Title page

Chemogenetic approaches to explore the functions of Free Fatty Acid Receptor 2

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Abstract

Short Chain Fatty Acids (SCFAs) are generated in large amounts by the intestinal microbiota. They activate both the closely related G protein-coupled receptors FFA2 and FFA3 that are considered therapeutic targets in diseases of immuno-metabolism. Limited and species selective small molecule pharmacology has restricted understanding of the distinct roles of these receptors. Replacement of mouse FFA2 with a Designer Receptor Exclusively Activated by Designer Drug form of human FFA2 (hFFA2-DREADD) has allowed definition of specific roles of FFA2 in pharmacological and physiological studies conducted both *ex vivo* and *in vivo*, whilst overlay of murine disease models offers opportunities for therapeutic validation prior to human studies. Similar approaches can potentially be used to define roles of other poorly characterised receptors.

Keywords

Short chain fatty acid, G protein-coupled receptor, Designer Receptor Exclusively Activated by Designer Drugs, transgenic mice.

Main text

G protein-coupled receptors for short chain fatty acids

Many components of ingested food, and metabolites generated from these, are now well appreciated to act as homeostatic regulators rather than only as sources of energy [1]. In many cases, specific cell surface **G protein-coupled receptors** (**GPCRs**) (see **Glossary**) act as transducers to allow such metabolites to act in this way [1]. A pair of GPCRs, Free Fatty Acid receptor 2 (**FFA2**) and Free Fatty Acid receptor 3 (**FFA3**)[2-3] were shown now more than 15 years ago to be activated by **short chain fatty acids** (**SCFAs**) with chain length of between **C2** (acetate) and **C4** (butyrate)(**Figure 1**) [4-6]. Although certain other GPCRs can also be activated by SCFAs (e.g. [7]), FFA2, and to a lesser extent FFA3, has been considered widely as a potential therapeutic target given its tissue and cell expression profile that includes pancreatic islets, adipocytes, enteroendocrine and various immune cells [2, 8-12]. In recent times, progress on understanding roles of this receptor, which initially relied heavily on knock-out mouse models or on the provision of SCFAs in the drinking water of animal models, has slowed. In part this reflects that knowledge is limited on the extent to which externally provided SCFAs actually alter concentrations of these ligands in the gut and the systemic circulation. Indeed, it has been suggested that provision of acetate in drinking water of mice has little effect on systemic levels [13], although this is both a widely used and convenient means of delivery. Despite these comments, alternative strategies for delivery of either C3 (propionate) or C4 to the colon as the corresponding inulin-esters has been shown to be effective in both mouse [14-15] and human [16] and offer new insights into the broader roles of SCFAs. Moreover, because of the close proximity of the genes encoding FFA2 and FFA3 in mice (at chromosomal location 7 A3), as is also the case in human (at chromosomal location 19q13.1), it is vital to assess if knock-out of FFA2 in mice may also produce consequences for expression of FFA3 [17].

Synthetic ligands for FFA2 and their limitations

Antagonists

Antagonists are vital pharmacological tools to assess the relevance of activation of GPCRs by either synthetic or endogenous **agonists**. This is particularly the case where endogenous agonists may activate multiple GPCRs or have additional effects that are non-receptor mediated. The best characterised FFA2 antagonist is 4-[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid (**GLPG0974**) [18] (**Figure 1**). This compound was initially shown to inhibit C2-induced human neutrophil migration and activation, but even in these early studies lack of affinity at both rat and mouse FFA2 was noted. Although it was therefore impossible to assess the potential for translation of GLPG0974 using rat or mouse models of disease it was progressed through safety studies in humans [19] but subsequently failed to show efficacy in patients with mild to moderate ulcerative colitis [19]. A distinct series of FFA2 antagonists, represented by (*S*)-3-(2-(3-chlorophenyl)acetamido)-4-(4-(trifluoromethyl)phenyl) butanoic acid (**CATPB**) (**Figure 1**), also lacks affinity at mouse and rat orthologues of FFA2 despite also having high affinity at human FFA2 [20]. The key molecular determinant of these species differences appears to the presence of either lysine (human) or arginine (rat/mouse) at amino acid 65 (residue position 2.60), which is predicted to be located close to the extracellular end of transmembrane domain 2 of the receptor. Conversion of this residue from arginine to lysine in mouse FFA2 allowed both GLPG0974 and CATPB to function as blockers of receptor activation produced by the C3 SCFA propionate [21]. Moreover, this receptor mutant provided a high affinity binding site for [^{3}H]GLPG0974, whereas wild type mouse FFA2 does not bind [^{3}H]GLPG0974 with measurable affinity [21]. In support of the key role for this residue, the reciprocal switch in human FFA2, to encode arginine at this position, all but abolished measurable affinity of both GLPG0974 and CATPB [21].

Although indirect, homology modelling provides a possible explanation of these differences. Such models highlight the potential for a hydrogen bond between one of the two amide-carbonyls of GLPG0974 and Lys^{65} of human FFA2, whilst Lys^{65} may also participate in a hydrogen bond with the single amide-carbonyl of CATPB [21]. Although challenging to consider without an atomic level structure of FFA2 to facilitate virtual docking studies [22], there may be molecules within the chemical families of patents that disclosed GLPG0974 and CATPB that are able to interact with similar and high affinity with human and rodent forms of FFA2 but, if so, these have not been reported. A third series of FFA2 antagonists has been described, that includes 4-[4-(dimethylamino)phenyl]-N-(3,5-dimethylphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxamide (**BTI-A-404**)(**Figure 1**). However, BTI-A-404 displays substantially lower affinity at human FFA2 than either GLPG0974 and CATPB and like these compounds does not act as an antagonist at mouse FFA2 [23]. It is not clear if a switch from Arg^{65} to Lys in mouse FFA2 would promote affinity for BTI-A-404. Whilst recent advances in genome editing suggest that it should be relatively straightforward to generate a **transgenic mouse line** (**Box 1**) in which mouse FFA2 is replaced by Arg^{65}Lys mouse FFA2, and that this would be anticipated to result in SCFA-induced, FFA2-mediated effects now being sensitive to CAPTPB, GLPG0974 (and even potentially BTI-A-404), such a mouse line has not yet been reported.

Although BTI-A-404 has not been used in subsequent studies, GLPG0974 has been employed to help define roles of FFA2 in human cells and tissues [24-26]. Surprisingly, however, given its lack of affinity for rat and mouse FFA2, GLPG0974 has also been reported to be effective in blocking a potentially FFA2-mediated effect in rat small intestine [27] and in both a mouse model of type II diabetes [28] and the probiotic effects of *Leuconostoc mesenteroide* in mice [29]. Such effects of GLPG0974 in rodent models are incompatible with the known lack of affinity of this ligand at mouse and rat FFA2 and must, therefore reflect unknown '**off-target**' actions of GLPG0974. Discrepancies between *in vitro*

studies and apparent *in vivo* efficacy of species selective 'antagonists' at a further poorly characterised receptor, GPR35, have also recently been highlighted [30], emphasising the importance of solid underpinning knowledge of ligand pharmacology prior to use in animal models. CATPB has been similarly useful in helping to define FFA2-mediated effects in human neutrophils [31, 33], and possible roles for FFA2/FFA3 heterodimers [34] but would be equally unable to block effects mediated by FFA2 in wild type mice or in rats.

Agonists

Orthosteric agonists

As noted earlier, SCFAs are the endogenous agonists of FFA2. Unsurprisingly, given their small size, they have very modest potency but, particularly in the case of acetate, concentrations in the lower gut are predicted to achieve significant receptor occupancy. Across C2 to C4 the SCFAs are more potent at human FFA2 than at the mouse orthologue [35]. Although analysis has been restricted to a limited number of additional species orthologues, bovine FFA2 displays both higher potency for C4 than human FFA2 and also responds to longer chain length fatty acids, including C6 and C7, that have no appreciable potency at the human orthologue [36]. This variation in potency of SCFAs at species orthologues creates challenges in using the SCFAs directly. Particularly for *in vivo* studies it is difficult ensure that dosing provides '**on-target**' coverage of the receptor without generating additional effects via either the FFA3 receptor or by various non-receptor-mediated mechanisms. Although initial efforts to identify small carboxylic acids that had significant selectivity between FFA2 and FFA3, and *vice versa*, provided some promising directions for future development [37], such compounds have been more widely used to assess roles for FFA3 due to the paucity of synthetic ligands that are more potent and selective for this receptor (see [38] for review). Larger molecules including 3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid and (*R*)-3-

(cyclopentylmethyl)-4-(cyclopropyl-(4-(2,6-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid have been shown to act as selective **orthosteric** agonists of FFA2 that lack potency at FFA3 as well as at the long chain fatty acid receptors FFA1 and FFA4 [20]. A further and distinct FFA2 selective agonist (2S,5R)-5-(2-chlorophenyl)-1-1(2′-methoxy-[1,1′-biphenyl]-4-carbonyl)pyrrolidine-2-carboxylic acid [39] has been used *in vivo* after IP injection into mice. An extension of this chemistry developed substituted thiazolidine FFA2 agonists including, TUG-1375, reported to have improved potency, reduced lipophilicity, and favorable physicochemical and pharmacokinetic properties [40]. Moreover, as this compound is able to induce migration of human neutrophils and to inhibit lipolysis in murine adipocytes, it clearly has cross-species activity [40]. TUG-1375 and other orthosteric FFA2 agonists have been used to date, however, only sparingly in efforts to define the therapeutic potential of this receptor. Whether this reflects limited availability of some compounds to the academic community or a lack of information related to their pharmacodynamic and pharmacokinetic properties is unclear.

Allosteric agonists

The first described synthetic FFA2 activators were phenylacetamide-based **allosteric** FFA2 agonists [41-42]. Particularly 4-chloro-α-(1-methylethyl)-N-2-thiazolyl-benzeneacetamide (**4-CMTB**) (**Figure 1**), and the related [(*S*)-2-(4-chlorophenyl)-3,3-dimethyl-*N*-(5-phenylthiazol-2-yl)butanamide] (**compound 58**)(**Figure 1**) have been used more extensively than the orthosteric agonists described above to explore the pathophysiology and potential therapeutic potential of targeting FFA2 [3, 38]. For example, both compounds have been reported to limit Influenza A virus entry into cells [43], potentially by promoting internalisation of FFA2 and hence limiting cell surface levels of the receptor. 4-CMTB has poor pharmacokinetic properties, including poor microsomal stability, hence limiting its use [41]. A related molecule (compound 44, [41]) was, however, shown to lower plasma free

fatty acid levels in wild type mice after intraperitoneal injection at 10 mg.kg^{-1}. However, because a dose only two times greater than the effective one produced non FFA2-mediated regulation of free fatty acid levels in FFA2 receptor knock-out mice [41] this compound is also challenging to use *in vivo*. Such limitations, in addition to a lack of suitability for oral dosing, restricts enthusiasm for direct use of such compounds. A distinct FFA2 allosteric agonist *N*-[3-(2-carbamimidamido-4-methyl-1,3-thiazol-5-yl)phenyl]-4-fluorobenzamide (**AZ1729**) (**Figure 1**) [44] has attracted interest because it displays distinct signaling '**bias**' in that whilst it is able to promote FFA2-mediated functions that reflect activation of G_i-G proteins, unlike SCFAs, it does not promote engagement of the receptor with G_q-G proteins [44-45]. Although details of the mode of binding of both the phenylacetamides and AZ1729 remains undefined these compounds likely bind to separate and distinct sites because co-addition of AZ1729 and compound 58 produces co-operative effects on NADPH-oxidase activity in human neutrophils [33].

DREADD receptors

The concept of altering the orthosteric binding pocket of a GPCR by mutation to greatly reduce or eliminate the potency of binding of the endogenous agonist(s) whilst, in parallel, generating potency for a distinct agonist has been most effectively developed and demonstrated for members of the group of **muscarinic acetylcholine receptors** [46-49]. Such manipulations generate **Designer Receptors Exclusively Activated by Designer Drugs** (**DREADDs**) (**Figure 2**). In early days these were also sometimes described as **Receptors Activated Solely by Synthetic Ligands** (**RASSLs**). In the muscarinic receptor family alteration of two amino acids in the binding pocket reduces potency of acetylcholine markedly whilst generating a site that can effectively bind clozapine N-oxide, which now acts as a potent agonist [50]. As these sequence alterations appear to have limited effect on signalling profile or efficacy of the modified muscarinic receptors (e.g. [51]) they have been

used widely to explore downstream consequences of activating muscarinic receptor subtypes and signalling pathways [46-49]. Most usefully, when such a DREADD form of the muscarinic M_1 receptor was 'knocked-in' (see **Box 1**) to the genome to replace the wild type receptor in mice then, because the DREADD was unable to respond effectively to endogenous acetylcholine this mouse strain behaved as expected of an M_1-receptor 'knock-out' line [52]. However, if M_1-receptor mediated, functions were anticipated to be restored to these animals upon provision of the muscarinic DREADD agonist clozapine N-oxide [52] (**Figure 2**). Addition of clozapine N-oxide produced outcomes indicating a major role of the M_1-receptor in anxiety-like behaviours as well as learning and memory. Extension of this strategy to replace the M_1 receptor with an M_1-DREADD that is resistant to activation-mediated receptor phosphorylation (M_1-DREADD-PD) allowed demonstration that such phosphorylation plays key roles in driving both clinically relevant outcomes of receptor activation and in controlling adverse effects including epileptic-like seizures [52].

A human FFA2-DREADD

Inspired by the elegance of the DREADD concept and the effectiveness of such muscarinic receptor variants in unravelling potentially patho-physiologically relevant functions, Bolognini et al., [45] generated a knock-in mouse line in which a DREADD version of human FFA2 (hFFA2-DREADD) (**Figure 2**) replaced mouse FFA2. This built on initial development of the hFFA2-DREADD and its characterisation following expression in recombinant cell lines [36]. Alteration of residue position number 4.57 from Cys to Gly in hFFA2 resulted in the modified receptor responding to C6 and C7 fatty acids, which are larger than the fatty acid activators of wild type hFFA2, as well as to various related molecules. Further addition of a His-Gln alteration at residue position 6.55 to Cys 4.57 Gly hFFA2 generated a receptor that responded effectively to 2,4-hexadienoic acid (**sorbic acid**) (**Figure 1**) but not to the SCFAs C2 or C3 [36]. Importantly, sorbic acid also lacked agonist

activity at wild type hFFA2. At least when expressed in heterologous cell lines orthologues of FFA2 can regulate many downstream cellular functions, frequently by engaging with different heterotrimeric G proteins [3, 45]. For all end points that have been tested sorbic acid is able to control them, via hFFA2-DREADD, as effectively as SCFAs do via human FFA2 [45]. Subsequent studies showed sorbic acid to also lack activity at mouse FFA2 and at both human and mouse orthologues of the closely related SCFA-responsive GPCR FFA3 [45]. Although the muscarinic DREADD receptors still bind muscarinic antagonists, in many cases ligand affinity is reduced [50]. As noted above, no currently described antagonists can effectively block mouse or rat FFA2. This has hence restricted the use of rodent models of disease that might be predictive of therapeutic opportunity in human. However, both GLPG0974 and CATPB do block effects of sorbic acid, and of other hFFA2-DREADD orthosteric agonists (**Figure 1**), in transfected cell lines whilst [^{3}H]GLPG0974 binds with at least as high affinity to hFFA2-DREADD as to wild type hFFA2 [45].

Because sorbic acid is an orthosteric agonist at hFFA2-DREADD and only two residues within the orthosteric pocket were altered to produce the change in ligand selectivity, then, at least conceptually, the classes of allosteric agonists described above would also be expected to directly activate hFFA2-DREADD as effectively as they do hFFA2. However, co-operativity of effects between allosteric and orthosteric agonists can display '**probe-dependenc**e' [53-54] in which the effect of an allosteric ligand may be manifest to different levels, if at all, when employing distinct orthosteric agonists. Importantly thus, for a transgenic mouse line that is able to express hFFA2-DREADD to have the greatest utility in terms of therapeutic validation of FFA2 it is important to know the extent of co-operativity between the hFFA2-DREADD agonists and the full range of available allosteric FFA2 agonists. Results available to date highlight that this is akin to the extent of co-operativity between the allosteric agonists and SCFAs at wild type human FFA2 [45] and, although not reported to date, it is probable that pairs of chemically distinct allosteric agonists such as

AZ1729 and 4-CMTB will generate equivalent co-operativity at hFFA2-DREADD as they do at wild type hFFA2 [33]

Generation of a hFFA2-DREADD expressing mouse line

The hFFA2-DREADD receptor was thus used to replace mouse FFA2 and generate a 'knock-in' transgenic line in which the expression profile of hFFA2-DREADD is equivalent to FFA2 in wild type animals [45]. In addition, because many available anti-GPCR antisera are relatively poorly characterised, particularly for use in tissues and at endogenous expression levels, the **HA-epitope tag** sequence (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) was added at the intracellular C-terminal tail of the hFFA2-DREADD to generate hFFA2-DREADD-HA and allow effective immunolocalisation of the expressed protein (**Figure 3**).

As such, in both *ex vivo* and, potentially, *in vivo* settings, outcomes that reflect activation of hFFA2-DREADD (and thus FFA2 in wild type mice) were anticipated to be generated by sorbic acid, but not by SCFAs, and, because some effects of sorbic acid might be unrelated to hFFA2-DREADD-HA activation, effects of sorbic acid that are truly '**on-target**' should be blocked by concentrations of either GLPG0974 or CATPB compatible with calculated receptor occupancy [21]. Moreover, although effects of SCFAs in these animals could not reflect activation of the hFFA2-DREADD-HA they might reflect activation of FFA3. In addition, the strategy used by [45] also allowed the production of mice in which although the hFFA2-DREADD-HA construct replaced mouse FFA2 and was harboured at the correct genetic locus, it was not constitutively expressed. Lacking expression of both mouse FFA2 and hFFA2-DREADD-HA these animals provide the ideal FFA2 'knock-out' genetic control for 'off-target' effects of sorbic acid [45].

Identifying further hFFA2-DREADD ligands

During the production and optimisation of hFFA2-DREADD it was clear that in addition to sorbic acid a range of other ligands could activate hFFA2-DREADD, potentially in a suitably selective manner [36]. This was not unexpected because although SCFAs are the endogenously produced agonists of FFA2 (and FFA3) early studies had shown that a variety of small carboxylic acids could also activate hFFA2, and a number of these displayed substantial selectivity for activation of FFA2 over FFA3 [34] and *vice versa*. Barki et al., [55] therefore screened more than 1200 small molecules related to sorbic acid to identify additional molecules that would (i) activate hFFA2-DREADD, (ii) not activate wild type human or mouse FFA2, (iii) not activate mouse or human FFA3, and (iv) have effects at hFFA2-DREADD that were antagonised in a competitive fashion by CATPB or GLPG0974. Various compounds met these criteria, including a set of ligands closely related to 4-methoxy-3-methyl-benzoic acid (**MOMBA**). These are potentially as useful as sorbic acid in studies performed both *in vivo* and when using *ex vivo* tissue from hFFA2-DREADD-HA expressing mice [55].

Identification of hFFA2-DREADD-mediated functions

A range of studies had earlier indicated that anti-lipolytic effects of SCFAs in white adipose tissue were dependent upon and transduced by FFA2 [56-57]. Thus, Bolognini et al., [45] showed initially that sorbic acid was able to limit β-adrenoceptor-mediated lipolysis in tissue from the hFFA2-DREADD-HA animals (**Figure 3**), but not in wild type or in mice harbouring but not expressing hFFA2-DREADD-HA. Vitally, this effect of sorbic acid was prevented by the presence of either GLPG0974 or CATPB, in a concentration-dependent manner and, as anticipated, was transduced in a G_i-dependent manner because the effect of sorbic acid was blocked by prior treatment with pertussis toxin [45]. Although these were the anticipated outcomes, such studies provided key underpinning proof-of-concept for further use of this mouse line.

An unresolved aspect of roles of SCFAs in the lower gut is that production of, particularly acetate, by the microbiota is believed to result in very high concentrations of these SCFAs. Such concentrations would potentially be sufficient to activate FFA2 and the other SCFA responsive GPCRs, including FFA3, to a substantial level *in situ* and might limit further activation by a synthetic agonist. As noted earlier, it is unclear whether acetate or other SCFAs provided in the drinking water actually increase their concentrations *in vivo* [13]. In other studies antibiotics have been used to reduce local levels of SCFAs by eradicating the microbiota (e.g. [58]), whilst FFA2 knock-out animals provide further controls. In the hFFA2-DREADD-HA expressing mice, SCFAs cannot activate the DREADD, and sorbic acid is clearly delivered into the lower gut from intake via the drinking water at a concentration sufficient to activate hFFA2-DREADD-HA. This was demonstrated elegantly because the presence of sorbic acid in the drinking water caused an increase in **glucagon-like peptide-1** (GLP-1) measured directly in blood taken from the portal vein [45] (**Figure 3**). This must have reflected activation of hFFA2-DREADD-HA because sorbic acid did not produce such an effect in either wild type mice or in those transgenics in which expression of hFFA2-DREADD-HA was not induced [45].

The contribution of FFA2 versus FFA3, and indeed non-receptor mediated mechanisms, to release of enteroendocrine hormones such as **peptide YY** (PYY) in addition to GLP-1 [58-61] has remained an open question (see [62] for review). The hFFA2-DREADD-HA expressing mice allowed these questions to be re-explored. Firstly, anti-HA immunostaining showed the receptor to be co-expressed with each these hormones in only subsets of enteroendocrine cells [45, 55]. To assess if activation of the hFFA2-DREADD would promote release of these hormones, sorbic acid [45] or MOMBA [55] was used to treat either isolated colonic crypts, perfused into intact colonic preparations, or provided in the drinking water, and release of hormone measured in each of these *ex*- and *in-vivo* settings. Outcomes were consistent with release of these hormones by these agents reflecting activation of FFA2

rather than FFA3 or other non-receptor mediated effects, at least in part in contradiction with early studies that employed poorly characterised pharmacological tool compounds. Whilst various antisera suggested to identify FFA2 specifically are available and have been used to explore the expression profile of this receptor, full characterisation of these reagents has often been lacking [63-64] and many researchers are wary of using such anti-GPCR antisera. The HA-tag sequence present within the hFFA2-DREADD-HA construct has provided an alternative because high quality, highly characterised anti-HA epitope tag antibodies and antisera have been used for many years. Indeed, anti-HA staining has effectively illustrated hFFA2-DREADD-HA expression where this was previously unknown, for example in subsets of cells of dorsal root ganglia [55], with wild type mice providing suitable control tissue to confirm immune-specificity.

Looking forward

A substantial number of recent studies (e.g. [65-69]) have indicated novel and potentially important roles for FFA2 that are yet to be fully explored. These include the regulation of *Klebsiella pneumoniae* infection in the lung [65], the interplay between influenza infection and subsequent pulmonary *pneumococcal* superinfection [66] and in limiting respiratory syncytial virus infection [67]. Moreover, the expression of FFA2 in group 3 innate lymphoid cells has also highlighted an important role of this receptor in gut immunity [68], whilst a contribution to graft-versus-host disease [69] has also been uncovered. Follow up of these highly interesting studies has been restricted by the same challenges of ligand pharmacology of FFA2 in rodents as described earlier. The hFFA2-DREADD models offers direct ways to further define the roles of FFA2 in these settings and whether antagonist blockade could add additional insights. They can also be useful to better appreciate that not all effects of SCFAs reflect activation of this receptor (e.g. [70]). There are specific questions on the roles and importance or otherwise of FFA2 in pancreatic islets and such functions can be re-explored

by using chemogenetic approaches, including both the one described here, and modifications of it. For example, among the muscarinic DREADDs alterations of sequence to successfully limit signaling to either canonical G protein-mediated or non-canonical pathways (**Figure 2**) could easily be adapted to the hFFA2-DREADD to provide even more directed analysis of the contribution of different signals to physiological outcomes. This may be particularly helpful because at the current time only a single allosteric ligand, AZ1729, has been described to display substantial bias when activating downstream signals via both FFA2 and the hFFA2-DREADD. The plethora of roles suggested for FFA2 and efforts to manipulate the microbiota to favour beneficial health outcomes suggest many ways in which chemogenetic alterations of FFA2 can assist in unraveling whether conventional small molecule targeting of FFA2 may become a therapeutic reality.

Concluding remarks

The generation of a hFFA2-DREADD construct and its transgenic expression in mice has allowed a number of roles of FFA2 to be clarified and unexpected distribution patterns to be defined. This generates distinct **outstanding questions (see later)**. In a broader context a number of poorly characterised GPCRs have been screened to identify ligands with antagonist characteristics that can be used either directly as tool compounds or as starting compounds for medicinal chemistry programmes. In certain examples, because the human orthologue has been employed as the target, the identified hits have substantial, and in some cases virtually absolute, selectivity for the human orthologue compared to those of commonly studied rodents. FFA2 is an example. A common approach to overcome the lack of cross-species antagonists is to replace the mouse orthologue with a sequence encoding the human receptor. In the case of FFA2 this was extended to ensure that SCFAs are unable to activate the modified receptor whilst antagonists are still able to block the DREADD variant. This has provided clear insights into both anticipated, but also unexpected, roles of this receptor.

Variations of the approaches described here may be amenable for a variety of GPCRs where pharmacological variation in antagonist affinity between human and mouse receptor orthologues is extreme.

Acknowledgments

Work described in this article from the Milligan and Tobin teams is supported by the Biotechnology and Biosciences Research Council UK (**grant number BB/S000453/1**).

Disclaimer Statement

The authors have no competing interests to disclose, including any that relate to papers accepted for publication in this Journal

Resources

None

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Box 1. The generation and application of Designer Receptors Exclusively Activated by Designer Drugs

Endogenous ligands sometimes act on multiple related GPCRs. For example, serotonin is the endogenous agonist for sixteen distinct GPCRs, acetylcholine is the agonist for five muscarinic receptors and SCFAs activate at a minimum both FFA2 and FFA3. Thus, in a wild type animal it is frequently impractical to define the specific contribution of a single GPCR based only on the action(s) of an endogenous ligand. Moreover, such ligands can also produce effects by activating receptors other than GPCRs: e.g. serotonin is also the endogenous ligand for the ionotropic 5-HT_3 receptor and acetylcholine also activates ionotropic nicotinic receptors whilst SCFA can produce certain effects by non GPCR-mediated mechanisms. A Designer Receptor Exclusively Activated by Designer Drugs (DREADD) is a mutationally modified GPCR in which, either via random mutagenesis and molecular evolution or more specifically targeted mutagenesis, limited sequence alteration results in marked reduction or even elimination of affinity for the endogenous agonist (**Box 1, Figure I**). Vitally, however, these sequence alterations must also result in enhanced affinity/potency for one or more synthetic ligands that do(es) not have the ability to activate the wild type receptor (**Box 1, Figure I)**. Although not an inherent requirement it is an advantage if the DREADD retains affinity for known antagonists of the wild type receptor. Replacement of a wild type receptor with a corresponding DREADD in a transgenic knock-in mouse line (**Box 1, Figure I)** allows the DREADD to be expressed in the cells and tissues that would normally express the wild type receptor. As the DREADD is not activated by the endogenous agonists then these mice are anticipated to be

equivalent in function and behaviours as receptor ‘knock-out’ animals. However, functions that are dependent upon the DREADD (and by extension the wild type receptor in parental mice) should be restored in these mice upon provision of a DREADD specific agonist.

Figure Legends

Figure 1. Ligands at wild type and DREADD variants of human FFA2

Short chain fatty acids (C2-C4) are endogenously generated orthosteric activators of wild type FFA2 whilst sorbic acid and MOMBA are orthosteric activators of hFFA2-DREADD. Both 4-CMTB and AZ-1729 are allosteric agonists of both wild type FFA2 and hFFA2-DREADD. Compound 58 is also an allosteric activator of wild type FFA2 and although untested is anticipated to act in a similar way at hFFA2-DREADD. Both CATPB and GLPG0974 are high affinity orthosteric antagonists of both wild type FFA2 and hFFA2-DREADD and whilst BTI-A-404 is also likely to antagonise wild type FFA2 and hFFA2-DREADD any effect at hFFA2-DREADD has not been reported. As noted in the text none of these antagonists is able to block either mouse or rat FFA2 effectively.

Figure 2. Designer Receptors Exclusively Activated by Designer Drugs

Most of the characterised and published DREADDs are based on the five subtypes of muscarinic (M) acetylcholine receptor subtypes. The principal G protein-coupling profile is that M1, M3 and M5 receptors favour interactions with G_q-family G proteins whereas M2 and M4 favour coupling to Gi-family members. As muscarinic receptors are highly conserved in mammals then both human (h) and rat (r) sequences have been used. Because the orthosteric binding pocket is particularly well conserved across the subtypes then the same pair of DREADD mutations is sufficient to alter the ligand binding characteristics to greatly reduce potency of acetylcholine and, in parallel, greatly increase potency of the three ligands displayed. An additional mutation on the intracellular face of the rat M3 receptor uncouples this receptor from G protein interaction without altering agonist-induced interactions with an arrestin to generate an arrestin-biased DREADD (rM3Darr). See [46-47] for review. In addition to the hFFA2-DREADD described in the text a DREADD construct based on the

kappa opioid receptor (KORD) has also been described [48]. Further details on the hFFA2-DREADD described in the main text can be found in Figure 3 and [36, 44, 55]

Figure 3. Characteristics of hFFA2-DREADD-HA

As described in the main text, transgenic expression of hFFA2-DREADD-HA in mice has provided a means to detect receptor expression via the HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) sequence introduced in frame with the receptor C-terminal tails. Mutation of two residues in the orthosteric binding pocket have switched the nature of ligands with agonist activity and as the construct is based on human FFA2 the expressed receptor is blocked by antagonists including CATPB and GLPG0974 that lack affinity at mouse FFA2. Key physiological effects shown to be mediate by this construct in tissues of these transgenic mice are illustrated.

Box 1 Figure I. The generation and application of Designer Receptors Exclusively Activated by Designer Drugs

Designer Receptors Exclusively Activated by Designer Drug GPCRs (DREADDs) are generated by specific site mutations (**yellow circles**) in the orthosteric binding site (i.e. the binding site for the endogenous agonist). These DREADDs have much reduced, and sometimes negligible, affinity and/or efficacy compared to the wild type sequence, for the endogenous agonist (**blue circle**, **blue lines in insets**). Any response to the ligand cannot thus be transduced by the DREADD and must, therefore, be an '**off-target**' effect. DREADDs however are also engineered to possess substantial potency and efficacy for one or more synthetic ligands (**yellow triangle, yellow line in inserts**) that are inactive at the wild type receptor. In an animal transgenically expressing a suitable DREADD then the effect of the DREADD active synthetic ligand must be '**on-targe**t', i.e. mediated by the DREADD, if the synthetic ligand does not produce the same response in a wild type animal. Transgenic expression of such a DREADD

allows selective targeting of the specific receptor and hence provides a useful and potentially unique tool to study signalling pathways as well as physiological responses to activation of a single receptor subtype.

Glossary

Agonist: A chemical species that binds to a receptor and activates it, eliciting a biological response.

Antagonist: A chemical species that binds to a receptor preventing activation by an agonist.

Designer Receptor Exclusively Activated by Designer Drugs (DREADD): A G protein-coupled receptor in which alteration of one of more amino acids in the orthosteric binding pocket results in reduction or elimination of the potency and binding of the endogenous agonist and, in parallel, enhancement of potency (usually from a negligible level) to one or more synthetic small molecules.

Epitope-tag: a DNA sequence incorporated into a cDNA or DNA such that when transcribed and translated the encoded protein has an additional amino acid sequence that can be easily detected, usually with one or more highly characterised antibodies.

FFA2: Free Fatty Acid receptor 2. A member of the group of GPCRs that responds to short chain fatty acids including acetate and propionate.

FFA3: Free Fatty Acid receptor 3 A further member of the group of GPCRs that responds to short chain fatty acids.

Glucagon-like peptide-1 (GLP-1). An incretin (able to promote release of insulin) hormone produced and released from subsets of enteroendocrine cells in the lower gut in response to stimulation by various mediators including SCFAs.

G protein-coupled receptor (GPCR): integral membrane protein able to transmit a signal across the cell membrane in response to an external stimulus, to activate (usually) heterotrimeric guanine nucleotide binding proteins (G proteins). Characterised by a conserved structure comprising seven helical membrane-spanning domains, an extracellular N terminus, three external loops, three internal loops, and an internal C-terminal domain. Subdivided into classes based upon structural relatedness.

HA (haemagluttin): in this context an epitope tag sequence that encodes the amino acid sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala.

Ligand Bias: Ligands that are able to only effect a subset of signalling pathways that are controlled by the endogenous agonist(s) of the receptor in question.

Muscarinic acetylcholine receptors: A family of 5 related GPCRs whose endogenous ligand is acetylcholine.

On-target: An effect of a pharmacological ligand that is produced by interaction with the protein that the ligand is best understood to be able to regulate.

Off-target: An effect of a pharmacological ligand that is produced by means other than interacting with the protein or proteins that it is best understood to regulate.

Peptide YY (PYY): A further enteroendocrine hormone released in the lower gut in response to stimulation by various mediators including SCFAs.

Probe-dependence: the concept that an allosteric ligand will differ in the extent of co-operativity it is able to generate when using different orthosteric agonists.

Short Chain Fatty Acids (SCFAs): A group of molecules containing a carboxylic acid and 1 and 5 carbon atoms.

Receptors Activated Solely by Synthetic Ligands (RASSLs): An older and now less used term that is synonomous with DREADD.