Molecular Pharmacology Fast Forward. Published on March 2, 2017 as DOI: 10.1124/mol.116.107904
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TITLE PAGE

THE ORPHAN RECEPTOR GPR17 IS UNRESPONSIVE TO URACIL-NUCLEOTIDES AND CYSTEINYL-LEUKOTRIENES*

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Molecular Pharmacology Fast Forward. Published on March 2, 2017 as DOI: 10.1124/mol.116.107904
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RUNNING TITLE PAGE

Running Title: GPR17 is unresponsive to its assigned endogenous ligands

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Number of text pages: 35

Number of tables: 0

Number of figures: 10

Number of references: 58

Number of words in:

-Abstract: 232

-Introduction: 676 (excluding references)

-Discussion: 1468

List of nonstandard abbreviations: AUC, area under the curve; BRET, bioluminescence resonance energy transfer, CNS, central nervous system; CysLTs, cysteinyl-leukotrienes; CysLT1R, cysteinyl-leukotriene receptor 1; DMR, dynamic mass redistribution; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1 and 2; FCS, fetal calf serum; FGF, fibroblast growth factor; GPCR, G protein-coupled receptor; HTRF, homogeneous time resolved fluorescence; IP, inositol phosphate; LTC4, leukotriene C4, LTD4, leukotriene D4; MDL29,951, 2-carboxy-4,6-dichloro-1H-indole-3-propionic acid; PDGF-AA, platelet-derived growth factor-AA, P2Y receptors, purinergic G protein-coupled receptors.

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ABSTRACT

Pairing orphan G protein-coupled receptors (GPCRs) with their cognate endogenous ligands is expected to have a major impact on our understanding of GPCR biology. It follows that reproducibility of orphan receptor ligand pairs should be of fundamental importance to guide meaningful investigations into pharmacology and function of individual receptors. GPR17 is an orphan receptor characterized by some as dualistic uracil-nucleotide/cysteinyl-leukotriene receptor and by others as inactive towards these stimuli altogether. Whilst regulation of central nervous system (CNS) myelination by GPR17 is well established, verification of activity of its putative endogenous ligands has proven elusive so far. Herein we report that uracil-nucleotides and cysteinyl-leukotrienes do not activate human, mouse or rat GPR17 in various cellular backgrounds including primary cells, using eight distinct functional assay platforms based on label-free pathway-unbiased biosensor technologies as well as canonical second messenger or biochemical assays. Appraisal of GPR17 activity can neither be accomplished with co-application of both ligand classes, nor with exogenous transfection of partner receptors (nucleotide P2Y12, cysteinyl-leukotriene CysLT1) to reconstitute the elusive pharmacology. Moreover, our study does not support inhibition of GPR17 by the marketed antiplatelet drugs cangrelor and ticagrelor, previously suggested to antagonize GPR17. Whereas our data do not disagree with a role of GPR17 *per se* as orchestrator of central nervous system functions, they challenge the utility of the above proposed (ant)agonists as tools to imply direct contribution of GPR17 in complex biological settings.

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INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors in the cell. By functioning as sensors for extracellular stimuli, they are involved in a broad variety of physiological phenomena and, therefore, belong to the most common targets of pharmaceutical drugs although only a small percentage of GPCRs are targeted by current therapies (Rask-Andersen et al., 2011). For about 25% out of the more than 400 non-olfactory GPCRs, a defined physiologically relevant ligand is still lacking (Chung et al., 2008). These receptors are known as "orphan GPCRs". "Deorphanization" and identification of their *in vivo* roles is essential to clarify novel regulatory mechanisms and, consequently, to disclose novel drug targets. Whether GPR17 is such an orphan receptor is still a matter of debate (Davenport et al., 2013; Harden, 2013; Qi et al., 2013; Davenport et al., 2015). The original deorphaning report introduced the possibility that GPR17 might represent a dualistic receptor responding to two classes of endogenous signaling molecules: cysteinyl-leukotrienes (CysLTs) and uracil-nucleotides (Ciana et al., 2006). While this receptor ligand assignment agrees well with the phylogenetic classification of GPR17, which is just located intermediate between the nucleotide P2Y and the cysteinyl-leukotriene receptors, it is not yet accepted by the scientific community (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Davenport et al., 2013; Harden, 2013; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). More precisely, discordant findings have been reported on the nature of its cognate endogenous ligands with an ongoing controversy over their true identity (Bläsius et al., 1998; Heise et al., 2000; Ciana et al., 2006; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). To date, ten years after the original deorphaning report (Ciana et al., 2006), only a single independent study confirmed activation of GPR17 by uracil-nucleotides, yet failed to recapitulate activation by CysLTs (Benned-Jensen and Rosenkilde, 2010). Although these authors did indeed note statistically significant signaling upon challenge of GPR17 with uracil-nucleotides, they questioned the biological relevance of this receptor-ligand pairing because of the low efficacy responses observed in their functional assays (Benned-Jensen and Rosenkilde, 2010). Regardless, GPR17 deorphanization must be considered tentative

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at this point because the amount of contradictory reports (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014) clearly and continuously outnumbers the independent follow up studies (Benned-Jensen and Rosenkilde, 2010) and, therefore, leaves the original deorphaning report unconfirmed.

In principle, pairing of orphan GPR17 with its endogenous agonists are findings that ought to be considered particularly significant because they supply the scientific community with tools to explore pharmacology and function of this receptor. Endogenous agonists may also be employed to identify antagonist ligands that provide starting points for studying receptor pharmacology and physiology. With endogenous and surrogate (ant)agonist ligands at hand, fundamental questions related to GPR17 biology may in principle be addressed in various *in vitro/in vivo* paradigms. However, interpretational issues are connected with such studies if important conclusions are based on application of pharmacological tools that do not reliably activate or inhibit GPR17 with the disadvantage of misleading an entire research field.

Given these apparent ambiguities, we investigated potential interaction between orphan GPR17 and its putative endogenous ligands, cysteinyl-leukotrienes and uracil-nucleotides, along with a small molecule surrogate agonist MDL29,951 in great detail (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Moreover, as purported endogenous ligands were employed to identify GPR17 inhibitors (Ciana et al., 2006; Gelosa et al., 2014), we included these in our investigations. We undertook a highly systematic approach involving multi-tiered functional analysis of human, rat and mouse GPR17 in various recombinant, immortalized and primary cells in which GPR17 is abundant. We employed canonical biochemical and second messenger assays in conjunction with label-free biosensor platforms that sense pharmacologically-mediated alterations of cellular activity along all major GPCR signaling pathways thereby providing integrative and unbiased measures for receptor activity in whole living cells (Verdonk et al., 2006; Schröder et al., 2010; Schröder et al., 2011). Moreover, we also examined whether co-expression of GPR17 along with the nucleotide P2Y12 or the cysteinyl-leukotriene CysLT1 receptor (CysLT1R) might constitute a pharmacological entity displaying the elusive GPR17 pharmacology. In contrast to the original deorphaning report (Ciana et al., 2006) and the various follow-up studies from the

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same group (Lecca et al., 2008; Fumagalli et al., 2011; Daniele et al., 2014), and in agreement with previous elegant work by Qi and coworkers (Qi et al., 2013) we report herein that all proposed endogenous ligands are completely inactive on GPR17 orthologs from human, rat and mouse. Unfortunately, this also extends to the surrogate antagonists cangrelor and ticagrelor, two marketed antiplatelet medicines (Ingall et al., 1999; Nicholas, 2001; Qamar and Bhatt, 2016) that were previously reported to inhibit GPR17 (Ciana et al., 2006; Gelosa et al., 2014).

In view of these results, we strongly recommend to re-assess their value as pharmacological tools to probe physiology and function of orphan GPR17 as well as restrict their use to *in vitro/ex vivo/in vivo* applications that do not intend to elaborate on the cellular consequences that arise from stimulation or inhibition of this orphan receptor.

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MATERIALS and METHODS

Materials. Tissue culture media and reagents were purchased from Invitrogen (Karlsruhe, Germany). MDL29,951 was from Maybridge (Loughborough, UK). Ticagrelor was a kind gift from Astra Zeneca. All other laboratory reagents were obtained from Sigma-Aldrich (Steinheim, Germany), if not stated otherwise.

Cell Culture. Cell lines were kept in a humidified atmosphere with 5% CO_2 at 37°C. All media were supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Native and recombinant HEK293 and 1321N1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). For mGPR17-HEK293, rGPR17-HEK293, hP2Y14-HEK293 and FLAG-hCysLT1-HEK293 (hereafter referred to as hCysLT1-HEK293), the medium was supplemented with G418 (500 µg/ml) (InvivoGen, Toulouse, France), for 3HA-hGPR17-HEK293 (later named hGPR17-HEK293) with zeocin (56 µg/ml) (InvivoGen), for 3HA-hGPR17-*R*Luc-GFP^2-β-arrestin2-HEK293 (hGPR17-BRET-HEK293) and 3HA-hGPR17+FLAG-hCysLT1-HEK293 (hGPR17+hCysLT1-HEK293) with zeocin (56 µg/ml) and G418 (500 µg/ml), and for hGPR17-1321N1 and hP2Y12-1321N1 with G418 (800 µg/ml).

CHO-K1 cells were cultivated in DMEM with Nutrient Mixture F-12 (DMEM/F12). Medium for Flp-In T-REx CHO cells stably expressing hGPR17 (hGPR17-CHO) was supplemented with hygromycin B (500 µg/ml) and blasticidin (30 µg/ml). Expression from the Flp-In locus was induced by treatment with doxycycline (1 µg/ml) for 16-20 h. For hM1-CHO cells, G418 (200 µg/ml) was added to Ham's F-12 nutrient Mix with GlutaMAX.

Oli-neu cells were kindly provided by Prof. J. Trotter (University of Mainz) and cultured in Sato medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) horse serum, insulin (5 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), 1% (v/v) N2 supplement, sodium selenite (190 nM), gentamicin (25 µg/ml), triiodothyronine (400 nM), and L-thyroxine (520 nM).

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Differentiation and expression of GPR17 was induced by adding 1 µM of the EGFR tyrosine kinase inhibitor PD174265 (Calbiochem, Darmstadt, Germany) to the culture medium.

Primary rat OPC cultures were isolated by a differential detachment method from mixed glial cultures of postnatal Wistar rat cerebra, as previously described in (Chen et al., 2007; Hennen et al., 2013). OPCs were seeded in proliferation medium (Neurobasal medium supplemented with 2% (v/v) B27, 2 mM GlutaMAX, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml platelet-derived growth factor-AA (PDGF-AA), and 10 ng/ml basic fibroblast growth factor (FGF). For induction of spontaneous *in vitro* differentiation and GPR17 protein expression, medium was switched to growth factor-free Neurobasal medium.

[^{35}S]GTPγS Binding Assay. To estimate ligand-induced binding of [^{35}S]GTPγS to membranes of 1321N1 astrocytes, 5 µg of membranes isolated from hGPR17-1321N1 cells were incubated with 0.07 nM [^{35}S]GTPγS (Perkin Elmer, Homburg, Germany) in buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 3 µM GDP, 0.5 g/100 ml BSA) containing different concentrations of test compounds at room temperature for 1 h in a total volume of 500 µl in 96-well microplates (Thermo Scientific ABgene, Hamburg, Germany). Reactions were terminated by rapid filtration using a Tomtec harvester. Filters were washed twice with ice cold wash buffer (50 mM Tris, 5 mM $MgCl_2$) and filter-bound radioactivity was measured by solid scintillation. [^{35}S]GTPγS binding experiments with membranes isolated from hGPR17-CHO were performed as described in Célanire et al. with a few modifications (Celanire et al., 2009). Membranes (5 µg proteins) were incubated for 15 minutes at 25°C in 0.2 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, 100 mM NaCl, 3 µM GDP, 2 µg saponin, 1% DMSO and increasing concentrations of test compounds. Thereafter, 0.2 nM of [^{35}S]GTPγS were added to the samples and the incubation was prolonged for another 60 min at room temperature. Assays were terminated by rapid filtration through GF/C glass fiber filters and consecutive washes with at least 4 times the assay volume of ice cold 50 mM Tris HCl buffer (pH 7.4). Radioactivity on filters was determined by

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liquid scintillation. Nonspecific binding (NSB) was defined as the residual binding observed in the presence of 100 µM Gpp(NH)p.

Calcium Mobilization Assays. Intracellular Ca^{2+} mobilization was quantified with the calcium 5 assay kit. Cells were seeded at a density of 60,000 cells (hGPR17-HEK293, mGPR17-HEK293, rGPR17-HEK293, native HEK293) or 50,000 cells (hGPR17-1321N1, hM1-CHO, hGPR17-CHO) per well into black 96-well tissue culture plates with clear bottom. Twenty-four hours post seeding, cells were loaded with calcium 5 indicator dye for 45-50 min, stimulated and intracellular calcium flux was detected with the FlexStation 3 Multimode Benchtop Reader (Molecular Devices, Biberach an der Riss, Germany). For analysis of receptor inhibitors, these were injected 30 min prior to agonist application.

HTRF-Based cAMP accumulation and ERK1/2 phosphorylation Assays. Changes of the intracellular second messenger IP1 and levels of phosphorylated ERK1/2 were quantified with a Mithras LB 940 multimode reader (Berthold Technologies, Bad Wildbad, Germany) using the HTRF-IP1 kit and the HTRF Cellul'erk kit (Cisbio International, Codolet, France), respectively, according to the manufacturer's instructions and as described previously in detail. To estimate potency of inhibitors, cells were pre-incubated with antagonists for 30 min (Bock et al., 2012).

BRET^2 Assays. To estimate β-arrestin2 recruitment to hGPR17, hGPR17-BRET-HEK293 cells stably expressing hGPR17-*R*luc as energy donor and β-arrestin2–GFP^2 as energy acceptor were pre-incubated with compounds for different time-points (1.5 min, 5 min, 10 min, 15 min, 30 min and 60 min) before addition of the Renilla luciferase substrate (DeepBlueC coelenterazine (Gold Biotechnology, St. Louis, USA) and measurement of BRET signal using the Mithras LB 940 multimode reader. For detection of G protein subunit rearrangements, HEK293 cells were transiently co-transfected with cDNA for hGPR17, $G\alpha_{i1}$-91*R*luc, GFP^{10}-$G\gamma_2$ and the complementary subunit $G\beta_1$ using FuGENE HD transfection reagent (Promega, Mannheim, Germany). Forty-eight hours after transfection, BRET^2 between $G\alpha_{i1}$ and $G\gamma_2$ was

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detected 2 min after agonist exposure as described in detail previously (Gales et al., 2006; Bock et al., 2012).

Label-Free Assays (Dynamic Mass Redistribution (DMR), Bioimpedance (CellKey)). DMR was recorded using the Corning® Epic® (Corning, Corning, USA) biosensor or the PerkinElmer Ensight™ (PerkinElmer, Waltham, USA) in conjunction with the Cybi-SELMA semi-automated electronic pipetting system (Analytik Jena AG, Jena, Germany) as described previously (Schröder et al., 2010; Schröder et al., 2011; Simon et al., 2016). For bioimpedance measurements using the CellKey™ system (Molecular Devices, Biberach an der Riss, Germany), cells were seeded at a density of 12,000 cells (CHO and 1321N1) and 18,000 cells (HEK293) per well into 384-well microtiter plates and grown to confluence for 16 h (37°C, 5% CO_2). Before the assay, cells were washed twice with HBSS buffer containing 20 mM Hepes and incubated for at least 1 h in the DMR reader for temperature equilibration. The sensor plate was scanned for 5 min to record a baseline optical signature and then compound solutions were transferred into the biosensor plate to monitor bio-impedance changes for at least 1200 s. Effects of antagonists were observed for 1 h prior to agonist addition.

Data Analysis. Data points were fitted to both three-parameter (fixed Hill slope) and four parameter nonlinear regression isotherms using Prism 5.00 or 6.05 (GraphPad Software, San Diego, USA). Quantification of DMR and bioimpedance signals was performed by calculation of the maximum response or the area under the curve (AUC) between at least 0 and 1200 s. All data presented are mean values ± S.E.M. of n independent experiments, unless stated otherwise. Comparison between two experimental groups was performed with a two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test. P value significance thresholds were *P<0.05, **P<0.01, ***P<0.001 and ****P<0,0001. For statistical reasons n number of each individual compound in the different figure panels is listed in Supplemental Table 1 and 2.

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RESULTS

Uracil-nucleotides and cysteinyl-leukotrienes do not activate orphan GPR17 in three recombinant cell hosts stably expressing hGPR17

To study possible activation by uracil-nucleotides and cysteinyl-leukotrienes of GPR17, we initially employed two techniques: loading of hydrolysis-resistant [^{35}S]GTPγS onto the α subunit of heterotrimeric GTP binding proteins and mobilization of Ca^{2+} from intracellular stores. Both methods have been used successfully in the original deorphaning report (Ciana et al., 2006). However, incubation of membranes collected from 1321N1 astrocytoma cells that stably express the short isoform of human GPR17 (hGPR17) with the putative endogenous ligands did not unveil detectable [^{35}S]GTPγS incorporation. Lack of activity in response to these ligands was not due to deficient GPR17 expression or function because MDL29,951, a synthetic GPR17 agonist (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016) promoted robust and concentration-dependent increase of [^{35}S]GTPγS binding over basal levels (Fig. 1A). Because GTP exchange is an event proximal to receptor activation, we wondered whether a low degree of signal amplification might have precluded detection of low efficacy agonists in this assay. Akin to the results obtained from [^{35}S]GTPγS binding, effects of MDL29,951 were not mimicked by the endogenous ligands in fluorescence-based Ca^{2+} measurements despite the large degree of signal amplification in this assay (Fig. 1B). Because no evidence of GPR17 coupling to $G\alpha_i$ or $G\alpha_q$ upon stimulation with the putative endogenous ligands was obtained we took advantage of label-free whole cell recordings, which are powerful biosensor methods to report cell activation in real-time by stimulated GPCRs regardless of their primary signaling pathway (Verdonk et al., 2006; Schröder et al., 2010; Schröder et al., 2011). Yet, no indication of cell activity was observed in optical, dynamic mass redistribution (DMR)-based biosensor recordings for all five endogenous ligands contrasting with the robust and concentration-dependent DMR traces of cells exposed to MDL29,951 (Fig. 1C-I). Analogous results were obtained in label-free bioimpedance measurements where hGPR17-1321N1 cells were brought in contact with gold electrodes embedded in microtiter plates whereby the cell layer's change in electrical impedance was followed over time after application of the activating stimuli (Fig. 1J-P). MDL29,951 and all five endogenous ligands

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were completely inactive in label-free optical and impedance-based recordings on native 1321N1 cells, confirming GPR17 specificity of MDL29,951 action and ruling out that inactivity of endogenous ligands in hGPR17-1321N1 cells is due to compensation of activity profiles with opposing deflection in this cellular background (Fig. 2). We also examined the possibility that GPR17 function might be attenuated by uracil-nucleotides and cysteinyl-leukotrienes. To this end, hGPR17-1321N1 cells were pre-incubated with increasing concentrations of each endogenous ligand prior to stimulation with MDL29,951 at its EC_{80}. Supplemental Fig. 1 shows that the integrated cellular response observed with MDL29,951 was essentially unaffected by the presence of each compound. Thus, canonical second messenger and innovative label-free biosensor recordings do not indicate any type of functional interaction between UDP, UDP-glucose, UDP-galactose, LTC4 as well as LTD4 and orphan GPR17.

The same set of experiments was recapitulated in an independent cell line, hGPR17-CHO, to ensure that the negative results were not a consequence of the expression system. Akin to the results obtained with the hGPR17-1321N1 cells, a combination of membrane-based [^{35}S]GTPγS binding (Fig. 3A), intact-cell Ca^{2+} mobilization (Fig. 3B), real-time label-free DMR (Fig. 3C-I) and impedance-based assays (Fig. 3J-P) only permitted detection of cell activation by MDL29,951. Although we noted cellular activity in response to some high concentrations of UDP in both DMR and impedance recordings (Fig. 3F,M), these traces were not GPR17-specific because they also occurred in cells deficient in GPR17 expression (Supplemental Fig. 2D,J). Contrary to the lack of activity of all five endogenous ligands in hGPR17-1321N1 and -CHO cells, uracil-nucleotides and cysteinyl-leukotrienes markedly activated their cognate receptors P2Y14 and CysLT1, respectively, confirming both chemical integrity of the applied ligands as well as validity of assay design and performance (Fig. 4).

To exclude the possibility that the commonly used mammalian 1321N1 and CHO cell hosts lack cellular components that might be required for detection of agonist activity of the purported endogenous activators, we also investigated potential stimulation of GPR17 stably expressed in HEK293 cells. We initially examined cellular localization of GPR17 because we are aware of orphan receptors (e.g. GPR18) that show predominant intracellular expression in stably transfected but surface expression in transiently

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transfected HEK293 cells (Finlay et al., 2016). However, unlike GPR18, GPR17 displayed a considerable surface-resident population (Fig. 5A). In spite of significant surface expression, label-free DMR and impedance assays failed to report GPR17 activation in response to all five endogenous ligands (Fig. 5C-H and J-O). By contrast, prominent real-time activation profiles were evoked with MDL29,951 confirming functional expression of GPR17 in this cell line (Fig. 5B,I,H,O). Control experiments in which hGPR17 was not transfected showed no significant cell activation in both DMR and impedance recordings (Supplemental Fig. 3). These data ascertain MDL29,951 as GPR17-specific probe in the HEK293 cell background and both label-free platforms as suitable for unraveling potential biological effects set in motion via activation of this orphan receptor. Following our multi-tiered approach also in this expression system, we failed to detect agonist activity in Ca^{2+} and a bioluminescence resonance energy transfer (BRET)-based live cell $G\alpha_i$ assay conceived with $G\alpha_i$-*R*Luc as energy donor, GFP^{10}-$G\gamma_2$ as energy acceptor along with unlabeled $G\beta_1$ (Fig. 6A,B) (Gales et al., 2006; Bock et al., 2012). Activation of numerous GPCRs converges on the level of extracellular signal regulated kinases 1 and 2 (ERK1/2), a key signaling molecule which is consistently activated in cells by $G\alpha_q$, $G\alpha_i$, and some $G\alpha_s$ GPCRs but also in an arrestin-dependent, G protein-independent manner (Goldsmith and Dhanasekaran, 2007; Rajagopal et al., 2010; Rakesh et al., 2010; Reiter et al., 2012). Therefore detection of ERK1/2 phosphorylation might provide an advantage to capture signaling of the purported endogenous ligands via pathways that were not yet addressed experimentally. Robust phospho-ERK1/2 production was evoked by treating cells with MDL29,951 but not with endogenous stimuli which were completely inactive (Fig. 6C). These results contrast sharply with the prominent ERK1/2 phosphorylation we observed when all endogenous ligands were acting via their cognate receptors P2Y14 and CysLT1, respectively (Fig. 6D,E). In agreement with these findings, BRET-based detection of induced interaction between GPR17 and β-arrestin2 indicated strong activity of MDL29,951 but inactivity of all five endogenous ligands (Fig. 6F). As β-arrestin recruitment is perceived as distinct intracellular signaling route (Walters et al., 2009; Rajagopal et al., 2010; Violin et al., 2010; Reiter et al., 2012) our results exclude the possibility that uracil-nucleotides and cysteinyl-leukotrienes are biased towards β-arrestin over classical G protein pathways.

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Uracil-nucleotides and cysteinyl-leukotrienes do not activate mouse and rat GPR17

Since marked differences of ligand pharmacology have been encountered with species orthologs of GPCRs (Milligan, 2011; Hudson et al., 2013; Strasser et al., 2013), we investigated the possibility that uracil-nucleotides and cysteinyl-leukotrienes might eventually serve to stimulate rodent GPR17 orthologs. To this end, we compared label-free whole cell activity profiles evoked by MDL29,951 and the endogenous ligands in HEK293 transfectants stably expressing rat and mouse GPR17 (rGPR17, mGPR17, Fig. 7A,B). Marked and concentration-dependent activation of rGPR17 and mGPR17 was triggered with MDL29,951 ensuring accurate function of both orthologs in this expression system (Fig. 7C,I,J,P). Endogenous ligands were inactive at all applied concentrations (Fig. 7D-I and K-P). In agreement with inactivity of uracil-nucleotides and cysteinyl-leukotrienes on recombinant rodent GPR17 orthologs, the same activity pattern was replicated in the immortalized murine oligodendrocyte cell line Oli-neu (Fig. 8A-G) and in primary rat oligodendrocytes using whole cell DMR recordings (Fig. 8H-N). Both cell lines endogenously express GPR17 during differentiation and were functionally responsive to MDL29,951 in a concentration-dependent and GPR17-specific manner (Fig. 8A,G,H,N and ref. (Hennen et al., 2013; Simon et al., 2016)). We conclude that uracil-nucleotides and cysteinyl-leukotrienes are completely inactive on both native and recombinant rodent GPR17 orthologs and are, therefore, inappropriate to explore the function of this receptor in rodent cells or tissues.

The antiplatelet drugs ticagrelor and cangrelor do not inhibit human, mouse and rat GPR17

Because of the great discrepancies between the original deorphaning and essentially all subsequent studies including our own concerning responses of human, rat, and mouse GPR17 to uracil-nucleotides and cysteinyl-leukotrienes, we also reassessed antagonist activity of ligands previously reported to inhibit GPR17 (Ciana et al., 2006; Gelosa et al., 2014). Among these are cangrelor and ticagrelor, two drugs marketed for inhibition of platelet aggregation (Ingall et al., 1999; Nicholas, 2001; Qamar and Bhatt, 2016) and employed in extensive *in vivo* investigations by virtue of their purported capacity to inhibit GPR17 (Lecca et al., 2008; Ren et al., 2012). Yet, we noted that akin to literature on GPR17 agonists, literature on antagonists is both relatively limited and inconsistent (compare (Ciana et al., 2006) with

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(Hennen et al., 2013)). Interestingly, using three distinct functional assay platforms we found that cangrelor and ticagrelor were completely inactive as inhibitors of MDL29,951-stimulated human, mouse and rat GPR17 (Fig. 9). Pranlukast, on the contrary, an anti-asthma medicine originally described as CysLT1 receptor antagonist, fully reversed agonist action of an EC_{80} concentration of MDL29,951 in Ca^{2+} mobilization assays at either ortholog (Fig. 9A-C) and did so – albeit less efficiently - in the other assay platforms (Fig. 9D-I). As control, cangrelor and ticagrelor were examined for inhibition of their cognate P2Y12 receptor, function of which was effectively attenuated (Supplemental Fig. 4). Thus, our data suggest that the CysLT1 inhibitor pranlukast but not the antiplatelet drugs cangrelor and ticagrelor serve to attenuate function of MDL-activated GPR17.

Signaling complexes composed of GPR17 and a partner receptor do not explain its enigmatic pharmacology

As we failed to confirm inhibition of GPR17 by cangrelor and ticagrelor, we wondered whether the inhibition observed previously might have been due to co-expression of GPR17 and P2Y12 in the cell system under investigation. Following this line of thought, a GPR17-P2Y12 receptor signaling unit might also be the pharmacological entity responding to uracil-nucleotides and cysteinyl-leukotrienes. We chose whole cell phenotypic label-free DMR biosensor assays to test this assumption because co-expressed receptors may modulate each other's signaling profile via heteromer formation or cross-talk (Milligan and Bouvier, 2005; Ellis et al., 2006; Sedej et al., 2012; Vischer et al., 2015) but holistic label-free assays are pathway-unbiased and should, therefore, be ideally suited to visualize impacts of co-expression on quantitative or qualitative aspects of receptor signaling (Supplemental Fig. 5). In cells co-expressing both receptors we evoke distinct activity patterns with the GPR17 agonist MDL29,951, the P2Y12 agonist ADP but not cysteinyl-leukotrienes or UDP-glucose with the latter inducing only a hint of agonism at the very high concentration of 100 µM (Supplemental Fig. 5A-C). From these data we conclude that co-expression of GPR17 with P2Y12 in the same cell does not form the elusive molecular entity that helps to reconcile the inconsistent data surrounding GPR17 and its putative endogenous ligands. We then performed analogous experiments using HEK293 cells transfected to stably co-express GPR17 and

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CysLT1 (Supplemental Fig. 5D-F). Functional expression of each receptor was verified by robust whole cell activity profiles evoked upon application of individual receptor agonists (Supplemental Fig. 5D,E). Therefore, inability to trigger whole cell responses with uracil-nucleotides indicates that formation of a GPR17-CysLT1 signaling unit does not account for the elusive GPR17 pharmacology: agonist specificity of co-expressed receptors does not differ from that observed in the absence of a partner receptor.

Activation of GPR17 does not depend on the simultaneous presence of uracil-nucleotides and cysteinyl-leukotrienes

Finally, we also explored the possibility that cysteinyl-leukotrienes and uracil-nucleotides, functionally inert on GPR17 when applied alone, might evoke a cellular response upon co-administration. LTD4 and UDP-glucose, in particular, have already been co-administered to specifically target GPR17 *in vivo* with a novel paradigm on satiety control by GPR17 emerging from these experiments (Ren et al., 2012). Because this conclusion was centered in part on promotion of food intake by pharmacological ligands that do not activate GPR17 in their own right, we tested herein the theory that orphan GPR17 requires a cocktail of endogenous ligands to boost full functional activation. To this end we expressed GPR17 and CysLT1, alone and in combination and exposed each transfectant to LTD4 in conjunction with each uracil-nucleotide (Fig. 10A-D), but also to GPR17 agonist MDL29,951 (Fig. 10E-H), CysLT1 agonist LTD4 (Fig. 10I-L), or carbachol as viability control (Fig. 10M-P). Robust responses to the ligand mix were obtained only in cells expressing CysLT1 individually and in combination with GPR17 (Fig. 10C,D) but were not seen in cells expressing GPR17 or empty vector control (Fig. 10 A,B). Whole cell activity patterns therefore indicated substantial contribution of the CysLT1 receptor to the signal generated by the ligand mix (Fig. 10C,D) but are incongruent with the notion that co-administration of cysteinyl-leukotrienes and uracil-nucleotides suffices to trigger functional activation of GPR17. Because treatment of GPR17 cells with ligand mix did not evoke any functional response, we conclude, that cellular GPR17 activity does not depend on co-ordinated activation of a single receptor by two classes of endogenous ligands.

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DISCUSSION

Erroneous designation of orphan receptors and their cognate endogenous ligands may cause considerable confusion within the scientific community (Lauwers et al., 2006; Civelli et al., 2013; Rueda et al., 2016). GPR17 is such an orphan GPCR, pharmacology of which should be of great interest due to several recent studies that have implicated this receptor in demyelinating central nervous system diseases such as multiple sclerosis, but also in regulation of food intake and aging of the brain (Lecca et al., 2008; Ren et al., 2012; Marschallinger et al., 2015). All of these studies have in common that major conclusions were drawn based on utilization of ligands, activity of which at GPR17 is still ill-defined. Thus, there is pressing need to revisit the issues surrounding GPR17 pharmacology and authenticity of both activators as well as inhibitors proposed to target this receptor. If pharmacological tools, inert on GPR17, are being employed in extensive *ex vivo/in vivo* investigations to uncover physiological functions of this receptor, conclusions drawn from such studies may mislead an entire field (Lecca et al., 2008; Ren et al., 2012; Marschallinger et al., 2015).

Originally, GPR17 has been proposed as dualistic receptor responding to two distinct classes of signaling molecules: uracil-nucleotides and cysteinyl-leukotrienes (Ciana et al., 2006). While this receptor ligand assignment agrees well with the phylogenetic position of GPR17, located just intermediate between P2Y nucleotide and CysLT receptors (Bläsius et al., 1998; Ciana et al., 2006), variability of GPR17 responses in a ligand- and system-dependent manner is striking (Bläsius et al., 1998; Heise et al., 2000; Ciana et al., 2006; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Davenport et al., 2013; Harden, 2013; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). In spite of apparently identical experimental conditions only a single independent lab succeeded to recapitulate activity of uracil-nucleotides but not cysteinyl-leukotrienes on GPR17 (Benned-Jensen and Rosenkilde, 2010). Instead, constitutive $G\alpha_i$ activity was proposed for GPR17, a receptor feature that was not observed in the original deorphaning report and subsequent publications from this laboratory (Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009). The scenario seems even more ambiguous taking into account another independent study showing neither constitutive nor ligand-mediated activity of GPR17 (Maekawa et al.,

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2009). In fact, the latter revealed an intriguing, ligand-independent regulatory role of GPR17 suppressing function of the leukotriene CysLT1 receptor as dominant negative inhibitor within the context of a heterodimer. In this study, inactivity of both uracil-nucleotides and cysteinyl-leukotrienes on GPR17 in different cellular backgrounds was communicated but experimental data were not provided (Maekawa et al., 2009). In 2013, Qi and coworkers confirmed suppression of CysLT1 receptor function by co-expressed GPR17 but failed to recapitulate activation by all purported endogenous ligands when applied in a single high concentration (Qi et al., 2013). Apparently, researchers do not all observe activation of GPR17-mediated signaling events by the proposed endogenous signaling molecules and there is no consensus among these reports as to the ability of the individual ligand classes to activate this orphan receptor (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014).

From our perspective, it is highly unlikely that fundamental differences in molecular function are the bottleneck in GPR17 deorphanization; rather GPR17 utilizes the same basic molecular mechanisms as other family A GPCRs in terms of signal transduction (Hennen et al., 2013). Consistent functional activity of MDL29,951 across all tested cell lines, species and assay platforms attests to this (Hennen et al., 2013; Simon et al., 2016). Thus, GPR17 is either poorly behaved when it comes to functional assays with uracil-nucleotides and cysteinyl-leukotrienes or the purported endogenous ligands are simply inactive. Regardless, our data clearly rule out the possibility that orphan GPR17 accounts for the direct effects reported for uracil-nucleotides and cysteinyl-leukotrienes. Consequently, these ligands should not be used to probe physiology and function of GPR17 in complex biological settings. The same applies to ticagrelor and cangrelor: both P2Y12 inhibitors do not blunt function of GPR17. One might argue that lack of inhibition is a consequence of agonist usage in our study, but one may just as well challenge their purported GPR17 inhibition because both “antagonists” were identified when GPR17 was stimulated with inactive ligands (Ciana et al., 2006; Gelosa et al., 2014). MDL29,951, in contrast, is the first small molecule agonist reliably activating GPR17 irrespective of the cellular background and expression system (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Therefore, we argue that MDL29,951 currently

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represents the only pharmacological agonist probe likely to accelerate research surrounding pharmacology and function of this exciting orphan GPCR and its implication in CNS physiology.

It is well known that matching of GPCRs with their cognate ligands may be error prone because GPCRs cannot be expressed and analyzed in isolation but instead require rather complex cell-based assay systems harboring an array of endogenous receptors in addition to the overexpressed receptor of interest (Atwood et al., 2011). It follows that the readout of GPCR activation is not necessarily specific to the overexpressed receptor, but rather represents the sum signal produced by all GPCRs in the assay tube. Nevertheless, differences between the receptor overexpressing and the native cell system is interpreted as receptor-specific response (Reinscheid et al., 1995; Hirasawa et al., 2005; Wellendorph et al., 2005; Hennen et al., 2013). Even if a parental cell line is silent for a given stimulus, it is conceivable that it may become responsive if the stimulus encounters a novel pharmacological entity assembled between an endogenous and the overexpressed receptor. For this reason, it is recommended that orphan receptor ligand pairs be corroborated using both multiple assay formats and cell lines (Wilson et al., 1998; Kostenis, 2001; Wise et al., 2004). Even though the original deorphaning report investigated GPR17 activation by its newly assigned ligands in three distinct cell systems (Ciana et al., 2006), the majority of independent follow up studies does not agree with it (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). Herein, we employed all purported endogenous ligands, LTC4, LTD4, UDP, UDP-glucose, and UDP-galactose in a broad concentration range head-to-head with the known GPR17 agonist MDL29,951 on human and rodent orthologs using eight different functional assays platforms and five distinct cellular backgrounds. We took advantage of the known small molecule agonist MDL29,951 (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016) to ascertain functional GPR17 expression in all cell systems and included positive controls, P2Y14 and CysLT1R, to verify ligand integrity as well as assay design and performance. In spite of this broad effort, evidence in favor of ligand-mediated responses of uracil-nucleotides and cysteinyl-leukotrienes remained elusive across all assays, cell lines and receptor species. As we failed to recapitulate both activation of GPR17 by all proposed endogenous ligands and inhibition

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by the antiplatelet drugs ticagrelor and cangrelor, which target the nucleotide P2Y12 receptor (Ingall et al., 1999; Nicholas, 2001; Qamar and Bhatt, 2016),we speculated that the elusive GPR17 pharmacology may be explained by the absence of partner receptors lacking in our assays. Indeed, a heteromer involving P2Y12 and an unknown receptor has been postulated previously as complex transmitting the pro-inflammatory effects of the lipid mediator leukotriene E4 (LTE4) (Paruchuri et al., 2009). To test our assumption, we enriched cells with specific cDNAs coding for the nucleotide P2Y12 but also the cysteinyl-leukotriene CysLT1 receptor and examined whether novel pharmacological properties may be conferred onto GPR17 upon co-expression with these receptors. However, presence of neither receptor was sufficient to re-establish the purported GPR17 pharmacology. Whereas our data do not rule out dimerization between GPR17 and P2Y12 as well as CysLT1R per se, they clearly indicate that co-expression of GPR17 along with its phylogenetic neighbors is not sufficient to form the elusive "receptor pair" accounting for the enigmatic pharmacology of GPR17.

We are aware that our results have not provided an explanation for the contradictory data regarding GPR17 and the functional consequences ensued by purported endogenous and surrogate ligands. While it is conceivable that inconsistencies may in part be explained by utilization of different cell expression systems, cell growth conditions, receptor expression levels, and assay methodologies employed to assess receptor function, our comprehensive study based on numerous assay platforms that capture proximal and distal signaling events convincingly demonstrates that GPR17 is functionally expressed, yet completely inert towards uracil-nucleotides and cysteinyl-leukotrienes. In view of these results, we strongly recommend to consider GPR17 as orphan and anticipate our findings to stimulate future efforts into deorphanization of this receptor that is still in search of its true endogenous ligand(s).

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ACKNOWLEDGMENTS

We thank Marianne Vasmer-Ehses for expert technical assistance with conductance of DMR experiments and Corning Inc. as well as PerkinElmer for their support on the Epic®- and the Ensight™-DMR readers.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Simon, Merten, Gomeza, Kostenis, Mohr, Gillard.

Conducted experiments: Simon, Merten, Schröder, Vermeiren, Schrage, Hennen, Preis, Schmitt, Peters.

Contributed new reagents or analytic tools:

Performed data analysis: Simon, Merten, Schröder, Vermeiren, Schrage, Hennen, Preis, Schmitt, Peters.

Wrote or contributed to the writing of the manuscript: Simon, Merten, Kostenis.

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FOOTNOTES

*This work was funded by a grant from the German Federal Ministry for Education and Research (BMBF) within the BioPharma Initiative "Neuroallianz" [Grant 1615609B]. K.S. and E.K. are members of the German Research Foundation (DFG)-funded Research Training Group RTG1873.

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Note in memoriam:

We dedicate this manuscript to our coworker, colleague and friend Lucas Peters who deceased while this study was in progress.

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FIGURE LEGENDS

Fig. 1. Functional analysis of activated GPR17 in response to the small molecule surrogate agonist MDL29,951 and a panel of putative endogenous activators in hGPR17-1321N1 cells.

(A) GPR17-mediated G protein activation is captured by [^{35}S]GTPγS binding to membranes collected from hGPR17-1321N1 cells (n=3-5). (B) Concentration-effect curves of intracellular Ca^{2+} flux triggered by MDL29,951, UDP, UDP-gal, UDP-glc, LTC4 and LTD4 (n=3-5). (C-H) Label-free real-time traces of MDL29,951 (C), LTC4 (D), LTD4 (E), UDP (F), UDP-gal (G) and UDP-glc (H) recorded with an optical biosensor based on detection of dynamic mass redistribution (DMR). (I) Concentration-effect curves of compound-induced DMR responses from C to H (n=4-6). (J-O) Label-free bioimpedance sensing of MDL29,951 (J) and proposed endogenous ligands (K-O) in living hGPR17-1321N1 cells. (P) Concentration-effect relationships calculated from impedance recordings in panels J-O (n=4-6). Label-free recordings are shown as representative traces (mean + S.E.M.), each trace is an average of three replicates. Concentration-effect curves are mean values ± S.E.M..

Fig. 2. Label-free whole cell traces of the synthetic GPR17 agonist MDL29,951 and a panel of putative GPR17 stimuli in native 1321N1 cells. Label-free real-time traces of MDL29,951 (A, G), LTC4 (B, H), LTD4 (C, I), UDP (D, J), UDP-gal (E, K) and UDP-glc (F, L) recorded with an optical DMR-based biosensor (A-F) and by bio-impedance detection (G-L). Carbachol and PGE1 were included as viability controls. Signatures are shown as representative traces (mean + S.E.M., n=3) with each trace representing an average of three replicates.

Fig. 3. Purported GPR17 ligands but not MDL29,951 are inactive in stable hGPR17-CHO transfectants. (A) Ligand-induced [^{35}S]GTPγS incorporation reflects G protein signaling in membrane homogenates collected from hGPR17-CHO cells (n=3). (B) Release of intracellular Ca^{2+} triggered by MDL29,951, UDP, UDP-gal, UDP-glc, LTC4 and LTD4 (n=3-4). (C-H) Label-free real-time traces of MDL29,951 (C), LTC4 (D), LTD4 (E), UDP (F), UDP-gal (G) and UDP-glc (H) recorded with the

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Corning® Epic® biosensor based on detection of dynamic mass redistribution (DMR). (I) Concentration-effect curves of compound-induced DMR responses from C to H (n=4-7). (J-O) Label-free bioimpedance sensing of MDL29,951 (J) and proposed endogenous ligands- (K-O). (P) Concentration-effect relationships from the impedance assays depicted in J-O (n=3-8). Label-free signatures are representative traces (mean + S.E.M.), each trace is an average of three replicates. Concentration-effect curves are averaged data ± S.E.M..

Fig. 4. Uracil-nucleotides and cysteinyl-leukotrienes are robust activators of their cognate receptors P2Y14 and CysLT1, respectively. (A-C) DMR recordings of UDP (A), UDP-gal (B) and UDP-glc (C) in HEK293 cells stably expressing the human ortholog of the P2Y14 receptor. (D) Concentration-effect curves of the DMR traces in A to C are depicted (n=3). (E) Concentration-effect relationships of Ca^{2+} flux mediated by LTC4 and LTD4 on HEK293 cells stably expressing the hCysLT1 receptor (n=3-5). (F, G) Label-free real-time traces of LTC4 (F) and LTD4 (G) in hCysLT1 receptor expressing HEK293 cells. (H) Concentration-effect curves from the DMR assays depicted in F and G (n=3-4).

Fig. 5. Membrane-localized GPR17 is functionally responsive to MDL29,951 but not uracil-nucleotides and cysteinyl-leukotrienes in stable hGPR17-HEK293 transfectants. (A) Immunocytochemical localization of human GPR17 in hGPR17-HEK293 cells. Permeabilized cells were treated with an antibody directed against the C terminus of GPR17 and then incubated with a Cy3-conjugated goat anti-rabbit antibody. Scale bar 20 μm. (B-G) Label-free real-time traces of MDL29,951 (B), LTC4 (C), LTD4 (D), UDP (E), UDP-gal (F) and UDP-glc (G) recorded with an optical DMR biosensor. (H) Quantification of DMR responses as concentration-effect curves (n=4-5). (I-N) Activity of MDL29,951 (I) and putative endogenous activators (J-N) as determined in whole-cell label-free bioimpedance sensing. (O) Concentration-effect relationships from impedance assays depicted in I to N (n=4-8). Label-free signatures are shown as representative traces (mean + S.E.M.), measured in triplicates. Concentration-effect curves are mean values ± S.E.M..

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Fig. 6. Cellular responses of activated GPR17, P2Y14, and CysLT1 in receptor-proximal and –distal signaling events.

(A) Concentration-dependent Ca^{2+} flux triggered by the indicated GPR17 ligands in stable hGPR17-HEK293 cells (n=4-6). (B) hGPR17 activation-mediated conformational change of the $G\alpha_i$-subunit relative to the βγ-complex as determined in BRET[2] assays using HEK293 cells transfected to transiently co-express GPR17, energy donor $G\alpha_{i1}$-91*R*luc, energy acceptor GFP10-$G\gamma_2$ and complementary subunit $G\beta_1$ (n=4-8). (C) Time-course of ERK1/2 phosphorylation in stable hGPR17-1321N1 cells after treatment with the panel of ligands in the indicated concentrations (n=3-4). (D, E) Phosphorylation of ERK1/2 in native and P2Y14-HEK293 cells (D, n=5) as well as CysLT1-HEK293 cells (E, n=4) after stimulation with indicated concentrations of uracil-nucleotides and cysteinyl-leukotrienes. (F) Kinetics of ligand-induced β-arrestin2 recruitment conceived with HEK293 cells stably expressing hGPR17-*R*luc and GFP2–β-arrestin2 (n=3-4). Data shown are means ± S.E.M..

Fig. 7. Membrane-localized rodent GPR17 orthologs are unresponsive to uracil nucleotides and cysteinyl-leukotrienes in label-free DMR studies. (A, B) Immunocytochemical localization of mouse GPR17 (A) and rat GPR17 (B) in HEK293 cells stably expressing these receptors. Permeabilized cells were treated with an antibody directed against the C terminus of GPR17 and then incubated with a Cy3-conjugated goat anti-rabbit antibody. Scale bars 20 µm. (C-H) Label-free real-time DMR responses of MDL29,951 (C), LTC4 (D), LTD4 (E), UDP (F), UDP-gal (G) and UDP-glc (H) in HEK293 cells stably expressing mGPR17. (I) Concentration-effect curves calculated from the peak DMR responses (n=3-4). (J-O) Label-free DMR sensing of MDL29,951 (J) and putative endogenous stimuli (K-O) in rGPR17-HEK293 cells. (P) Concentration-effect relationships from DMR assays in J to O (n=3-5). Label-free signatures are means + S.E.M. of representative traces, each representing the mean of three replicates. Concentration-effect curves are mean values ± S.E.M..

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Fig. 8. Label-free whole cell DMR recordings of activated GPR17 in immortalized and primary rodent oligodendrocytes natively expressing the receptor. Label-free real-time DMR traces of GPR17 agonist MDL29,951 and the panel of putative stimuli in the immortalized oligodendrocyte line Oli-neu (A-F) and in primary rat oligodendrocytes (H-M). Oli-neu cells were pre-treated with 1 µM EGFR tyrosine kinase inhibitor PD174265 for 24 h to induce GPR17 expression (Simon et al., 2016). (G,N) Concentration-effect curves of ligand-induced DMR responses calculated from peak maxima in Oli-neu (G, n=3) and primary rat oligodendrocytes (N, n=3-7). Shown are individual DMR recordings (mean + S.E.M.) of triplicate determinations and concentration-effect curves (mean values ± S.E.M.) calculated for data as given in A to F and H to M, respectively.

Fig. 9. CysLT1 receptor antagonist pranlukast but not P2Y12-inhibitors cangrelor and ticagrelor interdict activation of MDL29,951-stimulated GPR17. The ability of cangrelor and ticagrelor, effective as antiplatelet drugs, to dampen signaling of GPR17 orthologs from human, rat, and mouse was compared with the reported GPR17 inhibitor pranlukast in assays measuring intracellular Ca^{2+} flux (n=4-8), IP1 accumulation (n=3-5) and label-free DMR (n=3-7). MDL29,951 was added to the cells at its EC_{80} in all cases. Data shown are means ± S.E.M..

Fig. 10. Concerted application of uracil-nucleotides and cysteinyl-leukotrienes does not result in functional activation of GPR17. HEK293 cells expressing GPR17 and CysLT1 alone and in combination were stimulated with LTD4 in conjunction with each uracil-nucleotide (A-D), GPR17 agonist MDL29,951 (E-H), CysLT1 agonist LTD4 (I-L), or carbachol as viability control (M-P) and cellular activity was monitored with a label-free DMR biosensor. Shown are traces (mean + S.E.M.) of a single experiment representative of three such experiments. Each trace is an average of three replicates.

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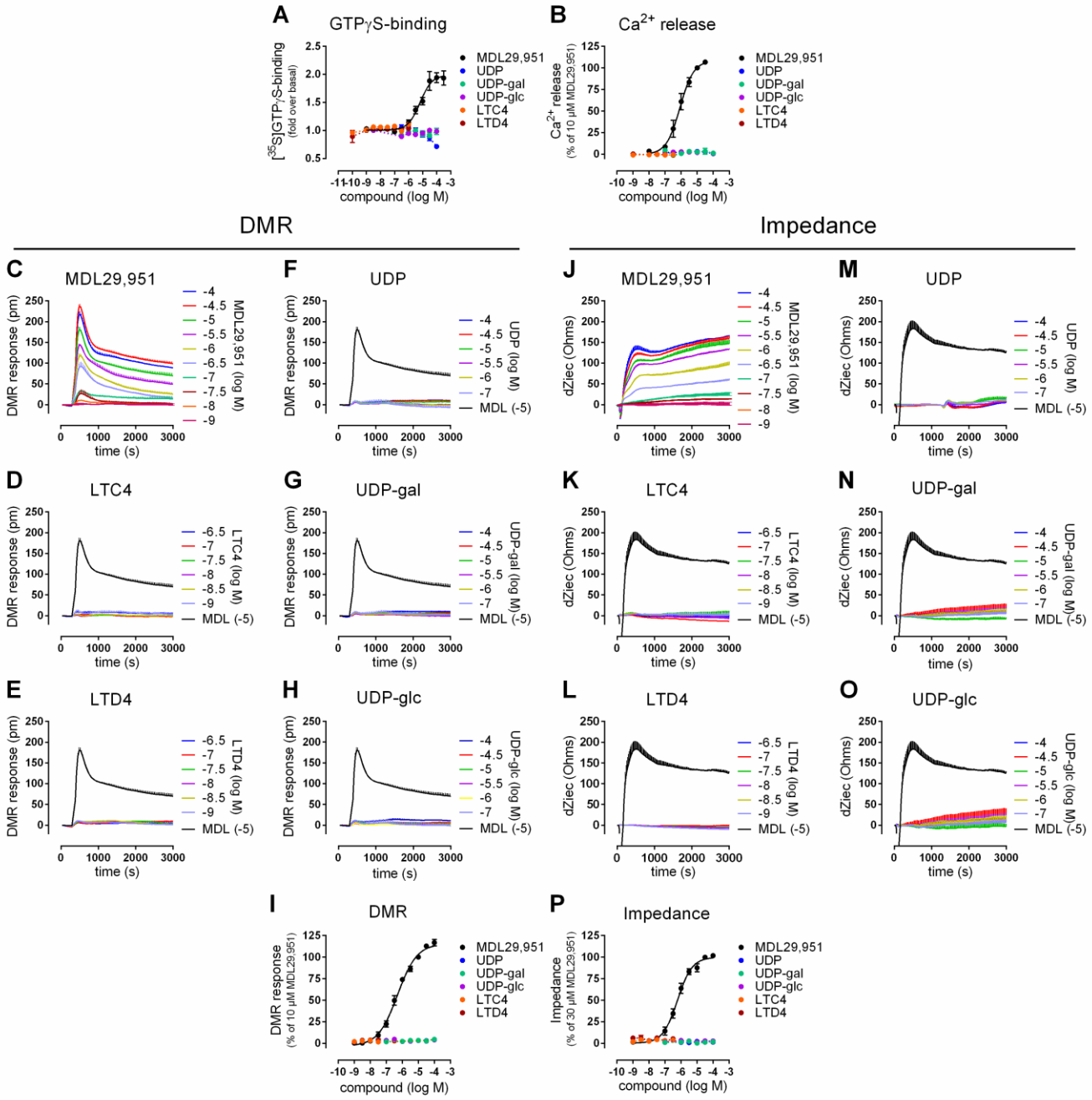

Figure 1

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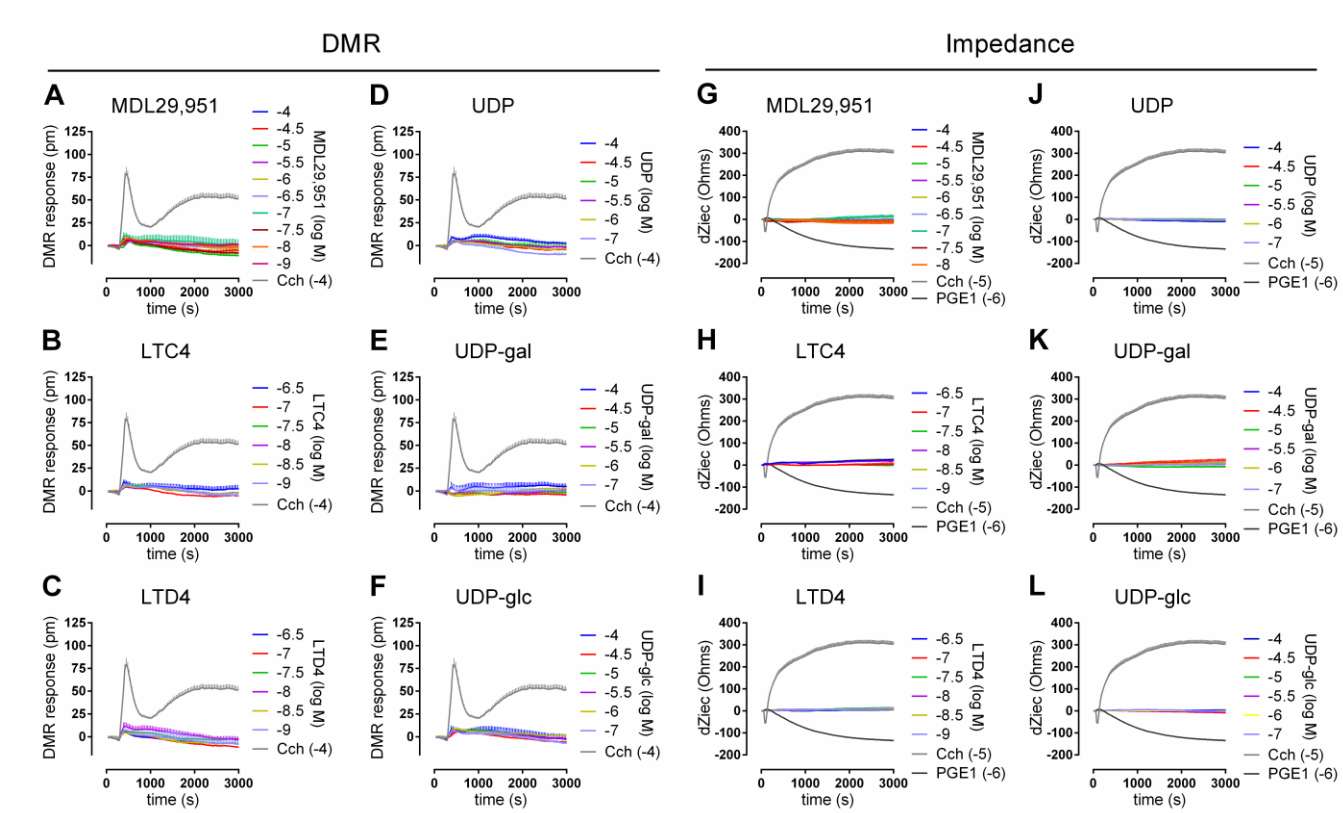

Figure 2

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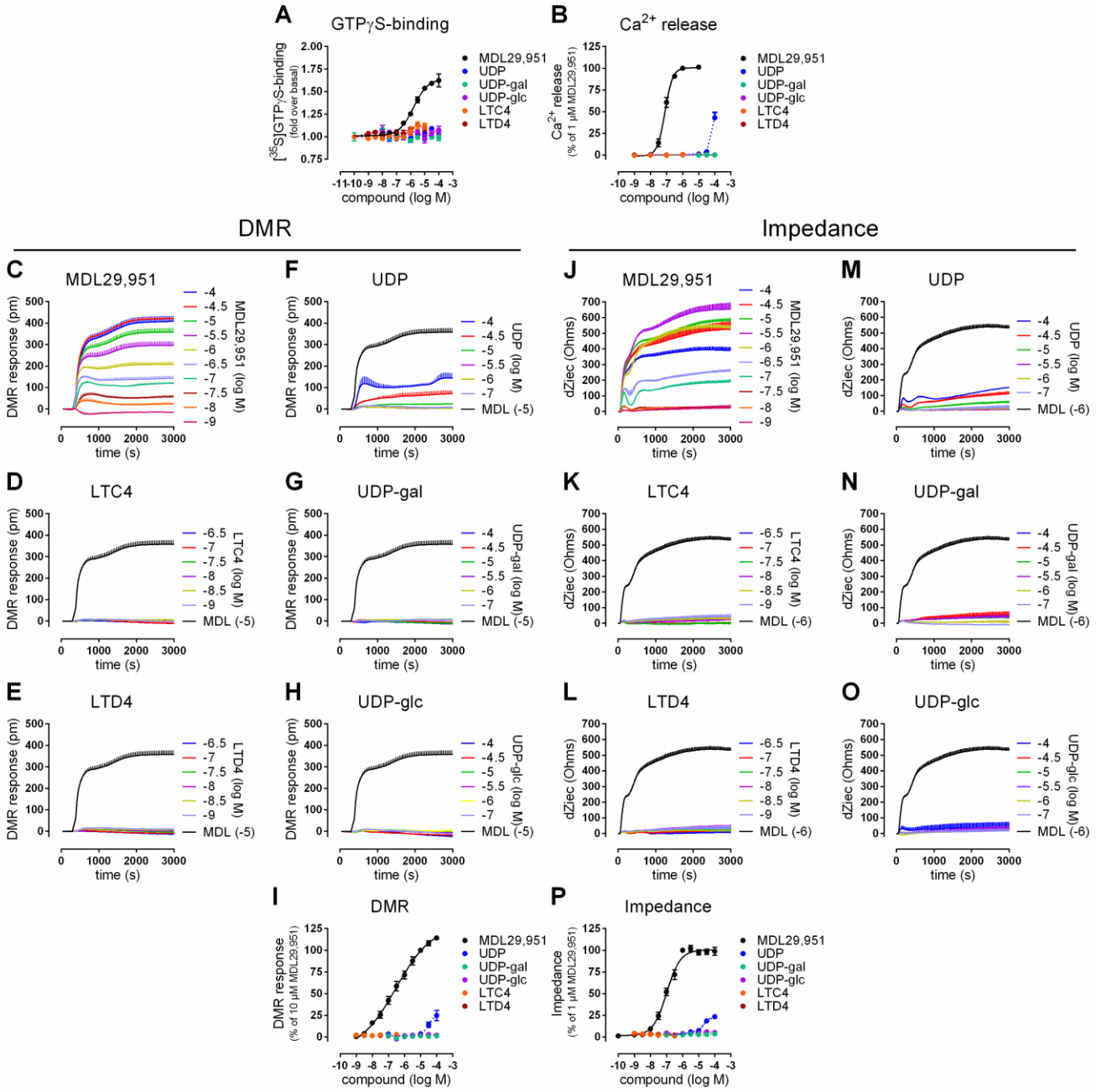

Figure 3

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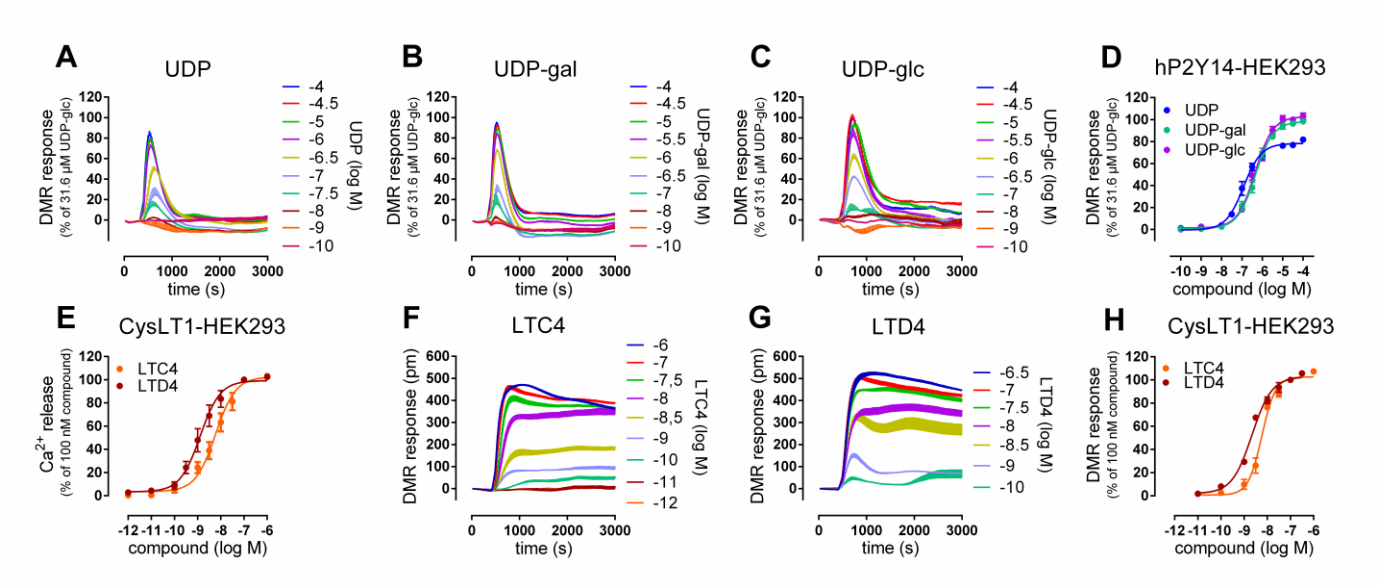

Figure 4

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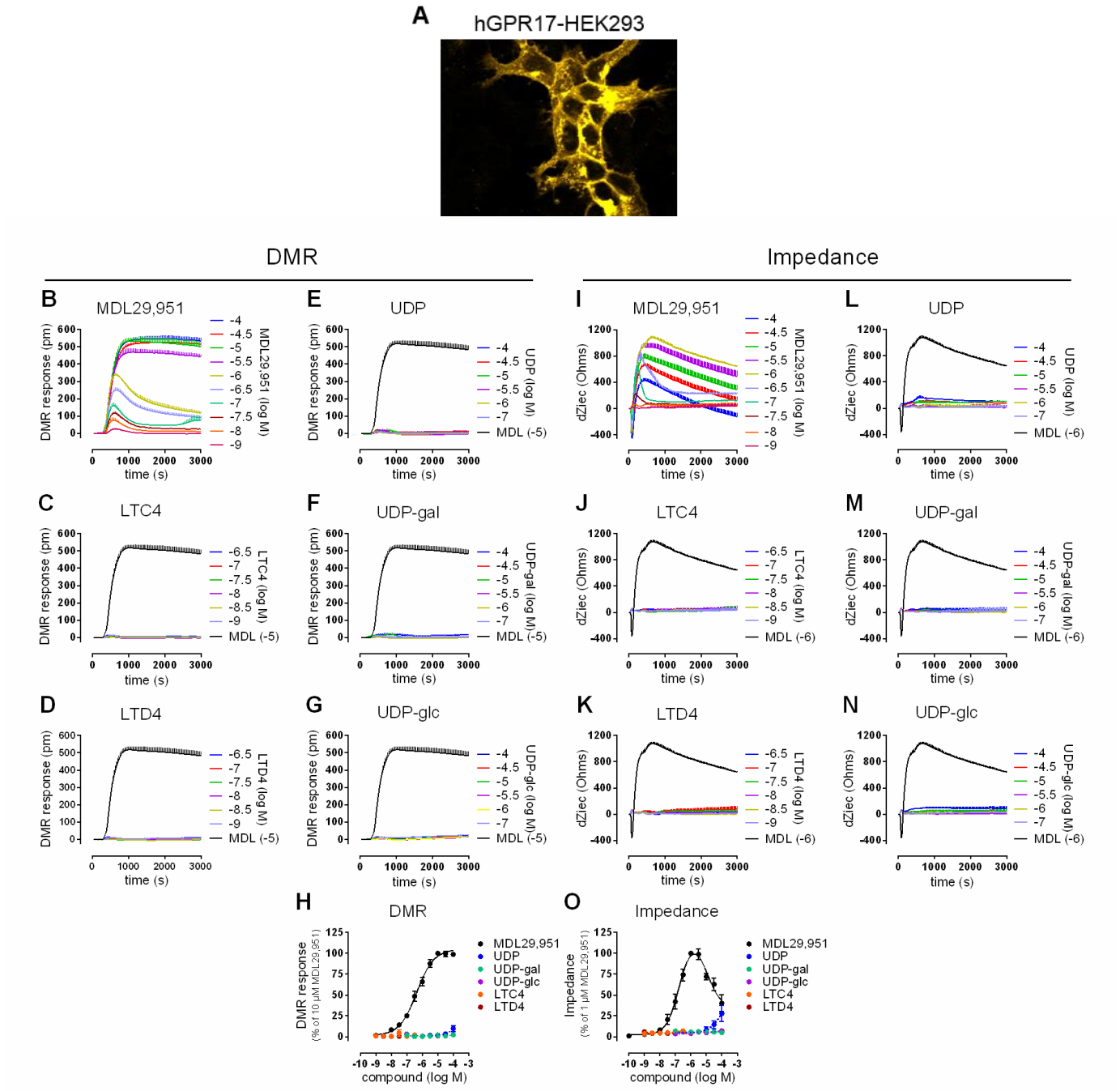

Figure 5

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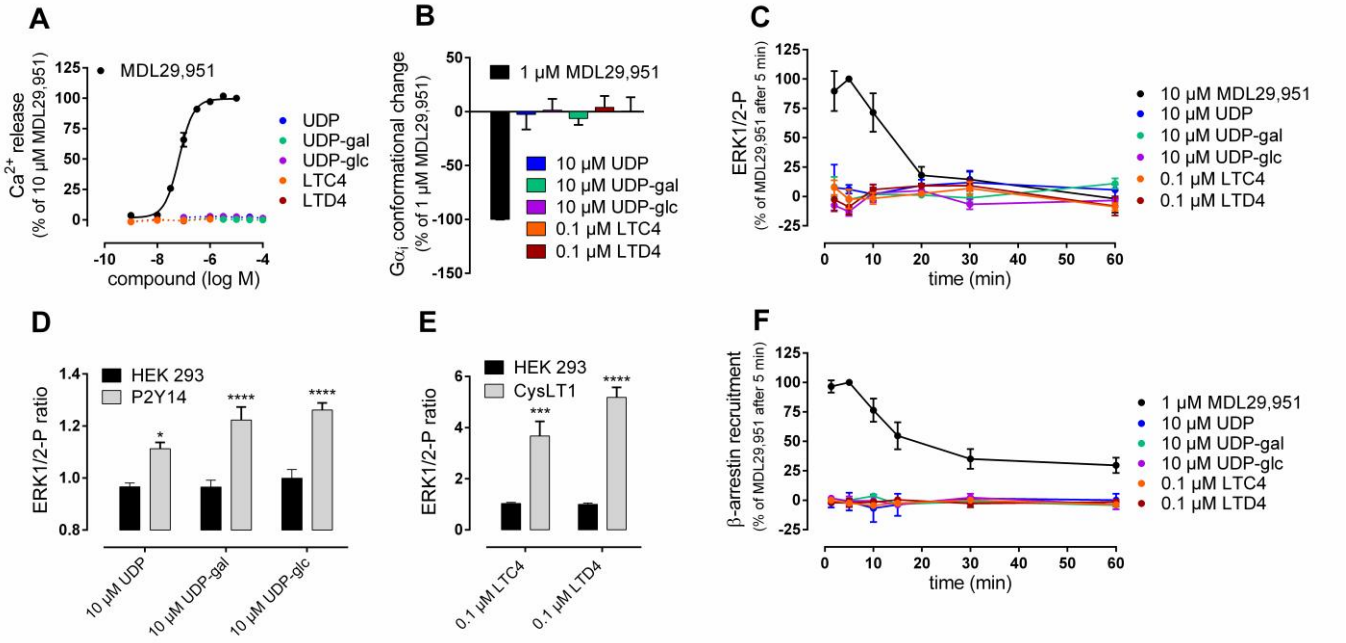

Figure 6

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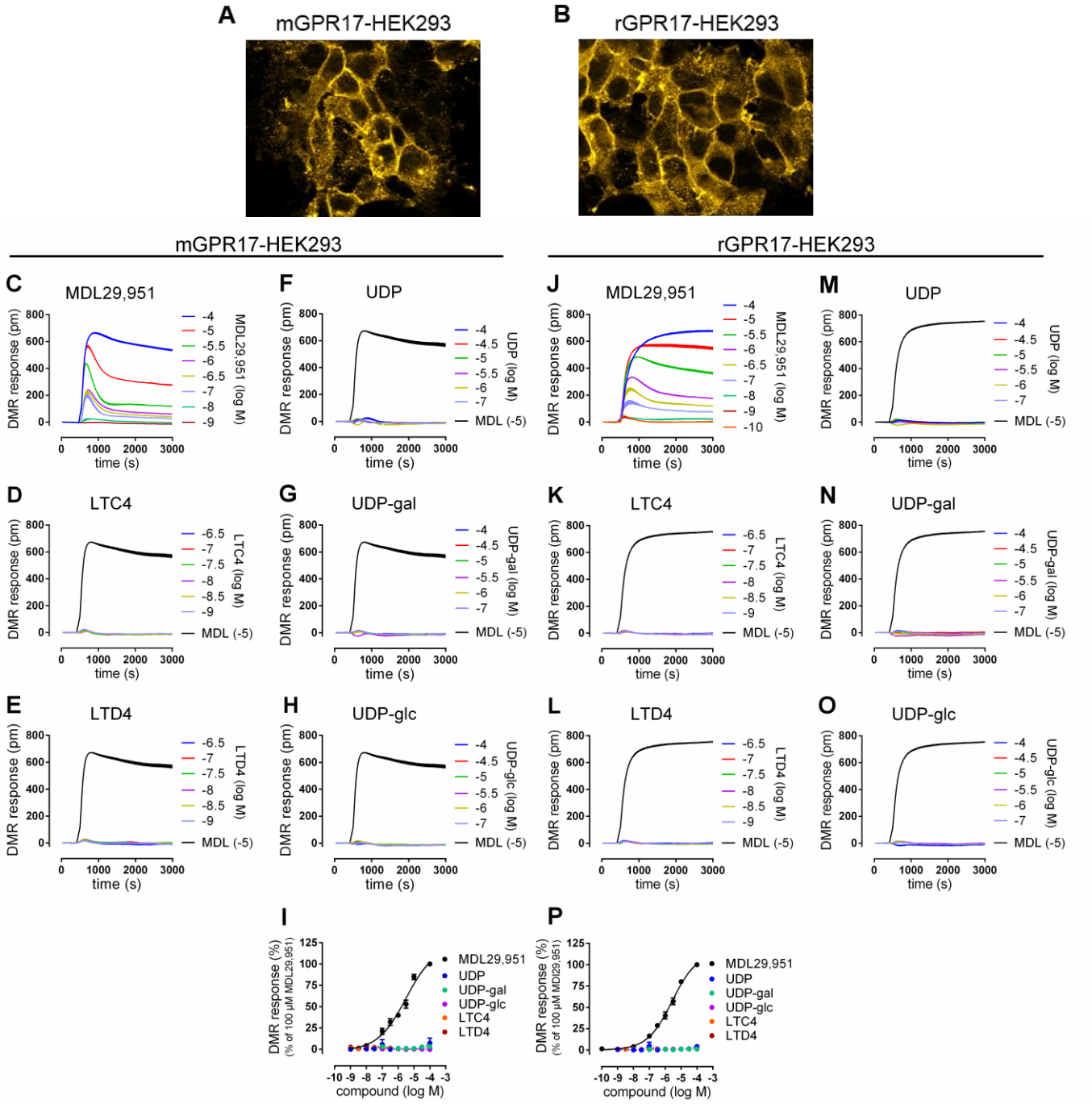

Figure 7

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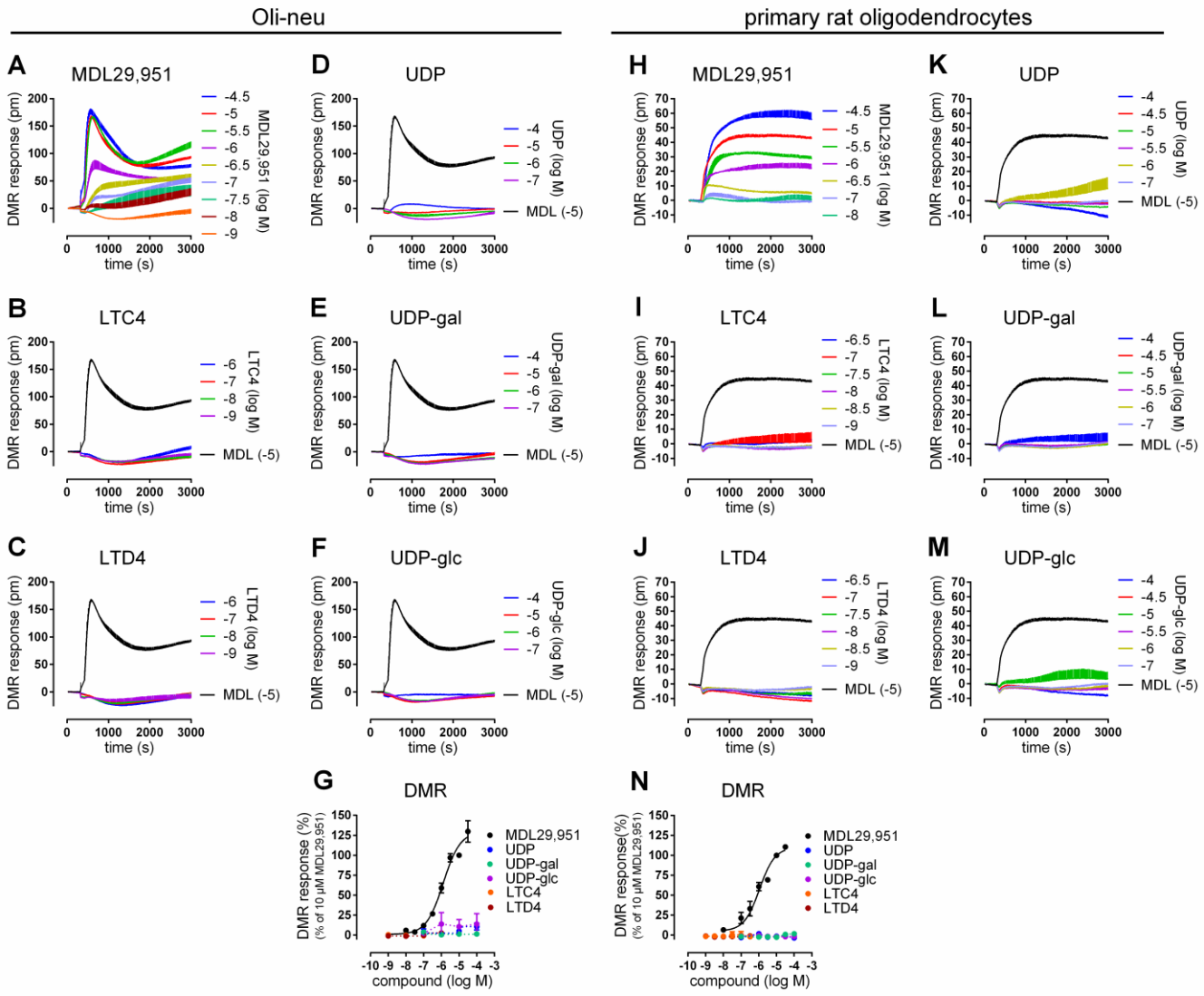

Figure 8

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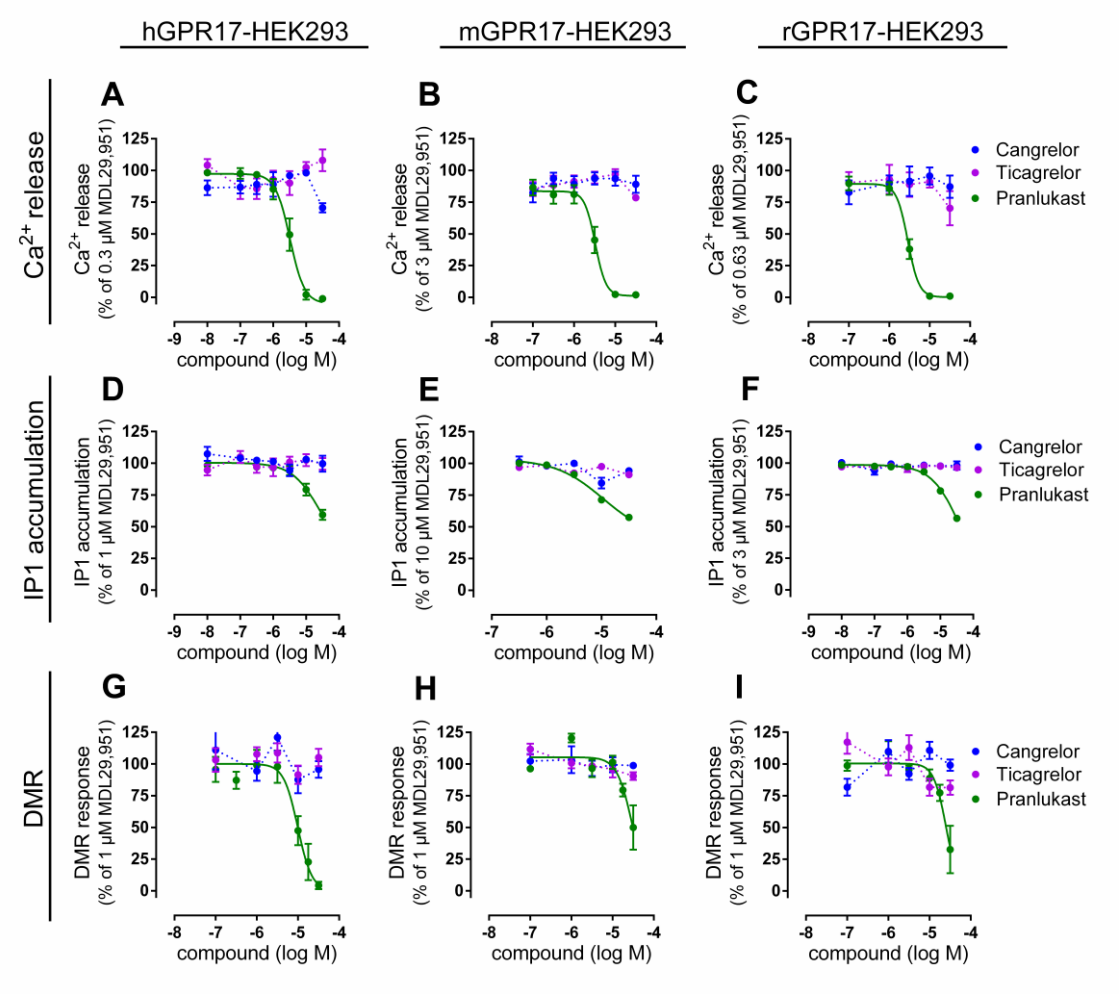

Figure 9

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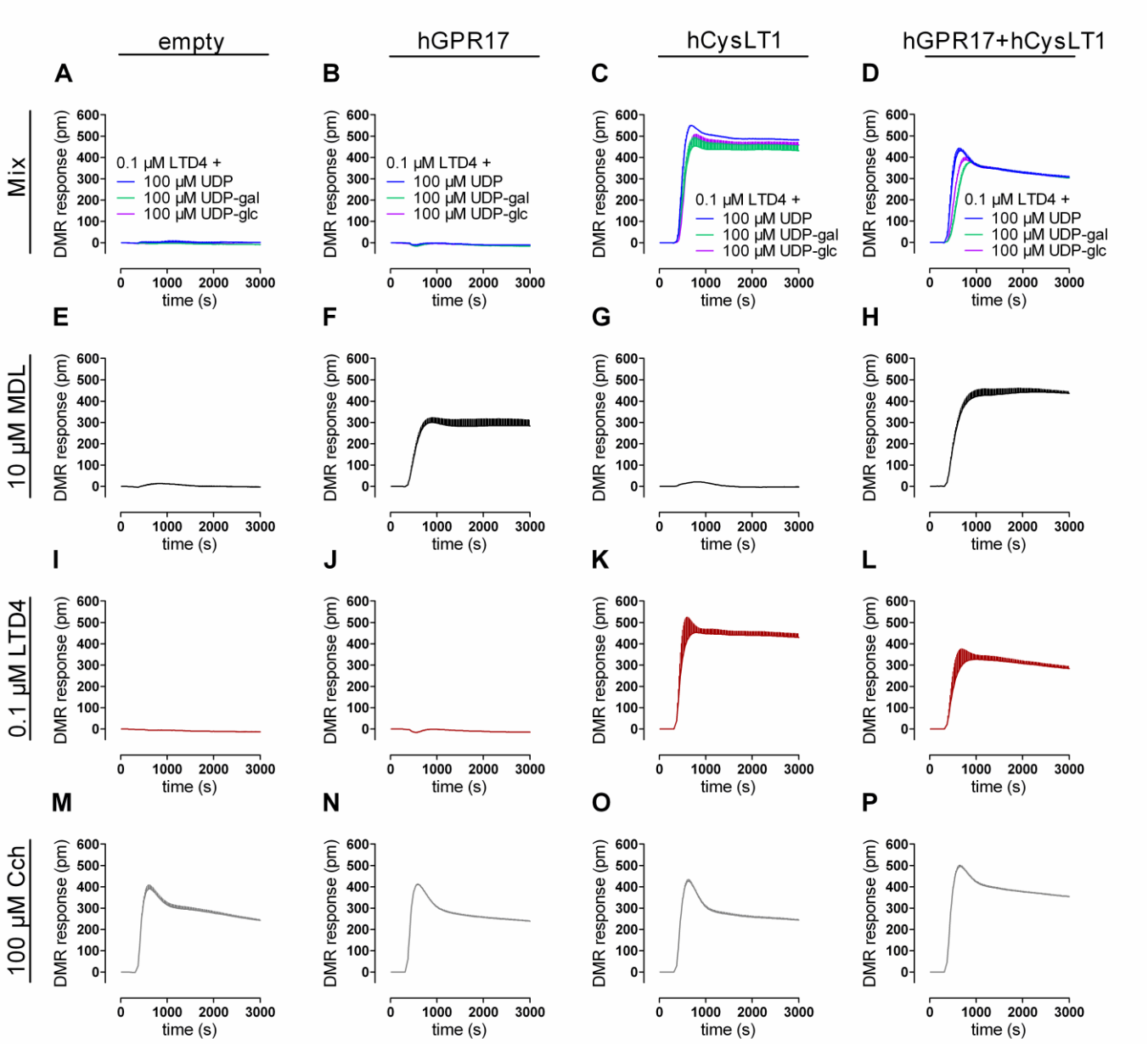

Figure 10