1521-0111/89/3/388–398$25.00
MOLECULAR PHARMACOLOGY
Copyright © 2016 by The American Society for Pharmacology and Experimental Therapeutics
http://dx.doi.org/10.1124/mol.115.102301
Mol Pharmacol 89:388–398, March 2016

MINIREVIEW

The Pharmacology and Function of Receptors for Short-Chain Fatty Acids

Daniele Bolognini, Andrew B. Tobin, Graeme Milligan, and Catherine E. Moss

Molecular Pharmacology Group, Institute of Molecular, Cell and Systems Biology, University of Glasgow, Glasgow, Scotland, United Kingdom (D.B., G.M.); and Medical Research Council Toxicology Unit, University of Leicester, Leicester, United Kingdom (A.B.T., C.E.M.)

Received November 2, 2015; accepted December 29, 2015

ABSTRACT

Despite some blockbuster G protein–coupled receptor (GPCR) drugs, only a small fraction (~15%) of the more than 390 nonodorant GPCRs have been successfully targeted by the pharmaceutical industry. One way that this issue might be addressed is via translation of recent deorphanization programs 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 predominantly activated by the short-chain 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 β-cells, FFA3 on neurons, and FFA2 on leukocytes and adipocytes means that the biologic role of these receptors likely extends beyond the widely accepted role of regulating peptide hormone release from enteroendocrine cells in the gut. Here, we review the physiologic 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 2–6 carbon atoms. Within the body, they are predominantly the by-product of the fermentation of nondigestible 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 (≥95%) (Topping and Clifton, 2001). In 2003, the previously designated 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, in relation to both the discovery of selective ligands and the physiologic role of these receptors. GPR41 and GPR43 were subsequently renamed FFA3 and FFA2, respectively (Stoddart et al., 2008b), 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;

These studies were funded in part by the Biotechnology and Biosciences Research Council [Grants BB/K019864/1 and BB/K019856/1].
D.B. and C.E.M. contributed equally to this work.
dx.doi.org/10.1124/mol.115.102301.

ABBREVIATIONS: AR420626, *N*-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide; Arg, arginine; BHB, β-hydroxybutyrate; CATPB, (*S*)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid; 4-CMTB, (*S*)-2-(4-chlorophenyl)-3- methyl-*N*-(thiazol-2-yl)butanamide; compound 1, 3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid; compound 6, 2-methyl-5-oxo-4-(3-phenoxyphenyl)-*N*-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide; DREADD, designer receptors exclusively activated by designer drug; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagonlike peptide-1; GLPG0974, -[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid; GPCR, G protein–coupled receptor; GSIS, glucose-stimulated insulin secretion; His, histidine; LE, ligand efficiency; NAM, negative allosteric modular; PAM, positive allosteric modulator; PMN, polymorphic nucleocyte; PTX, pertussis toxin; ROS, reactive oxygen species; SAR, structure-activity relationship; SCA, small, nonfatty acid, carboxylic acid; SCFA, short-chain fatty acid; sorbic acid, 2,4-hexadienoic acid; TM, transmembrane.

Stoddart et al., 2008b), cluster as a group of intronless sequences located in humans 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 physiologic 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., 2008b). 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, whereas, 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 for human FFA3, it is C3 = C4 = C5 > C2 > C1 (Milligan et al., 2009) (Fig. 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, used as a selective activator of FFA2, there is great need to identify more selective synthetic ligands that would allow improved discrimination between the biologic functions of FFA2 versus FFA3 both in vitro and in vivo.

The deorphanization 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., 2008a). Sequence alignment of

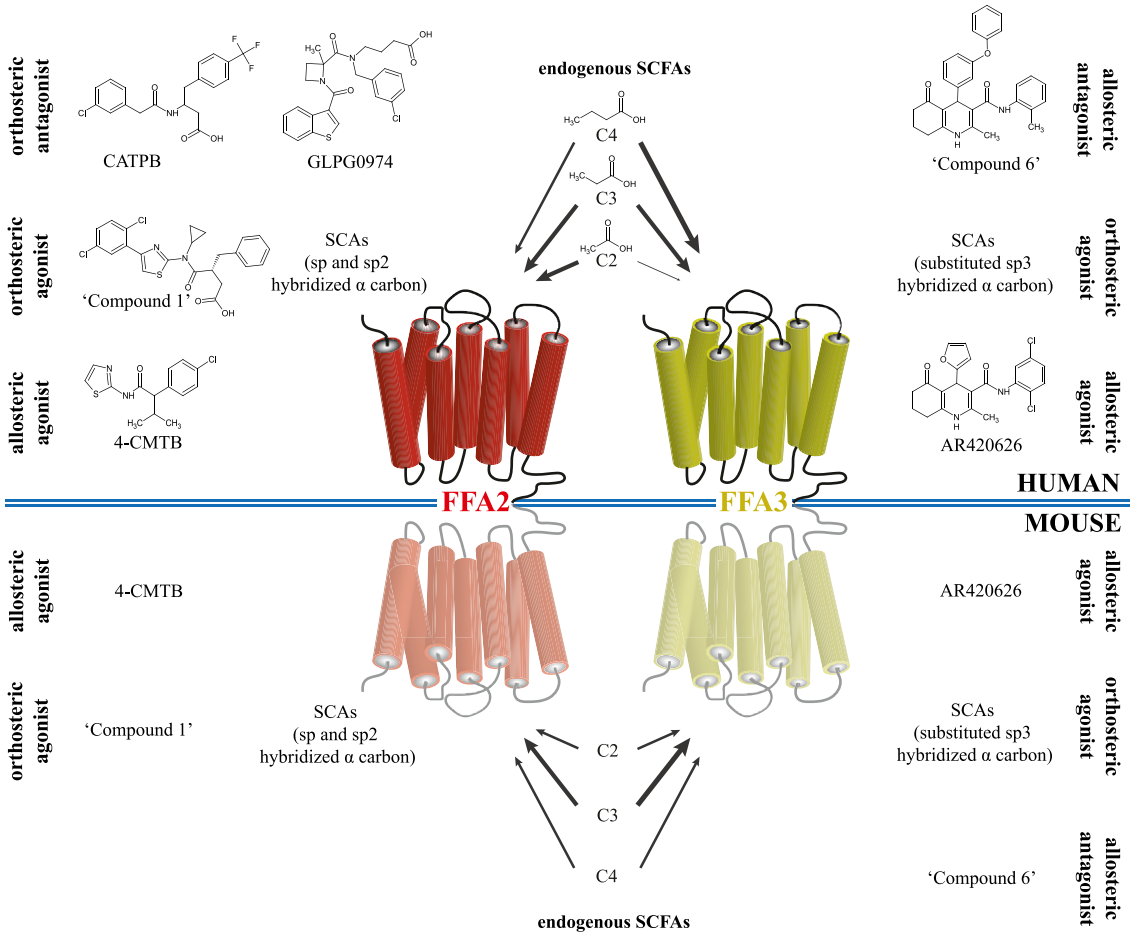

Fig. 1. Selectivity of ligands at human and murine FFA2 and FFA3. The main endogenous SCFAs (C2, C3, and C4) activate FFA2 and FFA3 with varying potency (denoted by the thickness of the arrow), and the rank order of activity is not maintained between human and mouse species orthologs (Hudson et al., 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 comprise allosteric agonists, such as AR420626, and allosteric antagonists, such as 2-methyl-5-oxo-4-(3-phenoxyphenyl)-*N*-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (compound 6) (Hudson et al., 2014). Both AR420626 and compound 6 maintain their activity at the murine species ortholog (Engelstoft et al., 2013; Nøhr et al., 2013; Hudson et al., 2014). Synthetic orthosteric agonists at FFA2 are represented by compound 1 (Hudson et al., 2013a) and SCAs with sp and sp^2 hybridized α carbon (Schmidt et al., 2011). Alternatively, SCAs with substituted sp^3 hybridized α carbon have a degree of selectivity for FFA3 (Schmidt 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, 2013a). 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., 2016).

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 used 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., 2008a). 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), and Arg in TM VII (7.35) (Stoddart et al., 2008a) (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 coordinating 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., 2008a). 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., 2008a). 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. Schmidt and colleagues (2011) established structure-activity relationships (SARs) of a group of small, nonfatty acid, carboxylic acids (SCAs) at FFA2. 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 with FFA3—the potency of such molecules was still very modest and too low to be useful as pharmacological tools for in vitro and, in particular, 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 δg (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 δg 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 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) (Fig. 1). This ligand was shown to cause activation of both $G\alpha_{i/o}$- and $G\alpha_{q/11}$-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 (discussed 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 plays an important role in the allosteric effect of 4-CMTB. Replacement of extracellular loop 2 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 $G\alpha_{i/o}$ and $G\alpha_{q/11}$ proteins (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Moreover, screens performed in strains of the yeast *Saccharomyces cerevisiae* containing different yeast/mammalian $G\alpha$ subunit chimeras also indicated an ability of FFA2 to interact with $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_{14}$ (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 $G\alpha_{i/o}$-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 β-arrestin-1 and, in particular, β-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, 2013a; Lee et al., 2013). To date, there are no published data regarding the ability of FFA3 to recruit arrestin isoforms.

Physiologic 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 (Le Poul et al., 2003) (Fig. 2). Recent studies have confirmed the presence of FFA2 in neutrophils, eosinophils (Maslowski et al., 2009), and in leukocytes of the lamina propria (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 SCFAs recruit PMNs via FFA2 to worsen tissue damage. Moreover, a "first in man" clinical trial with the FFA2 antagonist -[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid (GLPG0974; discussed later) for the treatment of ulcerative colitis did not provide any immediate beneficial effects, resulting in this study being terminated.

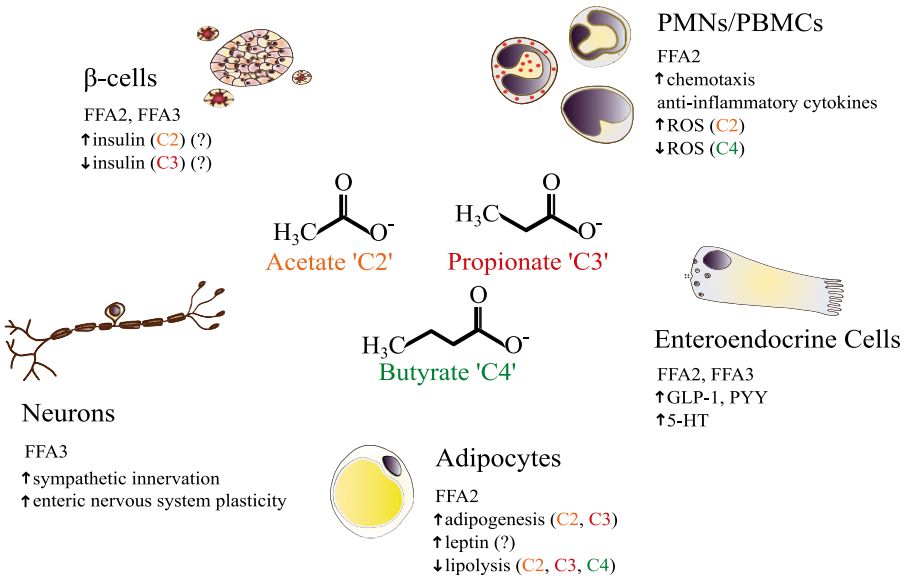

Fig. 2. Key actions of SCFAs at sites which express FFA2 and/or FFA3. The physiologic roles of FFA2 and FFA3 are complex and have yet to be completely elucidated. Details that remain contentious are marked with a ?. In PMNs/peripheral blood mononuclear cells (PBMCs), FFA2 causes chemotaxis down an SCFA 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, insulin secretion may be increased by acetate and decreased by propionate (Priyadarshini and Layden, 2015; Priyadarshini et al., 2015).

Clearly, FFA2 signaling in leukocytes is a complicated scenario that deserves further dissection and analysis.

Most reports suggest that SCFAs mediate a shift from proinflammatory to anti-inflammatory cytokine release from leukocytes. C2 and C4 can inhibit tumor necrosis factor-α 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). Neither FFA2 nor FFA3 knockout mice recruit Th1 cells in a rectal inflammation model (Kim et al., 2013). Finally, C3 can trigger the release of the anti-inflammatory interleukin-10 from regulatory T cells, and this happens in an 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 $G\alpha_{q/11}$. PMNs require calcium for chemotaxis toward *N*-formylmethionyl-leucyl-phenylalanine, 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 an FFA2-, p38-, extracellular signal-regulated kinase 1/2-, and phosphorylated Akt–specific manner, and this appears to be PTX-sensitive, implicating $G\alpha_{i/o,}$ in addition to $G\alpha_{q/11}$ (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 *N*-formylmethionyl-leucyl-phenylalanine via cAMP, and a PTX-sensitive release of G protein $G_{\beta\gamma}$ 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). This may reflect opposing signaling via FFA2 and $G\alpha_{q/11}$ and FFA3 and $G\alpha_{i/o}$. 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, whereas C4 inhibits, the various functions of innate immune cells. This may explain conflicting results using mixes of SCFAs, although nonreceptor 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 (discussed later), the low potency and pleiotropic effects of the SCFAs mean that, without access to new, well characterized, and highly selective synthetic ligands, unraveling 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) (Fig. 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 labeled 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 colocalizes with gastrin and ghrelin in the stomach, and completely colocalizes with cholecystokinin, glucose-dependent insulinotropic polypeptide (GIP), and secretin in the proximal small intestine and with peptide tyrosine tyrosine, neurotensin, and glucagonlike peptide-1 (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), peptide tyrosine tyrosine (Lin et al., 2012; Psichas et al., 2015), and GIP (Lin et al., 2012) release. However, reports as to whether oral SCFAs 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 enterochromaffin cells to trigger 5-hydroxytryptamine 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 total parenteral nutrition. 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) (Fig. 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 reported that, whereas 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 inhibits insulin secretion via $G\alpha_{i/o}$ 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 (discussed later) at FFA2/3. For example, FFA2 agonist–mediated enhancement of GSIS activation is reported to occur via phospholipase C-beta (Priyadarshini et al., 2015). Perhaps FFA2-dependant effects of C2 in the islet are biased to $G\alpha_q$ over $G\alpha_{i/o}$. Studies in FFA2 knockout 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 knockon 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 (Fig. 2). Two of the original deorphanization 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 knockout lines have been reported to show 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 knockout mice (Ge et al., 2008). Reports suggest that FFA2 activation reduces insulin sensitivity in the adipocyte by $G_{\beta\gamma}$-mediated inhibition of Akt phosphorylation downstream of the insulin receptor (Kimura et al., 2013).

SCFAs can drive adipocyte differentiation from stem cells (adipogenesis). C2 and C3 trigger the preadipocyte cell line 3T3-L1 to differentiate, and C3 increases expression of the adipogenesis markers peroxisome proliferator-activated receptor γ-2 and CCAAT/enhancer binding protein α. When these cells are treated with small interfering RNA against FFA2, expression of peroxisome proliferator-activated receptor γ-2 and aP2 (another adipocyte marker) decreases (Hong et al., 2005). FFA2 knockout mice have fewer 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 knockout and wild-type pups in the embryonic stage has even led one group to conclude that the effect of FFA2 in adipogenesis may be an in vitro artifact (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 knockout mice fed a high-fat diet (Bellahcene et al., 2013), although a different FFA2 knockout line had plasma leptin levels comparable to the wild type (Kimura et al., 2013). In vitro, SCFAs also increase leptin mRNA in bovine adipocytes (Soliman et al., 2007) and increases leptin secretion from primary murine adipocytes (Xiong et al., 2004) 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 β-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) (Fig. 2). FFA3 knockout 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. Coculturing cardiomyocytes and neurons together reveals an FFA3-dependent relationship where C3 can increase beat rate. This effect is PTX-sensitive, and involves $G_{\beta\gamma}$, phospholipase C-beta, and extracellular signal-regulated kinase 1/2 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 $G_{\beta\gamma}$ 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 physiologic function of FFA3 in these ganglia has yet to be described. Finally, FFA3 colocalizes 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 physiologic role(s) of a GPCR and its importance in pathophysiologic 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 using 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 coexpression of these two GPCRs in several tissues (discussed earlier) presents a major obstacle in understanding the pathophysiologic role of each receptor. This is further complicated by differences between the pharmacology of species orthologs of FFA2 and FFA3 (Fig. 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 (Fig. 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 nonreceptor-mediated effects, as well as the low potency and lack of selectivity of the SCFAs between FFA2 and FFA3, have 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 versus 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 SA (Gosselies, Belgium) (Hoveyda et al., 2010). From this series of ligands, Hudson et al., (2013a) 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 (Fig. 1). In a range of assays, including those reflecting FFA2-mediated $G\alpha_{i/o}$, $G\alpha_{q/11}$ and β-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., 2013a). 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., 2013a). Moreover, function of compound 1 is inhibited by the orthosteric antagonist (*S*)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid (CATPB) (Hudson et al., 2013a) in a competitive and surmountable fashion. However, compound 1 also contains 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 β-arrestin-2 recruitment assays, compound 1 did not also display competitive interactions with 4-CMTB (Hudson et al., 2013a). 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 $G\alpha_{i/o}$-dependent. Moreover, compound 1 has also been reported to induce GLP-1 release from the murine STC-1 enteroendocrine cell line (Hudson et al., 2013a). Importantly, although compound 1 displays reasonable potency at rodent orthologs of FFA2 (Hudson et al., 2013a), certain other compounds from this chemical series show markedly lower potency in rodents compared with humans (Hudson et al., 2013a). Although 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., 2013a). 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., 2013a).

Orthosteric Antagonist Ligands. Receptor antagonists, by blocking the action of either endogenous or synthetic agonists, routinely provide powerful tools to help define biologic roles of a receptor.

Interest in the biologic 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 shown 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 SA (Brantis et al., 2011). Among those compounds, CATPB (Fig. 1) inhibited effects of C3 in cells expressing human FFA2 in both guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding and calcium-based assays, with reported pIC_{50} values 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, CATPB binding to the orthosteric site of human FFA2 (Brantis et al., 2011). Subsequent experiments using guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate 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 contain an azetidine pharmacophore and were reported by Galapagos NV (Mechelen, Belgium) 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 GLPG0974 (Fig. 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, 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 program.

FFA2 antagonists could represent an extremely useful tool for preclinical 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 (Fig. 1). Recently, Sergeev and colleagues (2016) 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 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 that 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. 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 nonconserved regions of the receptor, and that the cooperativity 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 physiologic system (Kenakin and Miller, 2010; Wootten et al., 2013).

Currently, phenylacetamides, of which 4-CMTB (Fig. 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 $G\alpha_{i/o}$-, $G\alpha_{q/11}$-, and β-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., 2013b) 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 (San Diego, CA) (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, *N*-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (AR420626) (Fig. 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 biologic 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, 2014). This is in contrast to the behavior of the synthetic orthosteric ligands discussed earlier. 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 do not bind to these regions (May et al., 2007; Hudson et al., 2013b), it is helpful that the allosteric ligands described earlier do display function at rodent orthologs 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 preclinical studies are performed in animal models. In this regard, allosteric modulators at FFA2 could be a 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 physiologic processes without simultaneously driving signals that may be contraindicated. 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 to recruit arrestins (Brown et al., 2003; Stoddart et al., 2008a). Signal bias is certainly not restricted to synthetic ligands. For example, endogenous ligands for chemokine receptors clearly exert bias (Zweemer et al., 2014), and receptors, such as FFA2, that respond to multiple endogenously generated ligands may have the potential 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 physiologic implications of distinct signaling pathways to the biologic 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,

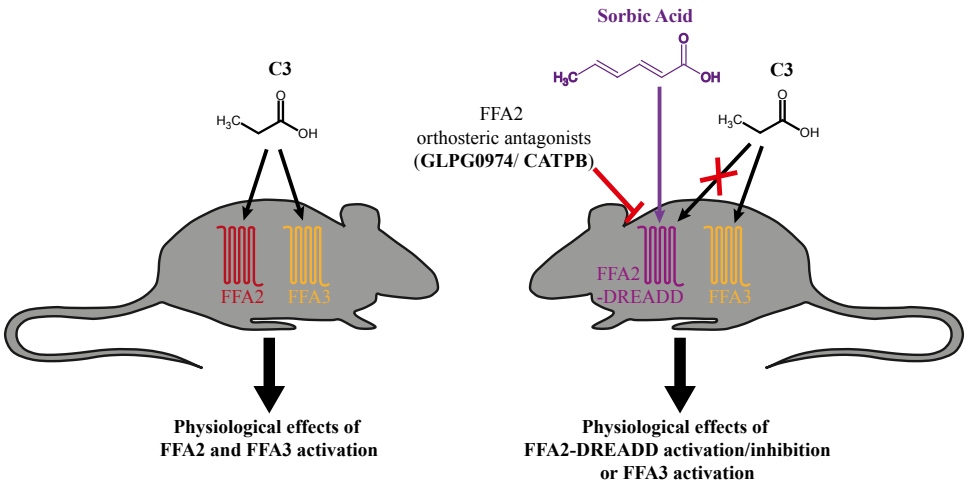

Fig. 3. DREADDs strategy for FFA2. In wild-type animals, FFA2 and FFA3 are both activated by SCFAs, i.e., propionate (C3). In tissues coexpressing both SCFA receptors, the physiologic response of C3 results from the activation of both FFA2 and FFA3. In mice engineered with the humanized FFA2-DREADD, the mutated FFA2 is solely activated by the administration of the nonendogenous ligand sorbic acid and inert to the endogenous ligand C3. Hence, the physiologic responses of C3 result only from FFA3 activation, whereas the responses of sorbic acid are 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.

$G\alpha_{q/11}$- and $G\alpha_{i/o}$-dependent pathways in beta cells seem to exert opposite effects relative to insulin secretion, with activation of $G\alpha_{q/11}$ signaling leading to increased levels of this hormone, and $G\alpha_{i/o}$, to diminished secretion (Winzell and Ahrén, 2007; Priyadarshini et al., 2015). Whether FFA2 activation in human pancreatic islets can signal through both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ pathways is still unclear. However, a biased agonist at FFA2 that would preferentially function through $G\alpha_{q/11}$, rather than $G\alpha_{i/o}$, would certainly be a valuable pharmacological tool to unravel the relative importance of FFA2 signaling pathways and their biologic role in pancreatic beta cells and potentially in type 2 diabetes.

Limits and Extensions to FFA2 and FFA3 Knockout Studies. As highlighted earlier, a useful technique for discovering the function of GPCRs is to create transgenic mouse knockout 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 knockout models. The FFA2 knockout mouse produced by Kimura et al. (2013) is heavier than the wild type, and has more adipose tissue, but another model shows no difference in weight gain between the wild type and knockout (Tang et al., 2015), whereas yet another actually shows the knockout is protective against weight gain on a high fat diet (Priyadarshini et al., 2015). Likewise, FFA3 knockout models have both increased adiposity (Bellahcene et al., 2013) and the same amount of adipose tissue as the wild type (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 knockout lines (Kimura et al., 2011; Tolhurst et al., 2012; Tang et al., 2015).

These discrepancies may be due to one of two reasons. First, as the endogenous ligands for FFA2 for FFA3 overlap between the two receptors, knockout of one may simply result in compensation by the other. Second, *FFAR2* and *FFAR3* in mice 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 downregulated in their FFA3 knockout model (Zaibi et al., 2010), whereas others have not checked for this issue.

Chemogenetic Approaches to Determining the Physiologic Function and Drug Responses of FFA2 and FFA3. Whereas gene knockout studies provide an approach for understanding the physiologic role of FFA2 and FFA3 and provide important indications of the physiologic impact and clinical potential of targeting these receptors, more sophisticated genetic approaches 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, and 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, which activates bovine FFA2 but not human FFA2, as well as a series of small synthetic compounds (Hudson et al., 2012a) (Fig. 3). Hence, these studies generated the first genuine FFA2-DREADD receptor, which possessed the following properties: 1) the FFA2-DREADD was no longer activated by endogenous SCFAs; 2) instead, this receptor mutant was activated by a synthetic chemical ligand (e.g., in this case, sorbic acid); and 3) the wild-type receptor (i.e., human FFA2) was not activated by the synthetic ligand (Hudson et al., 2012a) (Fig. 3).

This FFA2-DREADD opens up the possibility of using a chemogenetic approach akin to that successfully used by the muscarinic-DREADDs. Hence, by using gene-targeting techniques that replace the mouse FFA2 gene with the coding sequence for the FFA2-DREADD, 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, not only will researchers be able to define the physiologic 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 (Fig. 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 (Fig. 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 physiologic 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 knockout approaches have resulted in challenging data interpretation, perhaps due 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 define the physiologic importance of FFA2 versus FFA3.

Authorship contributions

Wrote or contributed to the writing of the manuscript: Bolognini, Moss, Tobin, Milligan.

References

Akiba Y, Inoue T, Kaji I, Higashiyama M, Narimatsu K, Iwamoto K, Watanabe M, Guth PH, Engel E, and Kuwahara A et al. (2015) Short-chain fatty acid sensing in rat duodenum. *J Physiol* **593**:585–599.

Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, and Venema K (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. *Biochim Biophys Acta* **1801**:1175–1183.

Alvarez-Curto E, Prihandoko R, Tautermann CS, Zwier JM, Pediani JD, Lohse MJ, Hoffmann C, Tobin AB, and Milligan G (2011) Developing chemical genetic approaches to explore G protein-coupled receptor function: validation of the use of a receptor activated solely by synthetic ligand (RASSL). *Mol Pharmacol* **80**: 1033–1046.

Armbruster BN, Li X, Pausch MH, Herlitze S, and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci USA* **104**:5163–5168.

Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, Smith DM, Cawthorne MA, Stocker CJ, and Arch JR (2013) Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. *Br J Nutr* **109**:1755–1764.

den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, and Bakker BM (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* **54**:2325–2340.

Bjursell M, Admyre T, Göransson M, Marley AE, Smith DM, Oscarsson J, and Bohlooly-Y M (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. *Am J Physiol Endocrinol Metab* **300**:E211–E220.

Böcker U, Nebe T, Herweck F, Holt L, Panja A, Jobin C, Rossol S, B Sartor R, and Singer MV (2003) Butyrate modulates intestinal epithelial cell-mediated neutrophil migration. *Clin Exp Immunol* **131**:53–60.

Brantis C, Ooms F, and Bernard J (2011) Euroscreen SA. Novel amino acid derivatives and their use as gpr43 receptor modulators. *PCT Int. Appl.* WO2011092284.

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, and Fraser NJ et al. (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278**:11312–11319.

Brown AJ, Tsoulou C, Ward E, Gower E, Bhudia N, Chowdhury F, Dean TW, Faucher N, Gangar A, and Dowell SJ (2015) Pharmacological properties of acid N-thiazolylamide FFA2 agonists. *Pharmacol Res Perspect* **3**:e00141.

Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, and Calignano A (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol* **17**:1519–1528.

Cavaglieri CR, Nishiyama A, Fernandes LC, Curi R, Miles EA, and Calder PC (2003) Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. *Life Sci* **73**:1683–1690.

Chen LW and Jan CR (2001) Mechanisms and modulation of formyl-methionyl-leucyl-phenylalanine (fMLP)-induced Ca2+ mobilization in human neutrophils. *Int Immunopharmacol* **1**:1341–1349.

Crouse JR, Gerson CD, DeCarli LM, and Lieber CS (1968) Role of acetate in the reduction of plasma free fatty acids produced by ethanol in man. *J Lipid Res* **9**: 509–512.

Dass NB, John AK, Bassil AK, Crumbley CW, Shehee WR, Maurio FP, Moore GB, Taylor CM, and Sanger GJ (2007) The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. *Neurogastroenterol Motil* **19**:66–74.

De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Bäckhed F, and Mithieux G (2014) Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* **156**:84–96.

Dewulf EM, Ge Q, Bindels LB, Sohet FM, Cani PD, Brichard SM, and Delzenne NM (2013) Evaluation of the relationship between GPR43 and adiposity in human. *Nutr Metab (Lond)* **10**:11.

Dong S, Rogan SC, and Roth BL (2010) Directed molecular evolution of DREADDs: a generic approach to creating next-generation RASSLs. *Nat Protoc* **5**:561–573.

Dranse HJ, Kelly ME, and Hudson BD (2013) Drugs or diet?–Developing novel therapeutic strategies targeting the free fatty acid family of GPCRs. *Br J Pharmacol* **170**:696–711.

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, and Nøhr MK et al. (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Mol Metab* **2**:376–392.

Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, Tian H, and Li Y (2008) Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. *Endocrinology* **149**:4519–4526.

Gee JM, Lee-Finglas W, Wortley GW, and Johnson IT (1996) Fermentable carbohydrates elevate plasma enteroglucagon but high viscosity is also necessary to stimulate small bowel mucosal cell proliferation in rats. *J Nutr* **126**:373–379.

Hattori H, Subramanian KK, Sakai J, Jia Y, Li Y, Porter TF, Loison F, Sarraj B, Kasorn A, and Jo H et al. (2010) Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis. *Proc Natl Acad Sci USA* **107**: 3546–3551.

He W, Miao FJ, Lin DC, Schwandner RT, Wang Z, Gao J, Chen JL, Tian H, and Ling L (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* **429**:188–193.

Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, and Lee HG et al. (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. *Endocrinology* **146**:5092–5099.

Hopkins AL, Keserü GM, Leeson PD, Rees DC, and Reynolds CH (2014) The role of ligand efficiency metrics in drug discovery. *Nat Rev Drug Discov* **13**:105–121.

Hoveyda H, E. Brantis C, Dutheuil G, Zoute L, Schils D, and Bernard J (2010) Euroscreen SA. Compounds, pharmaceutical composition and methods for use in treating metabolic disorders. *PCT Int. Appl.* WO2011076732.

Hudson BD, Christiansen E, Murdoch H, Jenkins L, Hansen AH, Madsen O, Ulven T, and Milligan G (2014) Complex pharmacology of novel allosteric free fatty acid 3 receptor ligands. *Mol Pharmacol* **86**:200–210.

Hudson BD, Christiansen E, Tikhonova IG, Grundmann M, Kostenis E, Adams DR, Ulven T, and Milligan G (2012a) Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. *FASEB J* **26**:4951–4965.

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, and Tikhonova IG et al. (2013a) Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. *J Biol Chem* **288**:17296–17312.

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, and Milligan G (2012b) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. *J Biol Chem* **287**:41195–41209.

Hudson BD, Ulven T, and Milligan G (2013b) The therapeutic potential of allosteric ligands for free fatty acid sensitive GPCRs. *Curr Top Med Chem* **13**:14–25.

Inoue D, Kimura I, Wakabayashi M, Tsumoto H, Ozawa K, Hara T, Takei Y, Hirasawa A, Ishihama Y, and Tsujimoto G (2012) Short-chain fatty acid receptor GPR41-mediated activation of sympathetic neurons involves synapsin 2b phosphorylation. *FEBS Lett* **586**:1547–1554.

Kataoka K, Ogasa S, Kuwahara T, Bando Y, Hagiwara M, Arimochi H, Nakanishi S, Iwasaki T, and Ohnishi Y (2008) Inhibitory effects of fermented brown rice on induction of acute colitis by dextran sulfate sodium in rats. *Dig Dis Sci* **53**: 1601–1608.

Kenakin T (2013) New concepts in pharmacological efficacy at 7TM receptors: IUPHAR review 2. *Br J Pharmacol* **168**:554–575.

Kenakin T (2015) The effective application of biased signaling to new drug discovery. *Mol Pharmacol* **88**:1055–1061.

Kenakin T and Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* **12**:205–216.

Kenakin T and Miller LJ (2010) Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol Rev* **62**:265–304.

Kim MH, Kang SG, Park JH, Yanagisawa M, and Kim CH (2013) Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology* **145**:396–406.

Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, and Tsujimoto G (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proc Natl Acad Sci USA* **108**:8030–8035.

Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, and Tani T et al. (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. *Nat Commun* **4**:1829.

Lane JR, Sexton PM, and Christopoulos A (2013) Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends Pharmacol Sci* **34**:59–66.

Layden BT, Durai V, Newman MV, Marinelarena AM, Ahn CW, Feng G, Lin S, Zhang X, Kaufman DB, and Jafari N et al. (2010) Regulation of pancreatic islet gene expression in mouse islets by pregnancy. *J Endocrinol* **207**:265–279.

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, and Van Damme J et al. (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem* **278**:25481–25489.

Lee SU, In HJ, Kwon MS, Park BO, Jo M, Kim MO, Cho S, Lee S, Lee HJ, and Kwak YS et al. (2013) β-Arrestin 2 mediates G protein-coupled receptor 43 signals to nuclear factor-κB. *Biol Pharm Bull* **36**:1754–1759.

Lee T, Schwandner R, Swaminath G, Weiszmann J, Cardozo M, Greenberg J, Jaeckel P, Ge H, Wang Y, and Jiao X et al. (2008) Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. *Mol Pharmacol* **74**:1599–1609.

Lemor A, Mielenz M, Altmann M, von Borell E, and Sauerwein H (2010) mRNA abundance of adiponectin and its receptors, leptin and visfatin and of G-protein coupled receptor 41 in five different fat depots from sheep. *J Anim Physiol Anim Nutr (Berl)* **94**:e96–e101.

Leonard JN, Chu ZL, Bruce MA, and Boatman PD(2006) Arena Pharmaceuticals. Gpr41 and modulators thereof for the treatment of insulin-related disorders. Patent number US 20080312277 A1.

Liaw CW and Connolly DT (2009) Sequence polymorphisms provide a common consensus sequence for GPR41 and GPR42. *DNA Cell Biol* **28**:555–560.

Lin HV, Frassetto A, Kowalik EJ, Jr, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, and Forrest G et al. (2012) Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS One* **7**:e35240.

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, and Artis D et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* **461**:1282–1286.

May LT, Leach K, Sexton PM, and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**:1–51.

McOrist AL, Abell GC, Cooke C, and Nyland K (2008) Bacterial population dynamics and faecal short-chain fatty acid (SCFA) concentrations in healthy humans. *Br J Nutr* **100**:138–146.

Mielenz M, Seybold C, and Sauerwein H (2008) Effects of short-term infusion with propionate on the mRNA expression of a putative G-protein coupled receptor 41 (GPR41) in adipose tissue of goats. *Livest Sci* **116**:328–331.

Milligan G, Stoddart LA, and Smith NJ (2009) Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3. *Br J Pharmacol* **158**: 146–153.

Milligan G, Ulven T, Murdoch H, and Hudson BD (2014) G-protein-coupled receptors for free fatty acids: nutritional and therapeutic targets. *Br J Nutr* **111** (Suppl 1): S3–S7.

Nakao S, Fujii A, and Niederman R (1992) Alteration of cytoplasmic Ca2+ in resting and stimulated human neutrophils by short-chain carboxylic acids at neutral pH. *Infect Immun* **60**:5307–5311.

Nilsson AC, Östman EM, Knudsen KE, Holst JJ, and Björck IM (2010) A cereal-based evening meal rich in indigestible carbohydrates increases plasma butyrate the next morning. *J Nutr* **140**:1932–1936.

Nilsson NE, Kotarsky K, Owman C, and Olde B (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochem Biophys Res Commun* **303**:1047–1052.

Nøhr MK, Egerod KL, Christiansen SH, Gille A, Offermanns S, Schwartz TW, and Møller M (2015) Expression of the short chain fatty acid receptor GPR41/FFAR3 in autonomic and somatic sensory ganglia. *Neuroscience* **290**:126–137.

Nøhr MK, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, and Han S et al. (2013) GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology* **154**:3552–3564.

Nordenfelt P and Tapper H (2011) Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* **90**:271–284.

Ohira H, Fujioka Y, Katagiri C, Mamoto R, Aoyama-Ishikawa M, Amako K, Izumi Y, Nishiumi S, Yoshida M, and Usami M et al. (2013) Butyrate attenuates inflammation and lipolysis generated by the interaction of adipocytes and macrophages. *J Atheroscler Thromb* **20**:425–442.

Patel DG and Singh SP (1979) Effect of ethanol and its metabolites on glucose mediated insulin release from isolated islets of rats. *Metabolism* **28**:85–89.

Pizzonero M, Dupont S, Babel M, Beaumont S, Bienvenu N, Blanqué R, Cherel L, Christophe T, Crescenzi B, and De Lemos E et al. (2014) Discovery and optimization of an azetidine chemical series as a free fatty acid receptor 2 (FFA2) antagonist: from hit to clinic. *J Med Chem* **57**:10044–10057.

Pratt VC, Tappenden KA, McBurney MI, and Field CJ (1996) Short-chain fatty acid-supplemented total parenteral nutrition improves nonspecific immunity after intestinal resection in rats. *JPEN J Parenter Enteral Nutr* **20**:264–271.

Priyadarshini M and Layden BT (2015) FFAR3 modulates insulin secretion and global gene expression in mouse islets. *Islets* **7**:e1045182.

Priyadarshini M, Villa SR, Fuller M, Wicksteed B, Mackay CR, Alquier T, Poitout V, Mancebo H, Mirmira RG, and Gilchrist A et al. (2015) An Acetate-Specific GPCR, FFAR2, Regulates Insulin Secretion. *Mol Endocrinol* **29**:1055–1066.

Psichas A, Sleeth ML, Murphy KG, Brooks L, Bewick GA, Hanyaloglu AC, Ghatei MA, Bloom SR, and Frost G (2015) The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. *Int J Obes* **39**: 424–429.

Puhl HL, 3rd, Won YJ, Lu VB, and Ikeda SR (2015) Human GPR42 is a transcribed multisite variant that exhibits copy number polymorphism and is functional when heterologously expressed. *Sci Rep* **5**:12880.

Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M, and Coughlin SR (2007) Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. *J Clin Invest* **117**:4034–4043.

Reimer RA and McBurney MI (1996) Dietary fiber modulates intestinal proglucagon messenger ribonucleic acid and postprandial secretion of glucagon-like peptide-1 and insulin in rats. *Endocrinology* **137**:3948–3956.

Robertson MD, Bickerton AS, Dennis AL, Vidal H, and Frayn KN (2005) Insulin-sensitizing effects of dietary resistant starch and effects on skeletal muscle and adipose tissue metabolism. *Am J Clin Nutr* **82**:559–567.

Sabirsh A, Bywater RP, Bristulf J, Owman C, and Haeggström JZ (2006) Residues from transmembrane helices 3 and 5 participate in leukotriene B4 binding to BLT1. *Biochemistry* **45**:5733–5744.

Säemann MD, Böhmig GA, Osterreicher CH, Burtscher H, Parolini O, Diakos C, Stöckl J, Hörl WH, and Zlabinger GJ (2000) Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J* **14**:2380–2382.

Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, and Yanagisawa M et al. (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci USA* **105**:16767–16772.

Sanière LRM, Pizzonero MR, Triballeau N, Vandeghinste NER, De Vos SIJ, Brys RCX, and Pourbaix-L'Ebraly CD (2013) Galapagos NV. Azetidine derivatives useful for the treatment of metabolic and inflammatory diseases. PCT Int. Appl. EP 2665704

Sawzdargo M, George SR, Nguyen T, Xu S, Kolakowski LF, and O'Dowd BF (1997) A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. *Biochem Biophys Res Commun* **239**:543–547.

Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, Ward RJ, Drewke C, Milligan G, and Kostenis E et al. (2011) Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. *J Biol Chem* **286**:10628–10640.

Sergeev E, Hansen H, Pandey SK, MacKenzie AE, Hudson BD, Ulven T, and Milligan G (2016) Non-equivalence of key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. *J Biol Chem* **291**:303–317.

Sethi JK and Vidal-Puig AJ (2007) Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. *J Lipid Res* **48**:1253–1262.

Shah JH, Wongsurawat N, and Aran PP (1977) Effect of ethanol on stimulus-induced insulin secretion and glucose tolerance. A study of mechanisms. *Diabetes* **26**: 271–277.

Sina C, Gavrilova O, Förster M, Till A, Derer S, Hildebrand F, Raabe B, Chalaris A, Scheller J, and Rehmann A et al. (2009) G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. *J Immunol* **183**: 7514–7522.

Smith C (2003) Drug target validation: Hitting the target. *Nature* **422**:341–, 343, 345 passim.

Smith NJ, Ward RJ, Stoddart LA, Hudson BD, Kostenis E, Ulven T, Morris JC, Tränkle C, Tikhonova IG, and Adams DR et al. (2011) Extracellular loop 2 of the free fatty acid receptor 2 mediates allosterism of a phenylacetamide ago-allosteric modulator. *Mol Pharmacol* **80**:163–173.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, and Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**:569–573.

Soliman M, Kimura K, Ahmed M, Yamaji D, Matsushita Y, Okamatsu-Ogura Y, Makondo K, and Saito M (2007) Inverse regulation of leptin mRNA expression by short- and long-chain fatty acids in cultured bovine adipocytes. *Domest Anim Endocrinol* **33**:400–409.

Soret R, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, and Neunlist M (2010) Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. *Gastroenterology* **138**:1772–1782.

Stitham J, Stojanovic A, Merenick BL, O'Hara KA, and Hwa J (2003) The unique ligand-binding pocket for the human prostacyclin receptor. Site-directed mutagenesis and molecular modeling. *J Biol Chem* **278**:4250–4257.

Stoddart LA, Smith NJ, Jenkins L, Brown AJ, and Milligan G (2008a) Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. *J Biol Chem* **283**:32913–32924.

Stoddart LA, Smith NJ, and Milligan G (2008b) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. *Pharmacol Rev* **60**:405–417.

Tang C, Ahmed K, Gille A, Lu S, Gröne HJ, Tunaru S, and Offermanns S (2015) Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. *Nat Med* **21**:173–177.

Tang Y, Chen Y, Jiang H, Robbins GT, and Nie D (2011) G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. *Int J Cancer* **128**:847–856.

Tappenden KA and McBurney MI (1998) Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. *Dig Dis Sci* **43**:1526–1536.

Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, and Gribble FM (2012) Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes* **61**:364–371.

Topping DL and Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev* **81**: 1031–1064.

Tunaru S, Lättig J, Kero J, Krause G, and Offermanns S (2005) Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). *Mol Pharmacol* **68**:1271–1280.

Ulven T (2012) Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. *Front Endocrinol (Lausanne)* **3**:111.

Urban DJ and Roth BL (2015) DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. *Annu Rev Pharmacol Toxicol* **55**:399–417.

Vieira AT, Macia L, Galvão I, Martins FS, Canesso MC, Amaral FA, Garcia CC, Maslowski KM, De Leon E, and Shim D et al. (2015) A Role for Gut Microbiota and the Metabolite-Sensing Receptor GPR43 in a Murine Model of Gout. *Arthritis Rheumatol* **67**:1646–1656.

Vinolo MA, Ferguson GJ, Kulkarni S, Damoulakis G, Anderson K, Bohlooly-Y M, Stephens L, Hawkins PT, and Curi R (2011) SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. *PLoS One* **6**:e21205.

Vinolo MA, Hatanaka E, Lambertucci RH, Newsholme P, and Curi R (2009) Effects of short chain fatty acids on effector mechanisms of neutrophils. *Cell Biochem Funct* **27**:48–55.

Wang Y, Jiao X, Kayser F, Liu J, Wang Z, Wanska M, Greenberg J, Weiszmann J, Ge H, and Tian H et al. (2010) The first synthetic agonists of FFA2: Discovery and SAR of phenylacetamides as allosteric modulators. *Bioorg Med Chem Lett* **20**:493–498.

Winzell MS and Ahrén B (2007) G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. *Pharmacol Ther* **116**:437–448.

Won YJ, Lu VB, Puhl HL, 3rd, and Ikeda SR (2013) β-Hydroxybutyrate modulates N-type calcium channels in rat sympathetic neurons by acting as an agonist for the G-protein-coupled receptor FFA3. *J Neurosci* **33**:19314–19325.

Wong JM, de Souza R, Kendall CW, Emam A, and Jenkins DJ (2006) Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* **40**:235–243.

Wootten D, Christopoulos A, and Sexton PM (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. *Nat Rev Drug Discov* **12**:630–644.

Xavier RJ and Podolsky DK (2007) Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**:427–434.

Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, and Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. *Proc Natl Acad Sci USA* **101**:1045–1050.

Yajima H, Komatsu M, Schermerhorn T, Aizawa T, Kaneko T, Nagai M, Sharp GW, and Hashizume K (1999) cAMP enhances insulin secretion by an action on the ATP-sensitive K+ channel-independent pathway of glucose signaling in rat pancreatic islets. *Diabetes* **48**:1006–1012.

Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJ, Smith DM, and Arch JR (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. *FEBS Lett* **584**: 2381–2386.

Zweemer AJ, Toraskar J, Heitman LH, and IJzerman AP (2014) Bias in chemokine receptor signalling. *Trends Immunol* **35**:243–252.

Address correspondence to: Daniele Bolognini, Molecular Pharmacology Group, Wolfson Link Building 253, University of Glasgow, University Avenue, Glasgow G12 8QQ, UK. E-mail: daniele.bolognini@glasgow.ac.uk or Catherine E. Moss, Medical Research Council Toxicology Unit, University of Leicester, Leicester LE1 9HN. E-mail: cm619@leicester.ac.uk