activation in human pancreatic islets can signal through both $G\pm_{q/11}$ and $G\pm_{i/o}$ pathways is still unclear. However, a biased agonist at FFA2 that would preferentially function through $G\pm_{q/11}$, rather than $G\pm_{i/o}$ would certainly be a valuable pharmacological tool to unravel the relative importance of FFA2 signaling pathway/s and their biological role in pancreatic beta cells and potentially in type 2 diabetes.

Limits and extensions to FFA2 and FF3 knock-out studies

As highlighted earlier, a useful technique for discovering the function of GPCRs is to create transgenic mouse knock-out models, and characterize the resulting phenotype(s). In the case of FFA2 and FFA3 however, the results of knocking out either receptor have proven to be conflicting. For example, a disparity is seen in adiposity between the different knock-out models. The FFA2 knock-out mouse produced by Kimura et al. is heavier than the wildtype, and has more adipose tissue (Kimura *et al.*, 2013), but another model shows no difference in weight gain between wildtype and knock-out (Tang *et al.*, 2015), while yet another actually shows the knock-out is

protective against weight gain on a high fat diet (Priyadarshini *et al.*, 2015). Likewise FFA3 knock-out models have both increased adiposity (Bellahcene *et al.*, 2013), and the same amount of adipose tissue as wildtype (Samuel *et al.*, 2008). Model-dependent differences have also been observed in glucose tolerance and insulin sensitivity in both FFA2 (Bjursell *et al.*, 2011; Tolhurst *et al.*, 2012; Priyadarshini *et al.*, 2015; Tang *et al.*, 2015) and FFA3 knock-out lines (Kimura *et al.*, 2011; Tolhurst *et al.*, 2012; Tang *et al.*, 2015).

These discrepancies may be for one of two reasons. Firstly, as the endogenous ligands for FFA2 for FFA3 overlap between the two receptors, knock-out of one may simply result in the compensation by the other. Secondly, *FFAR2* and *FFAR3* in mouse are located adjacent to each other on chromosome 7. Attempts to alter the transcript for one gene may affect the transcription of the other. One group has indeed reported that FFA2 was down-regulated in their FFA3 knock-out model (Zaibi *et al.*, 2010), while others have not checked for this issue.

Chemogenetic approaches to determining the physiological function and drug responses of FFA2 and FFA3

Whereas gene knock-out studies provide an approach for understanding the physiological role of FFA2 and FFA3 and provide important indications of the physiological impact and clinical potential of targeting these receptors, there are more sophisticated genetic approaches that can be adopted that can provide a direct measure of the impact of pharmacologically selective ligands. Work centered largely on the muscarinic receptor family has provided the framework for the development of a

chemogenetic approach where mutations introduced into the orthosteric binding site of receptors result in a loss of activity to the natural ligand but, instead, allows the receptor to be activated by a synthetic chemical ligand that is otherwise inert (Armbruster *et al.*, 2007; Dong *et al.*, 2010; Alvarez-Curto *et al.*, 2011; Urban and Roth, 2015). Such receptor mutants have been termed Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and have been used extensively to define G protein dependent *in vivo* responses (Urban and Roth, 2015).

Using the distinct endogenous ligand selectivity of bovine and human FFA2, two mutations introduced into the orthosteric binding site of human FFA2 reduced the response of the receptor to endogenous SCFAs by >100 fold. This receptor mutant was instead activated by sorbic acid, a naturally produced but not endogenously generated ligand, that activates bovine FFA2 but not human FFA2, as well as a series of small synthetic compounds (Hudson *et al.*, 2012a) (**Figure 3**). Hence these studies generated the first genuine FFA2-DREADD receptor which possessed the following properties (i) the FFA2-DREADD was no longer activated by endogenous SCFAs, (ii) instead this receptor mutant was activated by a synthetic chemical ligand (e.g. in this case sorbic acid) and (iii) the wild type receptor (i.e. human FFA2) was not activated by the synthetic ligand (Hudson *et al.*, 2012a) (**Figure 3**).

This FFA2-DREADD opens up the possibility of employing a chemogenetic approach akin to that successfully employed by the muscarinic-DREADDs. Hence, by using gene targeting techniques that replace the mouse FFA2 gene with the coding sequence for the FFA2-DREADD then the mutant receptor will be expressed at physiologically relevant levels and in the same cell types as the wild type FFA2. By

administration of sorbic acid to these FFA2-DREADD mutant mice researchers will not only be able to define the physiological role of FFA2 but also this approach will provide the first insights into the potential therapeutic response that can be expected of a drug that selectively targets FFA2 (**Figure 3**). Moreover, the FFA2-DREADD retains high affinity for the human specific antagonist ligands CATPB and GLPG0974. As such, on target, FFA2-DREADD mediated effects of sorbic acid will block such effects but not potential off target effects (**Figure 3**). It will be fascinating to see the results of such studies, not least as FFA3 will still be responsive to the endogenously generated SCFAs.

Conclusions

SCFA receptors are relatively newly discovered GPCRs. Emerging evidence suggests that these receptors are implicated in a variety of physiological functions and their pharmacological modulation could represent invaluable therapeutic targets. However, translational pharmacology has been limited by a paucity of selective ligands and by receptor species ortholog differences. Moreover, animal genetic knock-out approaches have resulted in challenging data interpretation, due perhaps to compensatory effects. Alternative and more refined strategies, such as the DREADD chemogenetic approach may hold great potential to unravel the impact of pharmacologically selective ligands at FFA2, and also define the physiological importance of FFA2 vs FFA3.

Authorship contributions

Wrote the manuscript DB, CEM, ABT, GM

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Footnotes

DB and CEM contributed equally to this work.

These studies were funded in part by grants [BB/K019864/1 and BB/K019856/1] from the Biotechnology and Biosciences Research Council (BBSRC).

Legends for Figures

Figure 1

Selectivity of ligands at human and murine FFA2 and FFA3. The main endogenous SCFAs (C2, C3 and C4) activate FFA2 and FFA3 with varying potency (denoted by the thickness of the arrow) and the rank order of activity is not maintained between human and mouse species orthologs (Hudson *et al.*, 2012b). Synthetic allosteric agonists at FFA2 are represented by phenylacetamides, where 4-CMTB is the most potent ligand (Lee *et al.*, 2008; Wang *et al.*, 2010; Smith *et al.*, 2011) and it maintains its activity across human and mouse FFA2 (Hudson *et al.*, 2012b). Allosteric modulators at FFA3 are represented by a class of synthetic ligands that comprises allosteric agonists, such as AR420626, and allosteric antagonists, such as 'compound 6' (Hudson *et al.*, 2014). Both AR420626 and 'compound 6' maintain their activity at the murine species ortholog (Nøhr *et al.*, 2013; Engelstoft *et al.*, 2013; Hudson *et al.*, 2014). Synthetic orthosteric agonists at FFA2 are represented by 'Compound 1' (Hudson *et al.*, 2013b) and SCAs with sp and sp^2 hybridized ± carbon (Schimdt *et al.*, 2011). Alternative, SCAs with substituted sp^3 hybridized ± carbon have a degree of selectivity for FFA3 (Schimdt *et al.*, 2011). The degree of selectivity of 'compound 1' and SCAs is relatively well maintained between human and mouse species orthologs (Hudson *et al.*, 2012b and 2013b). Synthetic orthosteric antagonists at FFA2 are represented by GLPG0974 and CATPB, however these compounds show affinity only for the human species ortholog (Hudson *et al.*, 2012b; Sergeev *et al.*, 2015).

Figure 2

Key actions of SCFAs at sites which express FFA2 and/or FFA3. The physiological roles of FFA2 and FFA3 are complex, and yet to be completely elucidated. Details that remain contentious are marked with a '?'. In PMNs/PBMCs FFA2 causes chemotaxis down a SCFA gradient, and elicits changes in ROS signaling and cytokine release (Säemann *et al.*, 2000; Cavaglieri *et al.*, 2003; Sina et *al.*, 2009; Vinolo *et al.*, 2009, 2011). In enteroendocine cells, FFA2 and FFA3 cause secretion of a variety of gut hormones (Lin

et al., 2012; Tolhurst *et al.*, 2012; Akiba *et al.*, 2015; Psichas *et al.*, 2015). FFA2 promotes adipogenesis, decreases lipolysis, and possibly increases leptin secretion from adipocytes (Xiong *et al.*, 2004; Hong *et al.*, 2005; Ge *et al.*, 2008; Zaibi *et al.*, 2010). FFA3 in neurons increases sympathetic innervation and increases plasticity in the enteric nervous system (Soret *et al.*, 2010; Kimura *et al.*, 2011). In the pancreas acetate may increase and propionate decrease insulin secretion (Priyadarshini and Layden, 2015; Priyadarshini *et al.*, 2015).

Figure 3

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) strategy for FFA2. In wild type-animals, FFA2 and FFA3 are both activated by SCFAs, i.e. propionate (C3). In tissues co-expressing both SCFA receptors, the physiological response of C3 results from the activation of both FFA2 and FFA3. In mice engineered with the humanized FFA2-DREADD, the mutated FFA2 is solely activated by the administration of the non-endogenous ligand, sorbic acid, and inert to the endogenous ligand, C3. Hence, the physiological responses of C3 results only from FFA3 activation, while the responses of sorbic acid is uniquely mediated by FFA2-DREADD activation. Moreover, the FFA2-DREADD retains high affinity for the human specific antagonist ligands CATPB and GLPG0974. As such, on-target FFA2-DREADD-mediated responses of sorbic acid will be blocked by these antagonists but not potential off-target effects.