Chemogenetics defines receptor-mediated functions of short chain free fatty acids

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Conflicts of interest

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Keywords

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Abstract

Differentiating actions of Short Chain Fatty Acids (SCFAs) at Free Fatty Acid receptor 2 (FFA2) from other free fatty acid-responsive receptors, and from non-receptor mediated effects, has been challenging. Using a novel chemogenetic and knockin strategy whereby an engineered variant of FFA2 (FFA2-DREADD) that is unresponsive to natural SCFAs but instead is activated by sorbic acid replaced the wild type receptor we determined that activation of FFA2 in differentiated adipocytes and colonic crypt enteroendocrine cells of mouse accounts fully for SCFA-regulated lipolysis and release of the incretin glucagon like peptide-1 (GLP-1) respectively. *In vivo* studies confirmed the specific role of FFA2 in GLP-1 release and also demonstrated a direct role for FFA2 in accelerating gut transit. Thereby we establish the general principle that such a chemogenetic knockin strategy can successfully define novel GPCR biology and both provide target validation and establish therapeutic potential of a 'hard to target' GPCR.

Introduction

The health benefits of a balanced diet rich in fibre are well established, however understanding of mechanisms underlying these benefits has only recently come to light with appreciation of the relationship between ingested food, the gut microbiome and health and well-being[1]. In the case of dietary fibre, fermentation of poorly digested carbohydrates by the gut microbiota produces prodigious amounts of SCFAs, particularly acetate and propionate[2]. These SCFAs, in addition to acting as an energy source, also have direct biological activities by acting in a hormone-like fashion through specific G protein-coupled receptors (GPCRs)[3-5]. The physiological roles of the two best characterised SCFA-activated GPCRs, FFA2 and FFA3 (previously known as GPR41 and GPR43 [3]), however are challenging to define as the potency of SCFAs is both low and similar between these two subtypes. Moreover,

pharmacological tools for these receptors are limited and not broadly available[6-7] and the only described FFA2 antagonists are 'human specific' and not effective at rodent orthologues[6-8]. Despite this, there is great interest in the potential therapeutic utility of targeting this receptor class and the FFA2 antagonist GLPG0974 ((4-[[1-(benzo[*b*]thiophene-3-carbonyl)-2-methylazetidine-2-carbonyl]-(3-chlorobenzyl)amino]butyric acid) has been assessed in 'first-in-man' studies designed to treat ulcerative colitis[9].

To address the physiological role of FFA2 we have adopted a chemogenetic approach pioneered largely using muscarinic acetylcholine receptors. In this approach mutations in the orthosteric binding site are introduced that prevent receptor activation by endogenous agonists but instead allows for activation by otherwise inert chemical ligands. Such mutant receptors, called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) have been widely used to probe signalling pathways that underlie both central and peripheral responses[10-15]. Here we have taken an alternative approach employing a novel FFA2-DREADD[16] knocked into the gene locus of the wild type receptor to generate a strain of mice that would mimic an FFA2-receptor knockout strain because the DREADD receptor is predicted to be unresponsive to endogenous SCFAs. However, unlike a gene knockout, phenotypic and functional deficits observed in the FFA2-DREADD-knockin mice should, theoretically, be rescued by a FFA2-DREADD specific ligand, in this case sorbic acid (SA), which exclusively activates the DREADD receptor[16].

Previously we generated a DREADD variant of human (h) FFA2[16] by substitution of two amino acids, located in the 4th and 6th transmembrane regions (Cys4.57Gly and His6.55Gln, position numbers as defined by the Ballesteros and Weinstein numbering system[17]). In so doing we took advantage of the observed variation in the chain length of fatty acids that act as agonists at certain species orthologues of FFA2 and linked this with homology modelling and mutagenesis to infer potentially important roles for these two

residues[16]. The hFFA2-DREADD variant no longer responded effectively to the SCFAs acetate and propionate but, rather, could be activated by a number of other ligands, including SA, which had no effect at the wild type receptor, nor at FFA3[16.] Importantly, the hFFA2-DREADD was not only activated by SA but, as shown here, is antagonised effectively by a pair of chemically distinct, human orthologue specific, FFA2 antagonists[8, 18-20].

By employing these tools here we tested the hypothesis that a DREADD-knockin mouse strain would induce phenotypic deficits that mimicked a knockout strain and that this deficit might be rescued by a DREADD ligand. Furthermore, we tested if the DREADD ligand response might be blocked by antagonists that are only active at the species orthologue from which the DREADD was derived. Thus, we report here that by using FFA2 as an exemplar receptor we were able to employ hFFA2-DREADD to probe the physiological role of FFA2 in the gut and white adipose tissue and also provide evidence for the general principle that employing DREADD-knockin mice can be an effective strategy to probe the function and therapeutic potential of 'hard to target' GPCRs.

Results

Properties of hFFA2-DREADD

Early studies on hFFA2 suggested that this receptor is able to interact, at least *in vitro*, with a broad range of G proteins[21]. We compared, therefore, the ability of propionate (C3) and SA to promote interactions of either wild type hFFA2 or hFFA2-DREADD across the range of mammalian G protein α subunits. Initially we employed a bioluminescence resonance energy transfer (BRET)-based biosensor strategy that reports on agonist-induced interactions between a *Renilla* luciferase (Rluc)-tagged form of the G protein-coupled receptor kinase (GRK)2 and a G protein β/γ complex containing a GFP_{10}-tagged form of the γ5 subunit[22]. This biosensor is based on the competition between Gα and GRK2 for

interaction with Gβγ upon over-expression of each individual unlabeled Gα subunit. The separation of Gβγ from the Gα subunit upon receptor activation produces an increase in BRET between GRK2-GFP_{10} and RlucII-Gγ5. Following co-transfection of HEK293 cells expressing the BRET biosensors with either hFFA2 or hFFA2-DREADD and each of thirteen distinct G protein α subunits addition of C3 to cells expressing hFFA2 resulted in enhanced BRET signal in the presence of all the G protein α subunits tested, except for $G\alpha_s$ (**Figure 1a**). The magnitude of the enhanced BRET signal varied between individual G protein α subunits but the wide-ranging capacity of hFFA2 to interact with different G proteins confirmed that agonist-activated hFFA2 can be pleiotropic in activation of G protein subtypes [8,21]. In cells expressing hFFA2-DREADD addition of SA resulted in an entirely equivalent profile of G protein interaction capacity and selectivity (**Figure 1b**). Thus, despite engineering the orthosteric binding site to generate a DREADD variant receptor this did not result in altered canonical, G protein-mediated signal transduction potential in response to the DREADD specific agonist. These studies established that, when acting at the hFFA2-DREADD receptor, SA is able to produce the same and full panoply of G protein interactions as when C3 binds to wild type FFA2.

To extend these comparisons we employed a second set of biosensors that instead directly sense separation induced by receptor activation between a G protein α subunit tagged with *Renilla* luciferase and the G protein γ1 subunit tagged with the fluorescent protein GFP_{10}. When co-expressed alongside wild type hFFA2, C3 promoted a reduction in BRET signal for the α subunit of each of G_q, G_{i1}, and G_{13}, but once again not for G_s (**Supplementary Figure 1a**). The same was true for SA when this sensor pairing was co-expressed with hFFA2-DREADD **(Supplementary Figure 1b)**. Parallel studies using the M_3 muscarinic acetylcholine receptor showed the agonist carbachol to cause activation of only G_q **(Supplementary Figure 1c**) and indicated that the broad range of G proteins activated by

the hFFA2 and hFFA2-DREADD receptors is not an artefact related to the assay biosensors employed.

We next investigated the signalling of these receptors specifically through G_i and $G_{q/11}$ respectively in cAMP and inositol monophosphate regulation assays that reflect directly activation of these G proteins (**Figures 1 c-f)**. As expected, at hFFA2 inhibition of cAMP and accumulation of inositol monophosphates was effectively induced by the endogenous SCFA C3 whereas SA showed essentially no activity (**Figures 1 c, e)**. In contrast, at hFFA2-DREADD SA produced potent regulation of both cAMP and inositol monophosphates whereas C3 was at least 1000 fold less potent **(Figures 1d, f)**.

In addition to signalling through heterotrimeric G proteins hFFA2 can recruit arrestin adaptor proteins[23-24]. C3, but not SA, was able to promote interactions between wild type hFFA2 and β-arrestin-2 in a concentration-dependent manner (**Supplementary Figure 2a**), whilst the opposite pattern of ligand-induced engagement was observed for interactions between hFFA2-DREADD and β-arrestin-2 (**Supplementary Figure 2b**). These studies markedly expanded the previously reported high selectivity of SA for hFFA2-DREADD and lack of activity of this ligand at wild type hFFA2[16].

hFFA2 antagonists inhibit hFFA2-DREADD activity

Two classes of high affinity synthetic FFA2 antagonists have been described[6-7]. Exemplars from both series, including CATPB ((*S*)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid) [18] and GLPG0974 [20] display high affinity at hFFA2 but not at rodent orthologues of FFA2 [6-8]. Here we assessed whether the ability of both CATPB and GLPG0974 to block wild type hFFA2 was preserved for hFFA2-DREADD. hFFA2 binds [^{3}H]GLPG0974 with high affinity (K_d = 7.5 nM)[19] and hFFA2-DREADD bound [^{3}H]GLPG0974 with similar high affinity (K_d = 2.2 ± 0.16 nM) (**Supplementary**

Figure 3a). Competition binding experiments against [^{3}H]GLPG0974 confirmed both GLPG0974 and CATPB interacted with similar affinities (pK_i) at each of wild type hFFA2 and hFFA2-DREADD **(Supplementary Figures 3b, c, Supplementary Table 1)**. Both antagonists were also able to inhibit, in a concentration-dependent fashion, recruitment of β-arrestin-2 to the appropriate receptor variants induced by EC_{80} concentrations of either C3 or SA (**Supplementary Figures 3d, e, Supplementary Table 1**).

Generation and characterisation of hFFA2-DREADD-HA mice

CRE-loxP technology was next employed to generate transgenic knockin mouse lines in which a section of exon 5 of the *Ffar2* gene, containing the coding sequence for the mouse receptor, was replaced by sequences encoding for hFFA2-DREADD. Furthermore, a loxP-STOP-loxP cassette was placed upstream of the receptor coding sequences whilst the receptor sequence was appended with an in-frame, C-terminal haemaglutinin (HA) epitope tag. Keeping the loxP-STOP-loxP cassette intact generated a strain that should express neither the wild type receptor nor the hFFA2-DREADD-HA receptor. Effectively this strain is an FFA2-knockout line, which we here term "MINUS-CRE". By crossing the MINUS-CRE strain with a whole body CRE-recombinase expressing strain, we generated mice where expression of hFFA2-DREADD-HA was driven from the endogenous mouse *Ffar2* promoter. Under these conditions hFFA2-DREADD-HA receptor should be expressed in cell types and at levels equivalent to wild type mouse FFA2, this mouse strain is termed hFFA2-DREADD-HA (**Supplementary Figure 4; see Methods section**).

Animals were genotyped based on the presence of the inserted transgene, whilst direct sequencing of the transgene validated the presence of the DREADD mutations and HA-epitope tag sequence (**Methods**). qRT-PCR performed using mouse FFA2 (**Figure 2a**) or hFFA2-DREADD-HA (**Figure 2b**) specific primers indicated that corresponding transcripts

showed quantitatively similar patterns of expression between wild type C57BL/6 mice and homozygous hFFA2-DREADD-HA expressing animals in a selection of tissues known to express FFA2 (white adipose tissue, colon, lung) but to be lacking in other tissues (liver and cortex) (**Figures 2a, 2b**). Moreover, as anticipated from the genetic design, transcripts corresponding to hFFA2-DREADD-HA were not detected in tissues from the MINUS-CRE strain of mice (**Figure 2c**). Because *Ffar2* and the closely related receptor gene *Ffar3* are linked at chromosomal location 7A3, certain lines of FFA2 or FFA3 knockout mice have been reported to have altered expression of the other receptor[25]. However, we confirmed that mRNA encoding FFA3 was expressed equally in colonic tissue of C57BL/6, MINUS-CRE, and homozygous hFFA2-DREADD-HA expressing mice (**Figure 2d**).

Previous studies from our laboratory[26] and others[27-29] have suggested a role for FFA2 in the release of the incretin GLP-1 from colonic crypts. Examination of the expression of hFFA2-DREADD-HA in the colon illustrated sparse expression within subsets of enteroendocrine cells of the colonic crypts **(Figure 3)** as determined by co-staining of the HA-tagged hFFA2-DREADD with chromogranin A, a broad-spectrum marker for enteroendocrine cells, particularly monoamine-storing enteroendocrine cells[30]. By contrast, specific HA-immunostaining was not detected in equivalent sections from either C57BL/6 **(Figure 3)** or the MINUS-CRE mice **(Supplementary Figure 5a)**. Expression of hFFA2-DREADD-HA was also detected in a broader range of cells. For example, clusters of submucosal cells in the duodenum were observed that were not co-stained with chromogranin A, indicating that they were distinct from entroendocrine cells and likely represent submucosal leukocytes **(Supplementary Figure 5b)**. Importantly, these cell clusters also could not be observed in equivalent sections from either C57BL/6 or the MINUS-CRE mice **(Supplementary Figure 5b)**.

hFFA2-DREADD-HA releases GLP-1 from colonic crypts

We next examined release of GLP-1 from isolated colonic crypt-containing preparations. These preparations responded to the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) with an approximately five-fold increase in release of GLP-1 over basal levels (**Figure 4a**). The extent of IBMX response was equivalent in crypt preparations derived from each of C57BL/6, hFFA2-DREADD-HA and the MINUS-CRE mice (**Figure 4a**). As anticipated from previous work[26, 28], C3 also promoted GLP-1 release from colonic crypts derived from C57BL/6 mice (**Figure 4b**), but SA did not (**Figure 4b**). By contrast, SA specifically promoted the release of GLP-1 from colonic crypts derived from hFFA2-DREADD-HA mice (**Figure 4c**) whilst C3 did not. Neither C3 nor SA was effective in preparations derived from the MINUS-CRE mouse strain (**Figure 4d**). These studies provided evidence that FFA2 mediates GLP-1 release from mouse colonic crypts. However, to confirm the effects of SA in tissues from the hFFA2-DREADD-HA expressing mice were not the result of undefined 'off-target' effects of SA, we employed the structurally distinct, human selective, FFA2 antagonists CATPB and GLPG0974 to potentially block the effects of SA. Although neither CATPB nor GLPG0974 affected basal GLP-1 secretion when added alone (**Figures 4e, 4f**), both effectively blocked the release of the incretin produced by SA in tissue derived from hFFA2-DREADD-HA mice (**Figures 4e, 4f**). By contrast, no effect of either antagonist was observed on C3-mediated GLP-1 release from wild type C57BL/6 mice (**Supplementary Figure 6**).

Sections of the colon from either C57BL/6 or hFFA2-DREADD-HA mice were next introduced into a tissue bath. Following initial flow of buffer through the lumen of the colon to establish baseline GLP-1 release, C3 was added to preparations from C57BL/6 mice **(Figure 4g)** whilst SA was added to preparations from hFFA2-DREADD-HA expressing mice **(Figure 4h)**. Each agonist markedly enhanced release of GLP-1 from the expected

mouse line within the first sampling period and this was maintained for at least 20 minutes **(Figures 4g, h)**. For hFFA2-DREADD-HA this was clearly an 'on-target' FFA2-mediated effect of SA because pretreatment with, and the maintained presence of, GLPG0974 completely prevented response to SA **(Figure 4h)**. Although it can be anticipated from these results that the effect of C3 in colonic tissue from wild type C57BL/6 mice is also via FFA2 this could not be be assessed directly because GLPG0974 does not have significiant affinity at mouse FFA2.

FFA2 and the regulation of lipolysis

To explore FFA2-mediated function in epididymal fat tissue, adipocytes were isolated from each of C57BL/6, hFFA2-DREADD-HA or MINUS-CRE mice, maintained in culture and allowed to differentiate for 8 days[26]. Importantly, Oil Red O staining indicated equivalent lipid accumulation in cells derived from each mouse strain (**Figure 5a**). Furthermore, the β-adrenoceptor agonist isoproterenol induced lipolysis to a similar extent and with equivalent potency in adipocytes derived from each strain (**Figure 5b**). In tests of FFA2 function adipocytes from C57BL/6 mice treated with C3 showed a significant reduction in isoproterenol-stimulated lipolysis (**Figure 5c**) whereas SA was without effect (**Figure 5c**). By contrast, an anti-lipolytic effect was produced by SA and not by C3 in adipocytes from hFFA2-DREADD-HA expressing mice (**Figure 5d**), whilst neither C3 nor SA was able to produce an anti-lipolytic effect in adipocytes derived from the MINUS-CRE line (**Figure 5e**). The effect of SA in adipocytes derived from hFFA2-DREADD-HA expressing mice was, moreover, concentration-dependent with $pIC_{50} = 4.63 \pm 0.18$ (**Supplementary Figure 7**). Importantly, consistent with the lack of activity of the human selective antagonists CATPB and GLPG0974 at mouse FFA2, neither of these agents was able to block C3-mediated anti-lipolytic effects in adipocytes derived from wild type C57BL/6 mice (**Supplementary**

Figures 8a, b). In contrast, both of these ligands, although without effect on lipolysis when added alone, effectively blocked the action of SA in adipocytes from hFFA2-DREADD-HA mice and, in each case, in a concentration-dependent manner (**Figures 5f, 5g)**.

To assess the contribution of different G protein sub-groups to the physiological end points transduced by hFFA2-DREADD-HA we pretreated isolated and cultured adipocytes with either pertussis toxin, which blocks G_i-mediated receptor signalling, or with FR900359 that blocks signalling via G_q-family G proteins[26]. Anti-lipolytic effects of SA were blocked fully by pre-treatment with pertussis toxin but unaffected by pre-treatment with FR900359 **(Supplementary Figure 9a)** demonstrating this to be a G_i-mediated effect. By contrast, in colonic tissue SA-mediated release of GLP-1 was fully prevented by pre-treatment with FR900359 **(Supplementary Figure 9b)** whilst not affected by pertussis toxin, indicating that GLP-1 release is via a G_q protein.

An in vivo role for FFA2 in GLP-1 release and gut transit

To determine if SA can act on hFFA2-DREADD *in vivo* we adapted methods that describe the release of GLP-1 into the portal vein of mice following intra-colonic administered C3 [31]. We confirmed that C3 (150 mM) administered into the colon of wild type animals resulted in significant increase in GLP-1 plasma levels in the portal vein, a response that was completely absent in control MINUS-CRE mice (**Figures 6a, b**). Intra-colonic administration of SA generated a similar increase in portal vein GLP-1 plasma levels when administered to hFFA2-DREADD-HA mice (**Figure 6c**). Importantly, no response to SA was measured in MINUS-CRE control animals (**Figure 6b**), demonstrating that in the hFFA2-DREADD-HA mice SA was operating via the hFFA2-DREADD receptor and that the response to SA mimicked the response seen for C3 at FFA2 in the wild type mice.

In a second test of the *in vivo* response of SA at hFFA2-DREADD we examined the potential role of FFA2 in gut transit. Previous *in vivo* studies had indicated that administration of SCFAs into the gut (gavage) accelerated gut transit [32]. However, a more recent *ex vivo* study on isolated colon showed the opposite, where C3, possibly acting via FFA2, decreased gut transit [33]. We resolved this issue using the hFFA2-DREADD-HA mice. Administration of C3 in the drinking water of wild type mice accelerated gut transit whilst SA had no effect (**Figure 6d and Supplementary Figures 10a, b, c**). In contrast SA stimulated a significant increase in gut transit in hFFA2-DREADD-HA mice (**Figure 6e and Supplementary Figures 10d, e**). This not only demonstrated unequivocally that SA activates the hFFA2-DREADD receptor *in vivo* but also that FFA2 activation mediates an increase in gut transit.

Discussion

GPCRs remain the most highly studied group of therapeutic drug targets and many ligands that modulate GPCR function are continuing to gain regulatory approval[34]. As such, finding novel ligands or re-purposing previously identified ones is integral to efforts to understand the biology and therapeutic potential, particularly of poorly studied GPCRs[35-36]. This has been approached by methods ranging from high throughput and virtual screening to the application of structure-based drug design. However, finding ligands that are highly selective between closely related GPCRs that can be used to define novel biology and in target validation remains a substantial challenge and a major barrier to advances in pharmacology and drug discovery. Here we have endeavoured to address this final issue by employing a DREADD receptor mutant to assess two fundamental questions; *(i)* can a DREADD mutant be utilised to distinguish the biological action of the target receptor from other closely related receptors (e.g. FFA2 vs FFA3) and *(ii)* can this approach give insights

into the action of selective pharmacological ligands at the target receptor in a way that can be used in target validation and drug design.

Our study is a substantial departure from the more common use of DREADDs (particularly muscarinic acetylcholine DREADDs) which have largely been to determine the importance of particular down-stream signalling pathways in physiological responses[12-15] and to link receptor activation to behavioural phenotypes[37]. In contrast, we have employed here a hFFA2-DREADD, knocked into the mouse *Ffar2* gene locus in a manner that replaces the wild type receptor with a DREADD mutant activated by the otherwise inert synthetic molecule SA with the aim to define specific functions of this receptor. Such DREADD knockin mice have not been used before and thus were untested as a strategy to probe the biological function of selected target receptors.

Importantly, we illustrate here that signalling through hFFA2-DREADD is indistinguishable from that of wild type hFFA2. Thus, SA promoted interactions of hFFA2-DREADD with the same panoply of G proteins as did the SCFA, propionate, via wild type hFFA2. More specifically, coupling of hFFA2-DREADD to down-stream signalling pathways mediated by G_q and G_i family G proteins, as well as interactions with β-arrestin-2 were equivalent to the wild type receptor. As such, activation of hFFA2-DREADD with SA faithfully reproduces the signalling repertoire produced by SCFAs when activating FFA2. This key proof-of-principle provided confidence that SA acting at hFFA2-DREADD-HA in tissues from knockin mice would act as an appropriate molecular probe to define biological roles of FFA2. Given that mFFA2 and the hFFA2-DREADD variant are able to interact with a wide range of G proteins, it will be interesting to explore the contribution of these to distinct physiological outcomes in these animals.

Given that the hFFA2-DREADD variant displayed near equivalent signalling to the wild type hFFA2 receptor we proceeded to generate a knockin mouse line expressing hFFA2-

DREADD-HA in place of wild type receptor. In this engineered line the tissue expression of hFFA2-DREADD-HA was the same as mFFA2 providing confidence that our model would faithfully reflect mFFA2 function and by inference possibly that of FFA2 in humans.

The first expectation of hFFA2-DREADD-HA knockin mice was that responses to SCFAs would be lost because *in vitro* experiments demonstrated that this mutant receptor was unresponsive to SCFAs. In this way the hFFA2-DREADD-HA knockin mice were expected to mimic FFA2-knockout mice (represented here by the MINUS-CRE control strain). This indeed was proven to be the case as the previously defined anti-lipolytic response to the SCFA C3 in isolated adipocytes, and the induction of GLP-1 release from colonic crypts, was lacking in the MINUS-CRE mice preparations. Moreover, because expression of FFA3 in the knockin mouse line was unaffected, the complete lack of response to SCFAs in these two tissue models also infers a lack of contribution of FFA3. This conclusion had previously been uncertain. FFA3 has been shown to be expressed by many enteroendocrine cells within the colon[28] and these same authors suggested that a poorly characterised, potentially FFA3 receptor selective, activator can promote GLP-1 release[28] more effectively than activation of FFA2. Moreover, studies by Tolhurst et al.,[27] had indicated that SCFA-mediated secretion of GLP-1 was compromised in both *Ffar2* and *Ffar3* whole body knockout mice. Our results instead show this effect to be mediated entirely by FFA2. As well as the stimulatory effect of the FFA2-DREADD agonist SA in tissue isolated from mice expressing hFFA2-DREADD-HA, this conclusion is further supported because the effect of SA can be defined unambiguously as being 'on-target' as it was completely blocked by exemplar compounds from two, chemically distinct, classes of human FFA2 specific antagonists that retain high affinity at hFFA2-DREADD. The complete selectivity of the antagonists CATPB and GLPG0974 for the human orthologue of FFA2 was also useful in that neither of these had any effect, either directly when added alone or in capacity to block

the effects of C3, in tissues from wild type mice. This contrasts with the muscarinic acetylcholine receptor DREADDs where standard antagonists such as atropine maintain high affinity at the modified receptor and also still bind to and block all subtypes of the muscarinic receptor family[10]. The capacity herein to define effects of SA as 'on-target' or 'off-target' is particularly attractive because small molecule ligands with modest potency at the 'on-target' site are frequently pleotropic in actions and it is well established that SCFAs can, for example, also act as epigenetic regulators. Indeed, in a recent study using isolated perfused rat colon as a model of colonic endocrine secretion Christiansen *et al*., suggested that effects of SCFAs on release of GLP-1 were not mediated by either FFA2 or FFA3[38]. As a number of other GPCRs are known to have antagonist ligands that are highly species orthologue selective then the proof-of-concept developed herein may well be appropriate to define both 'on-target' and 'off-target' effects of activators of other poorly characterised GPCRs by developing equivalent transgenic mouse lines.

A role of FFA2 in GLP-1 release from enteroendocrine cells was further supported by our *in vivo* studies where GLP-1 levels in the portal vein were seen to be increased following intra-colonic administration of SA in hFFA2-DREADD mice. Such a response was not produced in control mice. This not only confirmed a role for FFA2 in the regulation of GLP-1 release from the gut but importantly it established the general utility of the DREADD-model to study the biology of FFA2 in live animals. This general principle was further extended by investigation of a potential role of FFA2 in the regulation of gut transit. Earlier *in vitro* and *in vivo* studies reported conflicting findings where SFCAs, possibly acting at FFA2, regulated gut transit either in a positive[32] or negative[33] manner. By administration of SA in the drinking water we were able to resolve these contradictory studies by showing that SA accelerated gut transit in a manner that was completely dependent on the expression of the hFFA2-DREADD-HA receptor.

Although somewhat less contentious in terms of the identity of the receptor(s) involved[39-41], equivalent studies with tissue from the knockin mouse lines also provided unequivocal evidence that FFA2 is the receptor responsible for SCFA-mediated anti-lipolytic responses in white fat epididymal cells. Our previous studies established that SCFAs mediate anti-lipolytic effects through G_i-signalling and GLP-1 release through $G_q/_{11}$-signalling[26]. Linking these previous findings with the current studies determines that these physiological effects of SCFAs are both mediated via FFA2 and, more interestingly, we can conclude that FFA2 recruits distinct G proteins in a tissue-dependent fashion to drive specific physiological responses[26]. The promiscuous G protein-coupling capability of FFA2 that has also been observed in previous *in vitro* studies[21] may therefore illustrate a natural flexibility to recruit different G proteins to drive selected physiological responses in different settings.

In our study we have not only used hFFA2-DREADD to define novel biology of FFA2 but also provided evidence for the general application of other DREADD knockin mice to define the function of GPCRs for which selective ligands are not available. This approach also provides a means of testing the physiological roles and potential therapeutic outcomes of selectively activating the target GPCR. In this way, using FFA2 as an exemplar receptor, we have illustrated the broader potential of this approach to investigate both GPCR biology and drug target validation.

References

1. Barratt, M.J., Lebrilla, C., Shapiro, H.Y. & Gordon, J.I. The Gut Microbiota, Food Science, and Human Nutrition: A Timely Marriage. *Cell Host Microbe.* **22**,134-141 (2017).
2. McKenzie, C., Tan, J., Macia, L., & Mackay, C.R. The nutrition-gut microbiome-physiology axis and allergic diseases. *Immunol. Rev.* **278**, 277-295 (2017).

3. Stoddart, L.A., Smith, N.J., & Milligan, G. International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. *Pharmacol. Rev.* **60**, 405-417 (2008).
4. Bolognini, D., Tobin, A.B., Milligan, G., & Moss, C.E. The Pharmacology and Function of Receptors for Short-Chain Fatty Acids. *Mol. Pharmacol.* 89, 388-398 (2016).
5. Bindels, L.B., Dewulf, E.M., & Delzenne, N.M. GPR43/FFA2: physiopathological relevance and therapeutic prospects. *Trends Pharmacol. Sci.* **34**, 226-232 (2013).
6. Milligan, G., Shimpukade, B., Ulven, T., & Hudson, B,D. Complex Pharmacology of Free Fatty Acid Receptors. *Chem. Rev.* **117**, 67-110 (2017).
7. Milligan, G., Bolognini, D., & Sergeev, E. Ligands at the Free Fatty Acid Receptors 2/3 (GPR43/GPR41). *Handb. Exp. Pharmacol.* **236**, 17-32 (2017).
8. Sergeev, E., et al., A single extracellular amino acid in Free Fatty Acid Receptor 2 defines antagonist species selectivity and G protein selection bias. *Sci. Rep*. **7**, 13741 (2017).
9. Namour, F. et al., Safety, pharmacokinetics and pharmacodynamics of GLPG0974, a potent and selective FFA2 antagonist, in healthy male subjects. *Br. J. Clin. Pharmacol.* **82**, 139-148 (2016).
10. Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S., & Roth BL. 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 U S A.* **104**, 5163-5168 (2007).
11. Urban, D.J., & Roth, B.L. DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. *Annu. Rev. Pharmacol. Toxicol.* **55**, 399-417 (2015).
12. Smith, K.S., Bucci, D.J., Luikart, B.W., & Mahler, S.V. DREADDS: Use and application in behavioral neuroscience. *Behav. Neurosci.* **130**, 137-155 (2016).

13. Thiel, G., Kaufmann, A., & Rössler OG. G-protein-coupled designer receptors - new chemical-genetic tools for signal transduction research. *Biol. Chem*. **394**, 1615-1622 (2013).
14. Roth, B.L. DREADDs for Neuroscientists. *Neuron* **89**, 683-694 (2016).
15. Bradley, S.J., Tobin, A.B., & Prihandoko R. The use of chemogenetic approaches to study the physiological roles of muscarinic acetylcholine receptors in the central nervous system. *Neuropharmacol.* **136**(Pt C), 421-426 (2018).
16. Hudson, B.D. et al. Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. *FASEB J.* **26**, 4951-4965 (2012).
17. Ballesteros, J.A. & Weinstein, H. Integrated methods for the construction of three-dimensinal models and computational probing of structure-function relations in G proein-coupled receptors *Meth. Neurosci*. **255**, 366-428 (2013).
18. Hudson, B.D. et al. 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 (2013).
19. Sergeev, E. et al. 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 (2016).
20. Pizzonero, M., et al. 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 (2014).
21. Brown, A.J. et al., 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 (2003).

22. Karamitri, A. et al., Type 2 diabetes-associated variants of the MT_2 melatonin receptor affect distinct modes of signaling. *Sci. Signal.* **11**(545). pii: eaan6622 (2018).

23. Lee, S.U. et al., β-Arrestin 2 mediates G protein-coupled receptor 43 signals to nuclear factor-κB. *Biol. Pharm. Bull*. **36**, 1754-1759 (2013).

24. Ang, Z., Xiong, D., Wu, M., & Ding, J.L. FFAR2-FFAR3 receptor heteromerization modulates short-chain fatty acid sensing. *FASEB J*. **32**, 289-303 (2018).

25. Zaibi, M.S. et al., Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. *FEBS Lett.* **584**, 2381-2386 (2010).

26. Bolognini D, et al., A Novel Allosteric Activator of Free Fatty Acid 2 Receptor Displays Unique Gi-functional Bias. *J. Biol. Chem.* **291**, 18915-18931 (2016).

27. Tolhurst, G., et al., Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes.* **61**, 364-371 (2012).

28. Nøhr M.K., et al., 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 (2013).

29. Li, Y., Kokrashvili, Z., Mosinger, B., & Margolskee, R.F. Gustducin couples fatty acid receptors to GLP-1 release in colon. *Am. J. Physiol. Endocrinol. Metab.* **304**, E651-E660 (2013).

30. Engelstoft, M.S., et al., Research Resource: A Chromogranin A Reporter for Serotonin and Histamine Secreting Enteroendocrine Cells. *Mol. Endocrinol*. **29**, 1658-1671 (2015).

31. Psichas, A. et al., The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. Int J Obes (Lond) **39**, 424-429 (2015).

32. Richardson, A., Delbridge, A. T., Brown, N. J., Rumsey, R. D. & Read, N. W. Short chain fatty acids in the terminal ileum accelerate stomach to caecum transit time in the rat. ***Gut*** **32**, 266-269 (1991).

33. Tough, I. R., Forbes, S. & Cox, H.M. Signaling of free fatty acid receptors 2 and 3 differs in colonic mucosa following selective agonism or coagonism by luminal propionate. *Neurogastroenterol Motil*, e13454, doi:10.1111/nmo.13454 (2018)

34. Hauser AS et al., Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov.* **16**, 829-842 (2017).

35. Roth, B.L., Irwin, J.J., & Shoichet, B.K. Discovery of new GPCR ligands to illuminate new biology. *Nat. Chem. Biol*. **13**, 1143-1151 (2017).

36. Milligan, G. G protein-coupled receptors not currently in the spotlight: free fatty acid receptor 2 and GPR35. *Br. J. Pharmacol.* **175**, 2543-2553 (2018).

37. Viskaitis, P. et al., Modulation of SF1 neuron activity co-ordinately regulates both feeding behaviour and associated emotional states. *Cell Reports* **21**, 3559-3572 (2017).

38. Christiansen, C.B., et al., The impact of short chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. *Am. J. Physiol. Gastrointest. Liver Physiol*. **315**, G53-G65 (2018).

39. Ge, H., et al., 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 (2008).

40. Lee T, et al., Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. *Mol. Pharmacol*. **74**, 1599-609 (2008).

41. Tang C, & Offermanns S. FFA2 and FFA3 in Metabolic Regulation. *Handb Exp Pharmacol.* **236**, 205-220 (2017).

Author contributions

GM and ABT devised the programme of work and **GM, ABT and DB** wrote the paper with assistance from all other authors; **GM, ABT, DB** and **NB** designed the experiments, and **DB** (genetic characterisation, lipolysis, GLP-1 release, immunostaining, pharmacological characterisation), **AJB** (biosensor studies), **ES** (ligand binding studies), **CLG** (biosensor studies), **CEM (**GLP-1 release), and **NB** (GLP-1 release and gut transit) performed experiments. **BDH** developed the hFFA2-DREADD receptor and performed initial characterisation. **MB** directed the biosensor studies. **SJB** and **CM** oversaw the animal-based studies.

Competing Financial Interests

The authors declare that there are no competing financial interests

Figure Legends

Figure 1 G protein interaction patterns and signal regulation by specific agonists of hFFA2 and hFFA2-DREADD are indistinguishable

The ability of C3 (**a**) and sorbic acid (SA) (**b**), each at 10 mM, to promote interactions between either hFFA2 (**a**) or hFFA2-DREADD (**b**) and a range of mammalian G protein α subunits was assessed using a BRET-based GRK2 biosensor assay[22]. Potency and capacity of C3 (**circle)** or SA (**triangle**) to inhibit forskolin-stimulated cAMP levels in Flp-In™ T-REx™ 293 cells induced to express hFFA2 (**c**) or hFFA2-DREADD (**d**) were measured. Equivalent studies also measured the potency and capacity of C3 and SA to promote accumulation of inositol monophosphates (IP1) in Flp-In™ T-REx™ 293 cells induced to express hFFA2 (**e**) or hFFA2-DREADD (**f**). Data represent the mean ± SD (**a**, **b**) or SEM (**c** – **f**) (n = 2 **b**; n = 3 **a**; n = 6 **c - f**).

Figure 2 Initial characterisation of hFFA2-DREADD-HA expressing mice

mRNA isolated from the indicated tissues of C57BL/6 mice (**a**, **d**), those homozygous for expression of hFFA2-DREADD-HA (**b**, **d**) or the 'MINUS-CRE' line (**c, d**) was used in qRT-PCR studies with primers designed to recognise specifically mouse FFA2 (**a**) or hFFA2-DREADD-HA (**b, c**). Similar studies used primers to detect mouse FFA3 (**d**). Data are presented as 1/Δ Ct and are derived from studies conducted on mRNA isolated from at least two mice. Data represent the mean ± SEM.

Figure 3 Immunocytochemical detection of expression of hFFA2-DREADD-HA

Sections from colonic tissue isolated from C57BL/6 and hFFA2-DREADD-HA expressing mice were immunostained with an enteroendocrine cell marker (anti-chromogranin A, green) and an antibody for the hFFA2-DREADD-HA protein (anti-HA, red). Images were acquired

at 20x and 63x objectives and scale bars are shown. Solid arrows represent examples of cells that display co-localisation of anti-chromogranin A and anti-HA antibodies; dotted arrows represent examples that display localisation of only anti-chromogranin A, but not anti-HA antibodies. Representative images are shown.

Figure 4 Activation of hFFA2-DREADD-HA promotes release of GLP-1

Colonic crypt-containing preparations were generated from C57BL/6, and both MINUS-CRE and hFFA2-DREADD-HA expressing mice. Treatment with IBMX strongly promoted release of GLP-1, and to similar extents in each case (**a**). The ability of C3 or sorbic acid (SA) to promote release of GLP-1 was assessed in preparations from C57BL/6 (**b**), hFFA2-DREADD-HA (**c**) and MINUS-CRE (**d**) mice. Results are presented relative to the effect of IBMX (100 μM). The ability of CATPB (**e**) and GLPG0974 (**f**) to inhibit GLP-1 secretion induced by SA (**e**, **f**) in colonic crypts derived from hFFA2-DREADD-HA expressing mice is shown (**e, f**). Data represent the mean ± SEM (n = 3-5). *** $P < 0.001$, two-way ANOVA followed by Bonferroni post hoc test (**a**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for significance versus vehicle data; $^{\$\$\$}$ $P < 0.001$, one-way ANOVA followed by Bonferroni post hoc test (**b - f**). Intact colonic preparations from C57BL/6 (**g**) or hFFA2-DREADD-HA (**h**) mice were mounted in a tissue chamber and following perfusion with buffer alone, either C3 (10 mM) (**g**) or SA (1 mM) (**h**, **circles**) was perfused at the time indicated by the arrow. GLPG0974 (10 μM) was perfused for 15 mins before addition of SA and maintained throughout the exposure to SA (**h**, **squares**). Data represent the mean ± SEM (n = 3-4). * $P < 0.05$, ** $P < 0.01$, for significance versus vehicle (mean between values measured at 5, 10 and 15 min); one-way ANOVA followed by Bonferroni post hoc test (**g**, **h**). $^{\$}$ $P < 0.05$, $^{\$\$}$ $P < 0.01$, for significance of SA versus SA in the presence of GLPG0974; two- way ANOVA followed by Bonferroni post hoc test (**h**).

Figure 5 Activation of hFFA2-DREADD-HA is anti-lipolytic

Adipocytes were isolated and differentiated from epididymal fat taken from C57BL/6, hFFA2-DREADD-HA and MINUS-CRE mice. Oil Red O staining was used to measure relative levels of triglycerides (**a**). Varying concentrations of the β-adrenoceptor agonist isoproterenol (**isopr.**) stimulated lipolysis in these adipocytes (**b**). The ability of either C3 or sorbic acid (SA) to inhibit isoproterenol-mediated stimulation of lipolysis was assessed in adipocytes from C57BL/6 (**c**), hFFA2-DREADD-HA (**d**) and MINUS-CRE (**e**) mice. The antagonists CATPB (**f**) and GLPG0974 (**g**) reversed SA-mediated anti-lipolytic effects in adipocytes from mice expressing hFFA2-DREADD-HA. For both CATPB and GLPG0974 this was produced in a concentration-dependent fashion. Data are expressed as the percentage of lipolysis taking as 100% the maximal effect of isoproterenol 1 μM (**b**) or 5 nM (**c - g**). Results represent the mean ± SEM (n = 3-6). ** $P < 0.01$, *** $P < 0.001$, for significance versus isoproterenol-induced response; $^{\$\$}$ $P < 0.01$, $^{\$\$\$}$ $P < 0.001$, for significance versus SA-induced response; one-way ANOVA followed by Bonferroni post hoc test (**a, c - g**).

Figure 6 *In vivo* activation of hFFA2-DREADD-HA reveals roles in GLP-1 release and gut transit

(**a-c**) Total GLP-1 was assessed in portal vein blood samples collected 10 min after an intra-colonic injection of saline (vehicle), C3 (150 mM) or sorbic acid (SA, 15 mM) in isofurane-anaesthetized C57BL/6 **(a)**, MINUS-CRE **(b)** and hFFA2-DREADD-HA **(c)** mice. Data represent the mean ± SEM (n = 6-12). ** $P < 0.01$, **** $P < 0.0001$, two-tailed t test (**a** and **c**); one-way ANOVA followed by Bonferroni post hoc test (**b**).

(**d-e**) *In vivo* gastrointestinal transit was assessed in either C57BL/6 **(d)** or hFFA2-DREADD-HA **(e)** mice that were provided with free access to water, C3 (150 mM) or SA (15 mM) for 4 days after an intial acclimitisation phase. On the final day, mice were gavaged and

GI transit was measured. Data represent the mean ± SEM (n = 4-7). Significant reduction of GI transit time was observed in C57BL/6 with C3 (**d**) and in hFFA2-DREADD-HA with SA (15 mM) (**e**). * $P < 0.05$, ***$P < 0.001$ (one-way ANOVA followed by Bonferroni post-test for multiple comparisons (**d**) and two-tailed t test (**e**)). Note: the data presented in this **d** and **e** is the cumulative data of the individual mice that are presented in Supplementary Figure 8.

Legends For Supplementary Figures

Supplementary Figure 1

BRET biosensor reports G protein interaction patterns by measuring interactions between Gα and Gβγ subunits.

Cells were transfected with a BRET biosensor consisting of donor RlucII tagged Gα-subunits and an acceptor GFP10-Gγ1 subunit together with Gβ1 and either hFFA2 **(a)**, hFFA2-DREADD **(b)** or human M_3-muscarinic acetylcholine receptor (hM_3-R) **(c)**. The changes in BRET signal following receptor stimulation with either C3 (10 mM), sorbic acid (SA) (10 mM) or carbachol (100 μM) are shown. Data represent the mean ± SD (n = 3).

Supplementary Figure 2

β-arrestin 2 recruitment patterns in hFFA2 and FFFA2-DREADD are indistinguishable

The potency and capacity of C3 or SA to promote interactions between eYFP-tagged forms of hFFA2 (**a**) or hFFA2-DREADD (**b**) and NanoLuc® luciferase-tagged β-arrestin-2 was measured in HEK293T cells transfected transiently to co-express the arrestin and appropriate receptor variant. Data represent the mean ± SEM (n = 5).

Supplementary Figure 3

The hFFA2 antagonists CATPB and GLPG0974 each have high affinity for both hFFA2 and hFFA2-DREADD

The ability of varying concentrations of [^{3}H]GLPG0974 to bind specifically to hFFA2-DREADD in membranes from Flp-In™ T-REx™ 293 cells induced to express this construct is shown (**a**). Non-specific binding was defined in parallel experiments that also contained 10 μM GLPG0974. The ability of varying concentrations of CATPB (**squares**) and GLPG0974

(**diamonds**) to compete with [^{3}H]GLPG0974 to bind to hFFA2 (**b**) or hFFA2-DREADD (**c**) is shown. The ability of CATPB (**squares**) and GLPG0974 (**diamonds**) to inhibit interactions between hFFA2-eYFP (**d**) or hFFA2-DREADD-eYFP (**e**) and β-arrestin-2 NanoLuc® luciferase induced by EC_{80} concentrations of C3 (2.5 mM) (**d**) or sorbic acid (SA) (0.63 mM) (**e**) was assessed. Data represent means ± SEM (n = 3-4).

Supplementary Figure 4

Generation of hFFA2-DREADD-HA expressing mice

Transgenic knockin mice were generated by insertion of a loxP-stop-loxP cassette containing the hFFA2-DREADD-HA sequence in the coding exon of *Ffar2*. After homologous recombination and insertion of ES into blastocysts, the generated chimeric mice were bred with C57BL/6 or Cre-recombinase expressing mice in order to obtain MINUS-CRE mice and mice constitutively expressing the hFFA2-DREADD-HA construct.

Supplementary Figure 5

Expression of hFFA2-DREADD-HA is not detectable in MINUS-CRE mice

Studies akin to Figure 3 were performed on sections from colonic tissue isolated from mice expressing hFFA2-DREADD-HA and the MINUS-CRE line (**a**). Staining for each of anti-HA and anti-chromogranin A is displayed in representative images.

Similar studies were performed on sections of duodenal tissue (**b**). Submucosal clusters of HA-positive cells were detected in tissue from the hFFA2-DREADD-HA expressing line but no co-localisation with chromogranin A was observed. No anti-HA staining was detected in tissue from either C57BL/6 or the MINUS-CRE animals.

Supplementary Figure 6

GLPG0974 and CATPB are unable to block effects of C3 in colonic crypts from C57BL/6 mice

The antagonists CATPB and GLPG0974 were unable to reverse the effect of C3 on GLP-1 secretion in colonic crypts derived from C57BL/6 mice. Results are presented relative to the effect of IBMX (100 μM). Data represent the mean ± SEM (n = 3-4). *P < 0.05, for significance versus vehicle data; one-way ANOVA followed by Bonferroni post hoc test.

Supplementary Figure 7

Anti-lipolytic effect of sorbic acid at FFA2

Concentration-response curves for the anti-lipolytic effect of sorbic acid on isoproterenol-induced lipolysis (using 5 nM isoproterenol) in adipocytes derived from hFFA2-DREADD-HA expressing mice. Results are presented as % lipolysis with the effect of isoproterenol 5 nM recorded as 100%. Data represent the mean ± SEM (n = 5).

Supplementary Figure 8

Human selective FFA2 antagonists GLPG0974 and CATPB fail to block C3 responses in adipocytes derived from C57BL/6 mice

The hFFA2 antagonists CATPB (**a**) and GLPG0974 (**b**) were unable to reverse the effect of C3 on isoproterenol-induced lipolysis (isopr.) in adipocytes derived from C57BL/6 mice. Results are presented as % lipolysis with the effect of isoproterenol 5 nM recorded as 100%. Data represent the mean ± SEM (n = 3-4). *** P < 0.001, for significance versus isoproterenol-induced response; one-way ANOVA followed by Bonferroni post hoc test.

Supplementary Figure 9

Defining the G_i- and G_q-mediated responses to FFA2 in colonic crypts and adipocytes

The anti-lipolytic **(a)** and the GLP-1 response **(b)** of sorbic acid (SA) in adipocytes or colonic crypts respectively, derived from hFFA2-DREADD-HA mice following pretreatment with vehicle, pertussis toxin (PTX)(to inactivate G_i) or the G_q-inhibitor FR900359. Results are presented as % lipolysis with the effect of isoproterenol 5 nM recorded as 100% (**a**) or relative to the effect of IBMX (100 μM) (**b**). Data represent the mean ± SEM (n = 3-4). *P < 0.05, for significance versus vehicle data (**b**); *** P < 0.001, for significance versus isoproterenol-induced response (**a**); one-way ANOVA followed by Bonferroni post hoc test.

Supplementary Figure 10

***In vivo* activation of FFA2-DREADD reveals a role for FFA2 in gut transit**

In vivo gastrointestinal transit was assessed in either individual C57BL/6 (**a, b, c**) or hFFA2-DREADD-HA (**d, e**) mice that were provided with free access to water (black bars), C3 (150 mM) (grey bars) or sorbic acid (SA, 15 mM) (red bars) for 4 days after the acclimisation phase. Gut transit time of individual animals during acclimitisation phase (open bars). Note that these mice are used in the cumulative data shown in **Figures 6d** and **e**.