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Differential signalling by splice variants of the human free fatty acid receptor, GPR120[†]

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Running title: Signalling specificity of human GPR120 isoforms

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ABBREVIATIONS: 2-APB, 2-Aminoethoxydiphenyl borate; BG-AF488, Benzyl guanine-Alexa Fluor-488; BiFC, bimolecular fluorescence complementation; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMR, dynamic mass redistribution; FBS, fetal bovine serum; FFA, free fatty acid; GPCR, G protein-coupled receptor; GW9508 (4-[[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid); HBSS, Hepes buffered saline solution; HEK, human embryonic kidney; ICL, intracellular loop; Myr, myristic acid; OA, oleic acid; PBS, phosphate buffered saline; PTX, *Pertussis* toxin; TMD, transmembrane domain; TZD, thiazolidinedione.

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ABSTRACT

GPR120 is a long chain fatty acid receptor that stimulates incretin hormone release from colonic endocrine cells, and is implicated in macrophage and adipocyte function. The functional consequences of long (L) and short (S) human GPR120 splice variants, which differ by insertion of 16 amino acids in the third intracellular loop, are currently unknown. Here we compare signalling and intracellular trafficking of GPR120S and GPR120L receptors, using calcium mobilization and dynamic mass redistribution (DMR) assays, together with quantitative imaging measurements of β-arrestin2 association and receptor internalization. FLAG or SNAP tagged GPR120S receptors elicited both intracellular calcium mobilization and DMR responses in HEK293 cells, when stimulated with oleic acid, myristic acid or the agonist GW9508. Responses were insensitive to *Pertussis* toxin, but increases in intracellular calcium were attenuated by 2-APB (2-Aminoethoxydiphenyl borate), an inhibitor of store inositol trisphosphate receptors. Despite equivalent cell surface expression of SNAP-tagged GPR120L receptors, no specific calcium or DMR responses were observed in cells transfected with this isoform. However agonist stimulated GPR120S and GPR120L receptors both recruited β-arrestin2 and underwent robust internalization, with similar agonist potencies in each case. Following oleic acid induced internalization, neither GPR120 isoform recycled rapidly to the cell surface. In both cases confocal microscopy indicated receptor targeting to lysosomal compartments. Thus, the third intracellular loop insertion in GPR120L prevents G protein dependent intracellular calcium and DMR responses, but this receptor isoform remains functionally coupled to the β-arrestin pathway – providing one of the first examples of a native β-arrestin biased receptor.

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Introduction

It is now recognized that long chain free fatty acid (FFA) nutrients also exert important effects as signalling molecules, beyond the consequences of mitochondrial β-oxidation (Yaney and Corkey, 2003). As ligands for the transcription factor family of peroxisome proliferator-activated receptors (PPARs), they regulate expression of genes that influence metabolism (Varga et al., 2011). Two cell surface G protein-coupled receptors (GPCRs) are also capable of mediating rapid responses to long chain (C12-C22) saturated and unsaturated FFAs (Stoddart et al., 2008; Talukdar et al., 2011). FFA1, also known as GPR40, is predominantly expressed in pancreatic β-cells (Briscoe et al., 2003; Itoh et al., 2003). It mediates short term FFA effects in enhancing glucose stimulated insulin secretion (Briscoe et al., 2006; Briscoe et al., 2003; Itoh et al., 2003; Steneberg et al., 2005), and may also have longer term actions that contribute to β-cell dysfunction (Steneberg et al., 2005; Talukdar et al., 2011).

The second GPCR, GPR120, also binds C14-C18 saturated or C16-22 mono- and poly-unsaturated long chain FFAs (Hirasawa et al., 2005), but is not closely related in amino acid sequence to FFA1 (Fredriksson et al., 2003; Stoddart et al., 2008). This receptor is a nutrient sensor on colonic L-type enteroendocrine cells, where its stimulation by lumenal FFAs releases incretins such as glucagon like peptide-1 and cholecystokinin into the circulation (Hirasawa et al., 2005; Tanaka et al., 2008) – indirectly leading to increased insulin secretion. FFA1, which is co-expressed in similar cells, may contribute to these effects (Edfalk et al., 2008) and both FFA1 and GPR120 also act as FFA detectors in taste buds (Cartoni et al., 2010). However, independent effects of GPR120 have been identified in adipocytes

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and macrophages (Gotoh et al., 2007; Oh et al., 2010). In adipocytes, GPR120 activation increased glucose uptake and adipogenesis and GPR120 signalling in macrophages exerted anti-inflammatory actions (Gotoh et al., 2007; Oh et al., 2010). Similar *in vivo* dual effects of plasma ω3 FFAs, acting via GPR120, were confirmed through comparison of wild type and GPR120 knockout mice (Oh et al., 2010). Thus as novel diabetes treatments, GPR120 agonists might be unique in improving insulin sensitivity while also reducing "metabolic" inflammation implicated in the disease pathogenesis (Talukdar et al., 2011).

Oh et al. (2010) also revealed that GPR120 responses in adipocytes and macrophages relied on distinct cell signalling mechanisms. In adipocytes, enhancement of glucose uptake was dependent on $G_{q/11}$ protein activation. However the macrophage anti-inflammatory effect instead required the recruitment of β-arrestin adaptor proteins, which have classical roles in receptor desensitization and internalization, and are also signalling scaffolds (Gurevich and Gurevich, 2006). Internalized GPR120-β-arrestin2 complexes prevented pro-inflammatory cascades by sequestering a component of the inflammatory pathway in an inactive form (Oh et al., 2010). This functional requirement for different GPR120 signalling cascades raises questions about whether they may be activated selectively by agonist-GPR120 complexes in different cell types.

It is therefore intriguing that human GPR120 exists in two splice variants (Moore et al., 2009), which for example are co-expressed in colonic endocrine cells (Galindo et al., 2011). The short isoform (GPR120S) contains 361 residues, whilst the long isoform (GPR120L; NM_181745) contains 16 additional residues between

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positions 231 and 247 in intracellular loop 3 (ICL3, Fig. 1). For rhodopsin-like GPCRs ICL3 is a critical cytoplasmic element of the receptor involved in both G protein and β-arrestin recognition (Gurevich and Gurevich, 2006; Rosenbaum et al., 2009), and ICL3 insertion in GPR120L could result in significant changes in receptor signalling properties. Investigations of GPR120L pharmacology have in general required over-expression of promiscuous or chimeric Gα subunits (Galindo et al., 2011; Hirasawa et al., 2005). In the absence of this method of facilitating G protein coupling, most recent studies have focussed on human and rodent equivalents of GPR120S (Galindo et al., 2011; Moore et al., 2009; Oh et al., 2010; Tanaka et al., 2008).

Here we compare GPR120S and GPR120L receptors in the same defined recombinant system. We demonstrate that in HEK293 cells, GPR120L is impaired in G protein coupling compared to GPR120S, in response to FFAs and the synthetic agonist GW9508 (Briscoe et al., 2006), as assessed in Ca^{2+} signalling and label free dynamic mass redistribution (DMR) assays (Schroder et al., 2010). However, by using quantitative imaging analysis of β-arrestin recruitment and receptor internalization (Kilpatrick et al., 2010), we show that both GPR120 isoforms efficiently engage the β-arrestin pathway, and exhibit similar intracellular trafficking to lysosomes. Thus alternative splicing generates a GPR120 isoform with signalling specificity tailored towards arrestin dependent pathways.

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Materials and Methods

Materials. FFAs were purchased from Sigma Aldrich (Poole, U.K.), and were stored as 100 mM stock aliquots in DMSO. From these aliquots, fresh 1.8 mM experimental stocks were prepared daily in the appropriate media. GW9508 was from Tocris (Bristol, U.K), and pertussis toxin (PTX) was from Calbiochem (La Jolla, CA). Cell culture media and selection antibiotics were from Sigma Aldrich, Lonza (Wokingham, U.K.) or Invitrogen (Paisley, U.K.). Molecular biology reagents were from Sigma Aldrich, Promega (Southampton, U.K.), New England Biolabs (NEB, Hitchin, U.K.) or Fermentas (St.Leon-Rot, Germany).

Molecular biology. GPR120S (Genbank accession number BC101175) and GPR120L (NM_181745) receptor cDNAs were inserted between restriction sites BamHI (5') and XhoI (3') in the tetracycline repressor (TR) regulated expression vector pcDNA4 /TO (Invitrogen). cDNA sequences lacked the start Met codon, and included the stop codon when appropriate. Insertion of a Kozak element and appropriate coding sequences between KpnI and BamHI into GPR120 vectors generated receptors tagged at the N terminus with (i) the FLAG epitope tag (DYKDDDDK) or (ii) a 5HT3 receptor derived signal sequence followed by the SNAP tag (New England Biolabs, Hitchin, U.K.). For some receptor constructs, the 3' STOP codon was removed to also allow C terminal receptor fusion (between XhoI/XbaI, linker LE) to green fluorescent protein (GFP) containing superfolder mutations S30R and Y39N (Pedelacq et al., 2006). For bimolecular fluorescence complementation (BiFC) experiments FLAG tagged GPR120 cDNAs lacking STOP codons were transferred to pCMV FLAG (Stratagene (Agilent), Edinburgh, UK), and venus YFP fragment 155-238 (Yc) was inserted in frame between

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3' XhoI and ApaI sites (linker LE) –generating GPR120S-Yc and GPR120L-Yc fusion proteins. R99A (2.64 in Ballesteros-Weinstein numbering) and R178A (4.65) mutations in GPR120S were generated using QuikChange II site-directed mutagenesis (Stratagene). The identities of all constructs were confirmed by sequencing.

Cell culture. Cells were maintained in a humidified environment at 37^{o}C / 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), and passaged when confluent by trypsinization (0.25% w/v in Versene). Transfections into human embryonic kidney (HEK) 293 cells were performed using lipofectamine (Invitrogen) according to the manufacturer's instructions. For inducible expression GPR120 receptor cDNAs in pcDNA 4 TO were introduced into 293TR cells (Invitrogen, which are transfected with the TR protein to allow inducible expression), and mixed population stable lines were selected by resistance to blasticidin (TR vector, 5 μg ml^{-1}) and zeocin (receptor, plasmid; 20 μg ml^{-1}, both Invitrogen). BiFC cell lines were generated by selection of GPR120Yc transfected cells (G418, 0.8 mg ml^{-1}) on a clonal zeocin resistant HEK293 cell line expressing β-arrestin2-venus Yn (2-173) that we have previously described (Kilpatrick et al., 2010). Routine culturing of stable transfected cell lines included lower media concentrations of blasticidin (2.5 μg ml^{-1}), zeocin (5 μg ml^{-1}) or G418 (0.2 mg ml^{-1}) to maintain selection pressure. For receptor inducible expression cells were seeded onto experimental plates or coverslips 48 h prior to experiments, and as appropriate DMEM containing 1 μg ml^{-1} tetracycline was added 24 h later. Otherwise cells were seeded the day before experiments.

Fluo4 calcium mobilization assay. Confluent cell layers, on poly-D-lysine coated 96-well clear-bottomed black plates (Costar 3904, Fisher, Loughborough, U.K.),

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were loaded with the intracellular calcium indicator Fluo4. Briefly, loading was performed in DMEM / 10 % FBS containing 2.5 mM probenecid (Sigma), 1.5 µM Fluo4AM (Invitrogen) and 0.023% pluronic acid (Invitrogen; 45 min at 37^{o}C). Cells were washed and then incubated in Hepes buffered saline solution (HBSS; 25 mM HEPES, 10 mM glucose, 146 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM sodium pyruvate and 1.3 mM $CaCl_2$, pH 7.45) containing 2.5 mM probenecid and 0.02% FFA free bovine serum albumin (BSA; Sigma; 20 min at 37^{o}C). Where appropriate, inhibitors were added during this equilibration step. Fluo4 fluorescence was measured on a FlexStation II (Molecular Devices, Sunnyvale, CA, USA) for 165 s, with agonist additions (in HBSS/0.02%BSA, maximum 0.3 % DMSO) made from a compound plate at 15 s.

Automated imaging of receptor internalization and β-arrestin2 recruitment. Cells were used at 80 – 95 % confluence on poly-D-lysine coated 96-well thin-bottomed plates (Greiner 655090, Greiner Bio-One, Stonehouse, U.K.). For receptor imaging, SNAP-GPR120 cell lines were labelled in DMEM / 10%FBS containing 0.1 µM SNAPsurface benzyl guanine (BG)-AF488 (unless otherwise stated, NEB; 30 min at 37^{o}C). Cells were washed and then incubated in HBSS / 0.2 % BSA, containing FFA and synthetic agonists at the indicated concentrations (30 min at 37°C, unless otherwise stated). For measurement of receptor-arrestin BiFC, cells were directly treated with agonists in HBSS / 0.2 % BSA at 37°C at the times and concentrations incubated (see Kilpatrick et al., 2010 for more details).

Internalization and arrestin BiFC assays were terminated by fixation in 3% paraformaldehyde in PBS (PFA; 10 min at room temperature), followed after PBS washed by H33342 (5 µM; 20 min) treatment to stain cell nuclei. Images of BiFC

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fluorescence, or SNAP labelled receptors were acquired from four central sites per well on the IX Ultra confocal plate reader (Molecular Devices), using a Plan Fluor 40x NA0.6 extra long working distance objective, with 405 nm (H33342), 488 nm (BG-AF488 and BiFC) laser excitation (Kilpatrick et al., 2010). Supplemental Figure 1 also shows similar images acquired from the IX Micro plate reader (Molecular Devices) using the same protocol.

Dynamic mass redistribution (DMR). Cells were seeded on fibronectin coated 384-well plates (Corning 5042, Corning, NY, USA). Two hours prior to the experiment, plates underwent a 5 wash buffer exchange (HBSS/0.02% BSA/0.5% DMSO) and left to equilibrate (2h at 26^{o}C) in the Corning Epic Biosensor. If inhibitors were used, these were added 1h post buffer exchange, and plates were left to equilibrate for 1h. Agonists were added in quadruplicate and readings were taken every 40s for 50 min.

Receptor imaging using confocal microscopy. Cells at 50 – 70 % confluence on poly-D-lysine coated 8-well plates (Nunc Labtek, Fisher) were labelled with 0.5 µM SNAPsurface BG-AF488 as previously described, followed by treatment with vehicle or 300 µM oleic acid in HBSS/0.1% FFA free BSA (60 min at 37^{o}C). In addition, incubation media contained either 10 nM lysotracker red (Invitrogen; 60 min at 37^{o}C) throughout, or 5 µg ml^{-1} transferrin-AF633 (Invitrogen) was added 15 min prior to imaging. Live cell images were taken using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Welwyn, U.K.) using a 63x Plan-Apochromat NA 1.4 oil objective with Ar 488 nm (BG-AF488), HeNe 543 nm (lysotracker red) and 633 nm (transferrin-AF647) laser lines for excitation. Images were taken sequentially with band passes of 505-530 nm (BG-AF488), 560-615 nm (lysotracker red) and a long bandpass of 650 nm (transferrin-

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AF647). Pinhole diameter was set to 1 Airy unit for the longest wavelength, with the detector gain and amplifier offset adjusted to ensure images were not saturated; equivalent settings were maintained for control and agonist-treated cell images.

Data analysis. Calcium and DMR measurement data was analysed in terms of the peak agonist response (maximum-minimum), with data points from individual experiments being performed in triplicate or quadruplicate. Data was normalized (as % 300 μM oleic acid response) where comparisons were made between data sets from the same cell line and experiment set. For quantitative analysis of internalization and receptor-arrestin BiFC, a granularity algorithm (MetaXpress 2.0, Molecular Devices) detected vesicles of a specified diameter range in images from the IX Ultra confocal plate reader; the threshold intensity for classification was set with reference to the plate positive (300 μM oleic acid) and negative controls. Several parameters normalized to cell count (number of nuclei stained by H33342) were obtained (vesicle count, area, intensity per cell), for triplicate wells (12 sites per data group). These all provided similar results and in the figures, the normalized data from vesicle average intensity per cell is presented throughout. Comparisons of SNAP-GPR120S and SNAP-GPR120L expression were estimated by analysis of images acquired under identical settings, and from the same plate. The SNAPreceptor images (vehicle treated, from timecourse internalization experiments) were processed by the multiwavelength cell scoring algorithm (MetaXpress 2.0). Segmentation defined regions of the image covered by individual cells, based on both the location of H33342 stained nucei and boundaries of SNAPreceptor fluorescence staining. The mean pixel intensity within these regions (covering both plasma membrane and cytoplasm) was then averaged for all cells in

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each image. This measure of cellular fluorescence intensity was pooled between expts (mean ± s.e.m.) and used as an indicator of expression levels.

Concentration-response curves were fitted to the pooled data points (mean ± s.e.m.) by non linear least squares regression (GraphPad Prism 5.02, GraphPad software, San Diego, CA) and EC_{50} were quoted under circumstances where a maximum agonist response could be defined. Time course data was described by single phase association kinetics, following a manually adjusted latency period where appropriate. This latency accounted for the observation that FFA internalization and arrestin recruitment measurements showed a short delay (typically 2 - 5 min) after agonist addition before any response was observed. Two way analysis of variance (ANOVA) and Bonferroni post-tests were used to define statistical significance between concentration response relationships (GraphPad Prism).

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Results

Only the short GPR120 splice variant is coupled to calcium mobilization in HEK293 cells. We initially investigated signalling by FLAG tagged short and long GPR120 isoforms in HEK293TR cells, in which receptor expression was controlled by a tetracycline inducible promoter. After tetracycline treatment, GPR120-mediated responses were measured as peak increases in intracellular Ca^{2+} concentration (Fig. 2). The unsaturated C18:1 FFA oleic acid (OA), the saturated C14:0 FFA myristic acid (Myr) and the synthetic agonist GW9508 (Briscoe et al., 2006) were all full agonists in FLAG-GPR120S expressing cells (Fig. 2A), with respective pEC_{50} values of 4.7 ± 0.3, 4.2 ± 0.3 and 5.5 ± 0.3 (n = 4 - 5). In the absence of tetracycline induction no effects of OA, at up to 300 µM, were observed (Fig. 2A), indicating that FFA responses were mediated by the transfected GPR120S receptor. Surprisingly however, no Ca^{2+} signals were obtained from FLAG-GPR120L receptors expressed in the same system, to OA, Myr or GW9508 (Fig. 2B).

To confirm cell surface expression of the GPR120 isoforms, and to also study their intracellular trafficking, the N terminal FLAG epitope on the receptors was replaced with a signal sequence (from the 5HT3 receptor) followed by the SNAPtag protein. The SNAPtag is an 18 kDa protein based on the enzyme O^6-alkyl guanine DNA alkyltransferase, which can be covalently labelled by a range of fluorophores conjugated to its benzyl guanine (BG) substrate (Keppler et al., 2003). We first examined the expression of SNAP-GPR120S receptors that were also fused at the C terminus to GFP. Monitoring GFP fluorescence in induced 293TR SNAP-GPR120S-GFP cells

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demonstrated that the receptors were predominantly targeted to the plasma membrane. Thus addition of the N terminal SNAPtag did not adversely affect receptor processing and maturation (Supplemental Figure 1). Plasma membrane and some intracellular GFP immunofluorescence also colocalized with SNAP-tag labelling by membrane impermeant BG conjugated fluorophores, indicating cell surface expression with a low level of constitutive internalization (Supplemental Figure 1). The identification of the SNAP tagged receptors by BG fluorophores (0.1 – 1 μM) was specific, with little labelling in cells that had not been exposed to tetracycline. In these cells SNAP-GPR120S activation initiated transient Ca^{2+} responses with equivalent pEC_{50} values for OA (4.6 ± 0.2), Myr (4.2 ± 0.3), and GW9508 (5.5 ± 0.2) to those observed for the FLAG-GPR120S receptor (Fig. 2C, for example timecourses see Supplemental Figure 2). However as before, no calcium responses were observed in 293TR SNAP-GPR120L cells (Fig. 2D). Similar plasma membrane localization and overall expression levels for SNAP-GPR120S and SNAP-GPR120L receptors were observed using platereader imaging, after labelling the transfected 293TR cells with SNAP fluorophore (Fig. 2E and F, see also Fig 6).

Coupling of SNAP-GPR120S to intracellular Ca^{2+} elevations appeared independent of $G_{i/o}$ proteins, because agonist responses were unaffected by 18 h pretreatment with *Bordella pertussis* toxin (PTX; 100 nM; Fig. 3A and Supplemental Figure 2). The effect of 2-aminoethoxydiphenyl borate (2-APB, 50 μM) as an inhibitor of intracelullar calcium release was also examined (Maruyama et al., 1997), at a concentration greater than its IC_{50} value (42 μM) for store inositol 1,4,5-trisphosphate

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(IP_3) receptors, but below that reported to elicit non selective rises in cytoplasmic Ca^{2+} (> 90 μM; Maruyama et al., 1997). 2-APB significantly depressed maximal Ca^{2+} elevations to OA and GW9508 (Fig. 3B), and increased the transient nature of the responses measured (Supplemental Figure 2).

Two basic amino acids within the transmembrane domains (TMD) of GPR120S were also investigated as potential counterions which might interact with the FFA and GW9508 carboxylate groups. Arg 2,64 (Ballesteros-Weinstein numbering, or R99), at the top of TMD II (Fig. 1) has previously been implicated in agonist binding (Suzuki et al., 2008), and R2.64A substitution in the SNAP-GPR120S abolished both OA and GW9508 mediated calcium responses (Fig. 3C). However, R2.64A also reduced SNAP GPR120S receptor expression in 293TR cells, and surface SNAP-GPR120S R2.64A receptors labelled with cell impermeant BG fluorophores underwent substantial basal internalization in otherwise unstimulated cells (Fig. 3). Alanine mutation of Arg 4.65 (R178), at the top of TMD IV (Fig 1), did not alter SNAP-GPR120S expression or Ca^{2+} responses to GW9508 or OA (Fig. 3C).

Label free DMR measurements also reflect differences in signalling between GPR120S and GPR120L isoforms. We used DMR technology (Corning EPIC biosensor) to probe integrated functional responses to the GPR120 receptor splice variants in the same cells. This label free method measures signalling-induced changes in the distribution of proteins and other constituents, within cells grown on a specialized optical grating biosensor. Such changes produce small alterations in

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refractive index, and thus in the wavelength of reflected light following broad spectrum polarized illumination (Schroder et al., 2010). In addition to their amplitude, the profile of DMR measurements can indicate different types of agonist-stimulated GPCR signalling events, in particular by discriminating different Gα protein pathways (Schroder et al., 2010).

Figure 4 summarises DMR responses obtained from 293TR cells expressing SNAP-GPR120S or SNAP-GPR120L receptors, in comparison with non-transfected cells or those expressing the SNAP-GPR120S R2.64A mutant. ATP responses, mediated by endogenous P2Y receptors (predominantly $G_{q/11}$ coupled; Wirkner et al., 2004), were used as consistent positive controls. In tet treated 293TR SNAP-GPR120S cells the agonists OA, Myr and GW9508 all produced monophasic positive deflections in reflected wavelength (Fig. 4A), with potencies equivalent to the Ca^{2+} mobilization assay (Fig. 4B, Table 1). Maximal responses to GW9508 were somewhat greater than for the FFA agonists, though not significantly so. R2.64A mutation substantially reduced agonist responses, without altering their relative potencies (Fig 4C). Equally only small agonist responses (relative to ATP) were observed in SNAP-GPR120L cells, though these were greater than vehicle controls (Fig. 4D, 4E; Table 1). Similar low potency effects of GW9508 and OA were observed in non-transfected 293TR cells (Fig. 4F). Neither PTX (100 nM) nor 2-APB (50 μM) preincubation altered DMR responses to ATP or GPR120 FFA agonists, in either GPR120S or GPR120L expressing cells (Fig. 5, Table 2). However a small (3-fold) but significant reduction in GW9508 potency was observed following treatment with either inhibitor (Table 2).

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Both GPR120 splice variants undergo agonist stimulated endocytosis. As a further alternative readout of GPR120 activation, SNAP-tagged receptor internalization in response to different agonists was quantified by automated imaging and analysis. Figure 6 shows representative images, acquired on an IX Ultra confocal platereader, of the localization of BG-AF488 labelled SNAP-GPR120 receptors. In contrast to the Ca^{2+} and DMR assay findings, endocytosis of both SNAP-GPR120S and SNAP-GPR120L receptors was clearly observed following 300 µM OA pretreatment for 30 min. The fluorescence intensity within punctate compartments, predominantly intracellular, was quantified by granularity analysis to provide a measure of internalization on a cell-by-cell basis. As Figure 7 indicates 100 µM GW9508 stimulated internalization of SNAP-GPR120L ($t_{1/2}$ 21 ± 3 min, *n* = 4; following 2 min latency) and SNAP-GPR120S ($t_{1/2}$ 13 ± 1 min, *n* = 4; Fig. 7A) receptors. 300 µM OA responses increased internalization with $t_{1/2}$ of 20 ± 6 min for 293TR SNAP-GPR120S cells and 21 ± 3 min for SNAP-GPR120L cells (both *n* = 4, calculated following 5 min latency; Fig 7A). Concentration-response relationships, following 60 min agonist treatment (Fig. 7B), demonstrated a threshold of 30 µM for both OA and Myr stimulated internalization, and an approximate 3- 10 fold increased potency for GW9508 (pEC_{50} 4.4 ± 0.3 and 4.0 ± 0.4 for GPR120S and GPR120L respectively; n = 4) compared to both FFAs. Internalization of SNAP-GPR120S receptors in response to GW9508 or OA was unaltered by PTX pretreatment or by R4.65A mutation (both *n* = 4; data not shown). We also observed that TZDs elicited SNAP-GPR120S receptor responses (10 – 30 µM threshold concentrations) in both intracellular calcium mobilization (rosiglitazone, ciglitazone, and troglitazone) and

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endocytosis assays (ciglitazone, troglitazone). A fourth TZD, pioglitazone, was only active at 100 μM in the calcium mobilization assay (Fig. 8).

Both GPR120S and GPR120L receptors recruit β-arrestin2. GPCR endocytosis is often a downstream indicator of their interaction with β-arrestins. As a consequence the molecular association between GPR120 and β-arrestin2 was measured directly using a bimolecular fluorescence complementation (BiFC) assay that we and others have previously described (Auld et al., 2006; Kilpatrick et al., 2010). Its basis is that receptor and arrestin interaction brings complementary Yn and Yc fragments of yellow fluorescent protein (YFP) together to allow refolding and reconstitution of a fluorescent signal. In HEK293 cells stably co-transfected with GPR120-Yc and β-arrestin2-Yn (GPR120 A2), OA or GW9508 stimulation resulted in the development of receptor-arrestin BIFC in intracellular compartments (see Supplemental Figure 3) and the resulting fluorescence was then quantified by granularity analysis (Fig. 9). Timecourses for BiFC development in GPR120S A2 and GPR120L A2 cells were comparable in response to 30 μM GW9508 ($t_{1/2}$ 7 ± 1 min and 8 ± 1 min respectively (*n* = 5 - 6), no latency; Fig. 9A and B). Somewhat slower development of the GPR120L A2 300 μM OA response was observed ($t_{1/2}$ 18 ± 2 min (*n* = 5) compared to 9 ± 1 min (*n* = 6) for GPR120S, both after 5 min latency; Fig. 9A and B). Concentration response relationships demonstrated equivalent agonist potencies for GPR120S recruitment of β-arrestin2 compared to intracellular Ca^{2+} responses (Fig. 9C; GW9508 pEC_{50} 5.1 ± 0.1 and OA pEC_{50} 4.4 ± 0.1; *n* =4). Moreover, GW9508

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(pEC_{50} 5.1 ± 0.2, *n* =4) and OA (pEC_{50} 4.6 ± 0.2, *n* =4) also stimulated GPR120L β-arrestin2 interaction with similar potencies to GPR120S in this assay (Fig. 9D).

GPR120 receptors are internalized to non-recycling pathways associated with acidic late endosomes and lysosomes. The fate of internalized GPR120 receptors was investigated by confocal microscopy. Following 60 min vehicle or 300 μM OA treatment, SNAP-labelled receptors were imaged in live cells, in the presence of either 10 nM lysotracker red or 5 μg ml^{-1} transferrin-AF647. Lysotracker red is a marker of acidic organelles such lysosomes, while transferrin labelled receptors constitutively engage the clathrin mediated endocytosis and recycling pathway. As illustrated in the representative images presented in Figure 10, agonist-stimulated SNAP-GPR120S and SNAP-GPR120L receptors showed extensive colocalization with lysotracker red positive compartments, and also some distribution to compartments labelled by transferrin.

The pattern of distribution suggested a non-recycling trafficking phenotype for both receptors, and this was investigated using internalization analysis on the platereader. Cells were stimulated for 30 min with 300 μM OA, followed by two rinse washes and a final wash step of 5 – 60 in HBSS containing an increased concentration (0.1 %) of fatty acid free BSA. Figure 11 shows that there was a less than 25 % reduction in OA stimulated internalization over the 60 min wash period, for either 293TR SNAP-GPR120S or 293TR SNAP-GPR120L cells.

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Discussion

GPR120 is a long chain FFA receptor which regulates incretin hormone secretion, and has important modulatory roles for adipocyte and macrophage function (Hirasawa et al., 2005; Oh et al., 2010; Talukdar et al., 2011). As such GPR120 synthetic ligands offer a multi pronged approach to the treatment of obesity and type II diabetes. Understanding the pharmacology of GPR120 is complicated by the existence of two isoforms in the coding region of the human receptor, generated by alternative splicing, which are not apparent in lower primates and rodents (Moore et al., 2009). This study provides the first direct comparison between these splice variants in the same defined cell system, using a variety of approaches to detect G protein and β-arrestin dependent events. We demonstrated that the insertion of 16 amino acids in the third intracellular loop of GPR120L (Fig. 1) results in a receptor which fails to activate G proteins in HEK293 cells, whilst preserving arrestin recruitment and downstream internalization and trafficking to lysosomal compartments. This characterization of a GPR120L as an arrestin biased receptor has relevance for future studies, based on the recent critical role discovered for GPR120 arrestin-mediated pathways in immune cells (Oh et al., 2010).

GPR120S G protein coupling and pharmacology. GPR120S activation stimulated intracellular calcium mobilization and changes in DMR global cell responses through a PTX insensitive pathway, implicating the involvement of $G_{q/11}\alpha$ proteins. The time-profile observed for GPR120S DMR responses also adopted a signature appropriate for this type of G protein (Schroder et al., 2010). The absence of a PTX

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effect on GPR120S DMR measurements (other than a small reduction in GW9508 potency) contrasts with studies using FFA1, where the potential for $G_{i/o}\alpha$ as well as predominant $G_{q/11}\alpha$ coupling has been suggested (Itoh et al., 2003; Kotarsky et al., 2003), most convincingly by DMR (Schroder et al., 2010). However PTX insensitivity is characteristic of GPR120 signalling (Hirasawa et al., 2005; Oh et al., 2010), and siRNA knockdown of $G_{q/11}\alpha$ abolished GPR120 mediated glucose uptake in adipocytes (Oh et al., 2010). 2-APB had a partial effect on GPR120S Ca^{2+} responses, consistent with its potency for IP_3 receptor inhibition (Maruyama et al., 1997). DMR measurements were largely unaffected, suggesting involvement of additional arms of the $G_{q/11}$ dependent pathway, for example activation of protein kinase C.

The potencies of oleic acid and myristic acid, and the synthetic agonist GW 9508, in the GPR120S calcium and DMR assays were consistent with previous reports (Briscoe et al., 2006; Galindo et al., 2011; Hirasawa et al., 2005; Moore et al., 2009; Smith et al., 2009; Suzuki et al., 2008), and indicated that receptor modification with FLAG or SNAP epitope tags did not alter agonist activity. A 3-10 lower potency of FFAs, compared to some studies (Briscoe et al., 2006; Hirasawa et al., 2005), is likely to result from our inclusion of BSA (3 μM) in all assay media, which aids fatty acid solubility and limits the use of DMSO as a solvent, but also reduces FFA concentration (Hirasawa et al., 2005). An inducible expression system, as previously employed for a number of studies on FFA1 (Smith et al., 2009), demonstrated the specificity of GPR120 agonist responses. Furthermore we confirmed that mutation of TMD II Arg99 eliminated GPR120S receptor signalling, but not substitution of the second basic residue (Arg178)

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located at the top of TMD IV. This may support a role for Arg 99 in recognising FFA carboxylate anions, in an orthosteric binding site otherwise poorly defined by mutagenesis (Suzuki et al., 2008). However this mutant also substantially reduced cell surface expression, and led to extensive constitutive internalization of surface labelled SNAP GPR120S receptors. Thus some caution is necessary in interpreting the functional effects of R2.64A substitution only in the context of changes in agonist activity.

TZD PPARγ agonists stimulated GPR120S receptor signalling and internalization with low potency (threshold > 10 μM for calcium mobilization) The demonstration that TZDs, containing the 2,4-dione rather than a carboxylic acid group, can show GPR120 agonism, parallels their additional actions as FFA1 agonists (Hara et al., 2009; Kotarsky et al., 2003; Smith et al., 2009). In contrast to other tested TZDs including troglitazone, pioglitazone was without effect at 30 μM, and this ligand also exhibits the lowest potency amongst TZDs in activating FFA1 (Hara et al., 2009; Smith et al., 2009). Since pioglitazone retains PPARγ agonist activity equivalent or greater to troglitazone (Sakamoto et al., 2000), its relative position in experimental orders of potency should discriminate PPAR and FFA GPCR mediated actions of TZD ligands.

GPR120L as a β-arrestin coupled GPCR. Poor G protein coupling efficiency of GPR120L receptors was indicated by the absence of intracellular calcium and DMR responses, despite similar levels of cell surface expression to the GPR120S isoform. It is possible that HEK293 cells lack critical G protein subunits for GPR120L coupling –

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though $G_{q/11}\alpha$ is present, the mediator of GPR120 signalling in adipocytes (Oh et al., 2010). A specific impairment of G protein coupling is indirectly supported by previous investigations using the GPR120L splice variant. These required receptor fusion to the promiscuous G protein $G\alpha_{16}$ (Hirasawa et al., 2005), viral receptor overexpression (Briscoe et al., 2006), or over-expression of chimeric $G\alpha$ subunits (Galindo et al., 2011) in order to detect intracellular calcium signalling after activation. However both GPR120S and GPR120L recruited β-arrestin2 equivalently and underwent endocytosis in response to agonists. Quantitative imaging approaches demonstrated that both isoforms exhibited similar agonist potencies for each assay. Thus the perceived selectivity of GPR120L for arrestin signalling pathways is unlikely to result from a general impairment of GPR120L signalling, which becomes apparent only in the calcium assay format (Kenakin and Miller, 2010) – particularly given an increased receptor reserve here, compared to internalization and arrestin binding assays measured at the receptor. We conclude that GPR120L represents a GPCR whose signalling exhibits functional selectivity for arrestin-mediated pathways.

Molecular basis for GPR120 isoform coupling selectivity. The effect of the GPR120L splice insert might be predicted from the critical role that ICL3 plays for rhodopsin-like GPCRs, in forming part of the cytoplasmic cleft involved in $G\alpha$ recognition (Rosenbaum et al., 2009). Subtle alterations in G protein coupling specificity occur between dopamine D2S and D2L receptors (Lane et al., 2008; Senogles et al., 2004), while a splice insert in central ICL3 of the histamine H3 receptor influences its constitutive activity (Bongers et al., 2007). However the positioning of the

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GPR120L insert in the key juxtamembrane region between TMD V and ICL3 may account for more extensive changes in G protein signalling than have previously been observed for other GPCR splice variants. Mutagenesis studies in other GPCRs have indicated the critical importance of this region for G coupling specificity and activation (Cotecchia et al., 1992; Kostenis et al., 1997).

The absence of isoform-specific effects on β-arrestin association implies the structural elements involved in its recruitment to active GPR120 receptors differ from those required by the G protein. The recognition of active rhodopsin-like GPCRs by arrestin has been suggested instead to involve ICL2 (Marion et al., 2006), but the specific proline-based motif implicated is absent in GPR120. A second arrestin sensor domain binds phosphorylated Ser/Thr residues in ICL3 or in the C tail of GPCRs (Gurevich and Gurevich, 2006). Although additional potential phopshorylation sites are introduced into GPR120L by the amino acid insertion (Fig.1), our data, together with similar patterns of agonist-induced phosphorylation for both GPR120S and GPR120L (Burns and Moniri, 2010), indicate that they are not critical for initial β-arrestin association and internalization. The extent and duration of receptor phosphorylation could also influence subsequent intracellular trafficking of the receptor through recycling or degradative pathways, for example by altering the stability of arrestin association (Gurevich and Gurevich, 2006). However no differences were observed between GPR120 isoforms in this respect. Both variants were sorted to lysosomal compartments on internalization, and were unable to recycle rapidly to the cell surface after removal of agonist.

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DMR assays indicate G protein but not β-arrestin signalling by GPR120.

The absence of specific DMR responses mediated by SNAP-GPR120L receptors, beyond those also apparent in non-transfected 293TR cells, suggests that the DMR system is relatively insensitive to β-arrestin mediated signalling pathways. Indeed previous findings have indicated that GPCR DMR responses largely reflect G protein mediated events – whether this is assessed by blocking G protein activation, or by considering the minor contribution of downstream arrestin pathways, such as ERK activation (Schroder et al., 2010). It should also be noted that DMR assays are carried out at room temperature, and this would minimize any contribution that might be expected from GPR120 receptor clustering and endocytosis.

We have demonstrated that alternative splicing generates GPR120 receptors with altered coupling specificity between G protein and β-arrestin pathways. Isoform specific expression in different cell types might thus tune GPR120 signalling, for example to β-arrestin dependent mechanisms in macrophages (Oh et al., 2010). Moreover our observations on GPR120L, together with an initial investigation of the chemokine receptor CXCR7 (Rajagopal et al., 2010), provide early examples of native "G protein coupled" receptors which in fact display significant selectivity for β-arrestin recruitment.

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Authorship Contributions

Participated in research design: Brown, Holliday

Conducted experiments: Watson

Contributed new reagents or analytic tools: Brown

Performed data analysis: Watson

Wrote or contributed to the writing of the manuscript: Holliday, Watson, Brown

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Footnotes

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Legends to Figures

Fig. 1. The amino acid sequence of human GPR120S, also indicating the position of the additional 16 residues found only in ICL3 of the long isoform GPR120L (*). Putative N-linked glycosylation (white on black N) and palmitoylation sites (white on black C) are shown, together with the two basic Arg residues (black on grey R) mutated in this study.

Fig. 2. Intracellular calcium mobilization in response to GPR120S or GPR120L receptor activation. Peak intracellular calcium responses to increasing concentrations of OA, Myr and GW9508 were measured from fluorescence changes in Fluo4 indicator loaded 293TR, cells expressing FLAG-GPR120S (A), FLAG-GPR120L (B), SNAP-GPR120S (C) and SNAP-GPR120L receptors (D). OA responses were also assessed in cells without prior tetracycline induction of receptor expression (No Induction). Vehicle was medium containing 0.3 % DMSO. The curve fits for FLAG- or SNAP-GPR120S GW9508 responses (from panels A or C) are also reproduced as dotted lines for comparison in the GPR120L graphs B and D. Pooled data represent the mean ± S.E.M. of three to thirteen experiments performed in triplicate, which yielded pEC_{50} values quoted in the text. Panel E shows representative images of the cell surface expression of SNAP-GPR120S and SNAP-GPR120L receptors in induced 293TR cells (acquired on the IX Ultra platereader after labelling with membrane impermeant SNAP-AF488 dye, as Fig. 6). The average cellular fluorescence intensity was calculated from these images (see data analysis section in Materials and Methods) as a guide to the relative expression levels of the two isoforms, and the resulting pooled data (n = 6) is shown in histogram F. * $P < 0.05$ (unpaired Student's t-test).

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Fig. 3. The effects of PTX, 2-APB and R2.64A or R4.65A mutation upon GPR120S stimulated intracellular calcium mobilization. Tetracycline induced 293TR cells expressing SNAP-GPR120S were pre-treated with 100 nM PTX for 18 hr (A) or 50 μM 2-APB for 20 min (B), prior to Fluo4 loading and stimulation with OA or GW9508 (pooled data *n* = 4). In (C), agonist responses were compared with cells expressing SNAP-GPR120S R2.64A or SNAP-GPR120SA R4.65A receptors (*n* = 4). The insets show receptor labelling obtained with membrane impermeant SNAP label BG-AF488, imaged with the IX Ultra platereader (see Fig. 6 legend for full details). Vehicle or 300 μM OA treatment was for 30 min in these representative images.

Fig. 4. GPR120 mediated DMR responses. The effects of OA, Myr and GW9508, in comparison to the endogenous agonist ATP, were measured in cells expressing SNAP-GPR120S (A, C), SNAP-GPR120L (B, D), SNAP-GPR120S R2.64A (E) and non transfected 293TR cells (F). A and B show 50 min timecourses of the DMR changes observed (26°C) for agonists added at the indicated concentration. Pooled concentration-response curves (*n* = 6 - 9, in quadruplicate) constructed from the pooled data are shown in C-F, yielding pEC_{50} and R_{max} values quoted in Table 1.

Fig. 5. PTX or 2-APB pretreatment does not alter DMR responses. 293TR SNAP-GPR120S or SNAP-GPR120L cells were pretreated with PTX (A, C, D; 100 nM) or 2-APB (B, E, F; 50 μM) and peak DMR changes were then measured to OA, Myr and GW9508, together with the reference agonist ATP. The 50 min timecourses in A and B

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are shown in comparison to the equivalent OA and GW 9508 data in the absence of inhibitors (dotted lines, shown in full in Fig. 4A). Pooled data represent the mean ± S.E.M. of 3 - 6 experiments performed in quadruplicate, with agonist potency and maximal responses indicated in Table 2.

Fig. 6. Quantitative analysis of agonist-induced SNAP-GPR120S (A) and SNAP-GPR120L (B) internalization. An example experiment (*n* = 4) is illustrated in which 293TR cells induced to express each receptor isoform were compared on the same analysis plate. Cells were labelled with 0.1 µM membrane impermeant SNAPsurface BG-AF488 (30 min, 37°C), prior to stimulation with vehicle or 300 µM OA (60 min, 37°C). After fixation, cell nuclei were labelled with H33342. Representative images of H33342 and receptor labelling (from at least 4 experiments) are illustrated, representing a magnified portion (25 % area) of original images acquired and analysed by the IX Ultra platereader. A granularity algorithm (right panels) identified punctate receptor vesicles 2- 5 µm in diameter (white dots) and the cell nuclei (grey). This analysis quantified the extent of receptor internalization as described in Materials and Methods.

Fig. 7. Agonist stimulated internalization of GPR120S and GPR120L receptors. Endocytosis was assessed by granularity analysis of SNAP-tagged receptors expressed in 293TR cells and imaged using the IX Ultra, as described (Fig. 6). Panel A shows internalization time courses for 300 µM OA or 100 µM GW9508, pooled from experiments in which 293TR GPR120S and GPR120L cells were simultaneously treated with agonist on the same plate (*n* = 4). A 60 min agonist treatment period,

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following SNAP fluorophore labelling, was used to construct the concentration-response relationships to OA, Myr and GW9508 shown in B. In each case data represent the mean ± S.E.M. of 4 experiments performed in triplicate.

Fig. 8. GPR120S receptor responses to TZD ligands. Pooled data illustrate peak intracellular calcium mobilization responses (A, *n* = 4 – 5), or internalization following 60 min agonist treatment (B, *n* = 4, measured as Fig. 6), in tetracycline-induced 293TR SNAP-120S cells. In each case responses were normalized to 300 µM OA reference controls (100 %). Troglitazone and Pioglitazone caused changes in cell morphology at 100 µM, and so this concentration has been excluded from the concentration-response curves.

Fig. 9. Comparison of GPR120S and GPR120L association with β-arrestin2 using bimolecular fluorescence complementation. Stable transfected HEK293T cells co-expressed β-arrestin2-Yn and either GPR120S-Yc or GPR120L-Yc. This allowed agonist stimulated receptor-arrestin BiFC to be quantified in intracellular compartments by granularity analysis of the IX Ultra images (Supplemental Figure 3). A shows response timecourses to 30 µM GW9508 or 300 µM OA (*n* = 5 - 6), over 120 min at 37°C. Pooled OA and GW9508 concentration response curves (30 min treatment, *n* = 4) are shown in B with pEC_{50} values quoted in the text.

Fig. 10. Colocalization of GPR120 with markers for the clathrin mediated endocytic and late endosomal / lysosomal pathways, following internalization. 293TR expressing

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SNAP-GPR120S (A) or SNAP-GPR120L (B) receptors were visualized by live cell confocal microscopy following labelling with SNAPsurface AF488 ("Receptor") and incubation with vehicle or 300 µM OA (37°C, 60 min). Simultaneous images were acquired of the markers transferrin-AF633 (5 µg ml^{-1}), or lysotracker red (Lyso, 10 nM). Representative images of four independent experiments are shown, together with the overlay of the two channels. White arrows highlight examples of colocalized receptor and marker fluorescence (yellow).

Fig. 11. Internalized GPR120 receptors do not recycle rapidly to the cell surface. SNAP-GPR120S (A) and SNAP-GPR120L (B) recycling was quantified by granularity analysis of IX Ultra platereader images, as for Fig. 7. 293TR SNAP-GPR120S or SNAP-GPR120L cells were labelled with 0.1 µM SNAPsurface BG-AF488, followed by addition of vehicle or 300 µM OA (in HBS / 0.02 % BSA) for 30 min at 37^{o}C. Cells were washed twice with HBSS / 0.1% BSA), and then incubated in this media for 5-60 mines at 37^{o}C (the "wash period") before fixation. Pooled granularity data are normalized to 300 µM OA control responses without a wash step, and represent the mean ± S.E.M. of 5 experiments performed in triplicate.

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Table 1. Summary of DMR response data for 293TR cells expressing SNAP-GPR120S or SNAPGPR120L receptors.

Agonist	293TR Non transfected		293TR SNAP-GPR120S		293TR SNAP-GPR120L		293TR SNAP GPR120S R2.64A	
	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)
ATP	5.8 ± 0.2	100	5.0±0.1	100	5.2 ± 0.1	100	5.4 ± 0.1	100
OA	---	17 ± 4	4.6±0.2	58 ± 6	---	21 ± 5	4.5 ± 0.3	20 ± 4
Myr	---	2 ± 1	4.9±0.2	65 ± 5	---	16 ± 4	5.1 ± 0.2	19 ± 3
GW9508	---	6 ± 1	5.7 ± 0.1	104 ± 6	---	27 ± 12	5.4 ± 0.2	20 ± 2

pEC_{50} and R_{max} values (300 µM FFA, 100 µM GW9508 normalized to 100 µM ATP) were obtained from the pooled concentration response curves in Fig. 7. Data represent the mean ± S.E.M. of 6-9 experiments performed in quadruplicate.

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Table 2. Influence of 2-APB or PTX pre-incubation on GPR120 DMR responses.

	+ 50 µM 2-APB				+100 nM PTX			
Agonist	293TR SNAP-GPR120S		293TR SNAP-GPR120L		293TR SNAP-GPR120S		293TR SNAP GPR120L	
	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)
ATP	5.3 ± 0.2	89 ± 5	5.2 ± 0.1	81 ± 10	5.2 ± 0.1	92 ± 2	5.4 ± 0.1	94 ± 8
OA	4.5 ± 0.2	50 ± 13	---	20 ± 4	4.6 ± 0.2	49 ± 3	---	10 ± 2
Myr	5.0 ± 0.2	57 ± 9	---	9 ± 5	4.8 ± 0.2	53 ± 9	---	7 ± 2
GW9508	5.2 ± 0.2 **	107 ±11	---	37 ± 17	5.2 ± 0.1 *	107 ± 5	---	30 ± 14

Pooled concentration response curves (Fig. 8, *n* = 3) yielded the above pEC_{50} and R_{max} values (300 µM FFA, 100 µM GW9508 normalized to 100 µM ATP in control cells without inhibitor). Data represent the mean ± S.E.M. of two to nine experiments performed in quadruplicate. *P < 0.05, ** P < 0.01 compared to control GW9508 EC_{50} values in SNAP-GPR120S cells (Student's t-test; Table 1).

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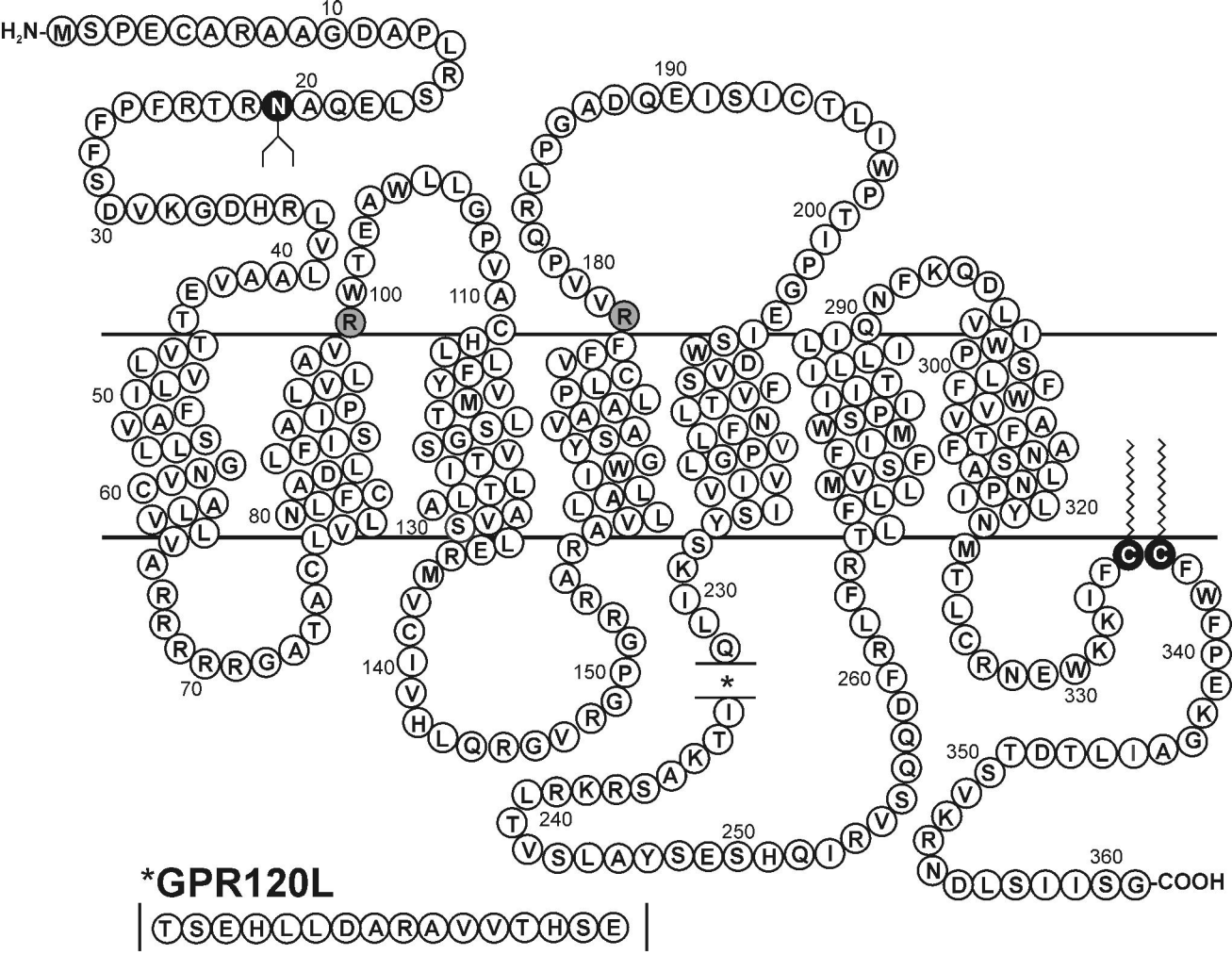
H2N-MSPECARAAGDAPLR
FPFRTRNAQELS
FFS
DVKGDHRLV
30
40
EVAALVT
TLVTLVV
50
IILVVAF
VALLSGN
60
CVNGVLA
VLAVL
70
ARRRRRGA
80
NLVLCATL
AWLLGPVACWTE
100
R
110
LHLFLMVTSGSLTVSITLTLALVAS
130
MREL
140
VCIVHLQRGVRGP
150
RARRRGVFFLCAAVLPVLAFLVLAVLWGLRQPVVR
180
190
ADQEISICTLIWPIT
200
IPGEWSIVDSVTLVFNLFLGPVIVYSI
230
SKILQ
*
IT
LRKRSAK
240
TVSLAYSESHQIRV
250
260
LIILLLITIWSPIMFVSFLTLRFLRFDQQS
290
NFKQDVLIPWISFLWFVVAFTFNASNLIPNYL
300
320
NMTLCRNEW
330
CCFFIKKFWFPEK
340
SVKRNDLSIISG-COOH
350
360
TDTLIAG
*GPR120L
TSEHLLDARAVVTHSE

Figure 1

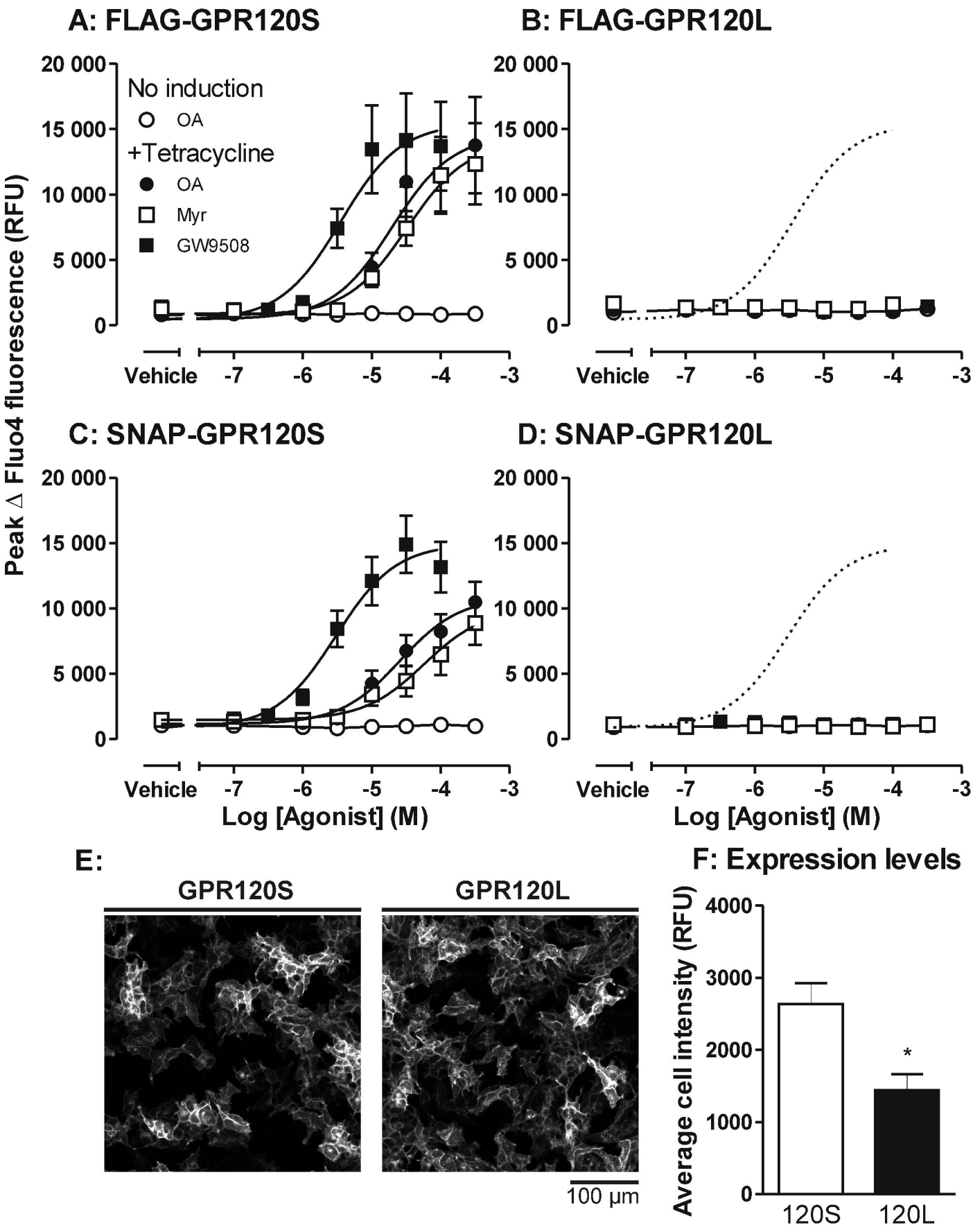
A: FLAG-GPR120S
B: FLAG-GPR120L
C: SNAP-GPR120S
D: SNAP-GPR120L
No induction
OA
+Tetracycline
OA
Myr
GW9508
Peak Δ Fluo4 fluorescence (RFU)
20 000
15 000
10 000
5 000
0
Vehicle
-7
-6
-5
-4
-3
Log [Agonist] (M)
E:
GPR120S
GPR120L
100 µm
F: Expression levels
Average cell intensity (RFU)
4000
3000
2000
1000
0
120S
120L
*

Figure 2

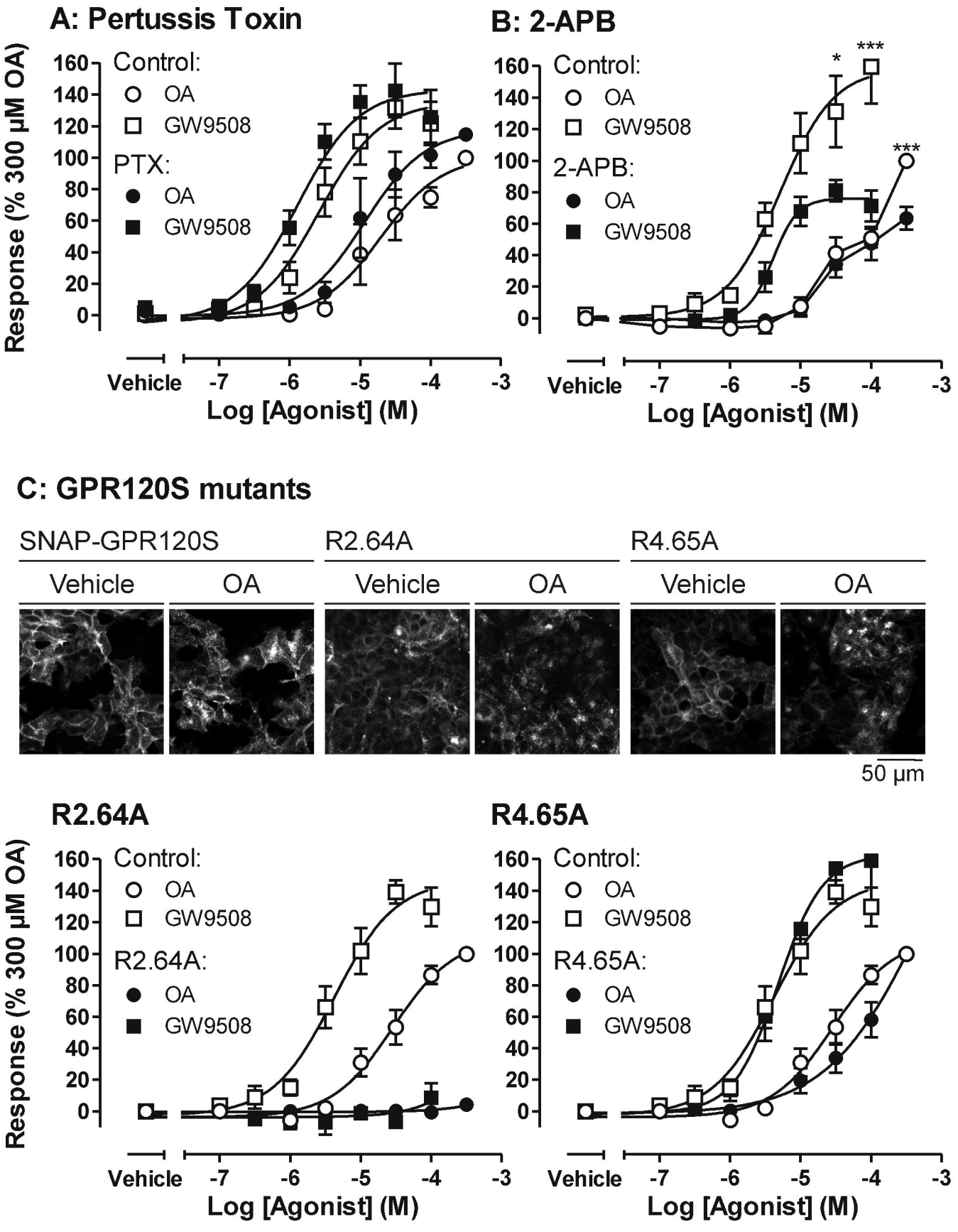
A: Pertussis Toxin
B: 2-APB
Response (% 300 µM OA)
Control:
OA
GW9508
PTX:
OA
GW9508
2-APB:
OA
GW9508
*
***
***
Vehicle
-7
-6
-5
-4
-3
Log [Agonist] (M)
C: GPR120S mutants
SNAP-GPR120S
R2.64A
R4.65A
Vehicle
OA
50 µm
R2.64A:
R4.65A:

Figure 3

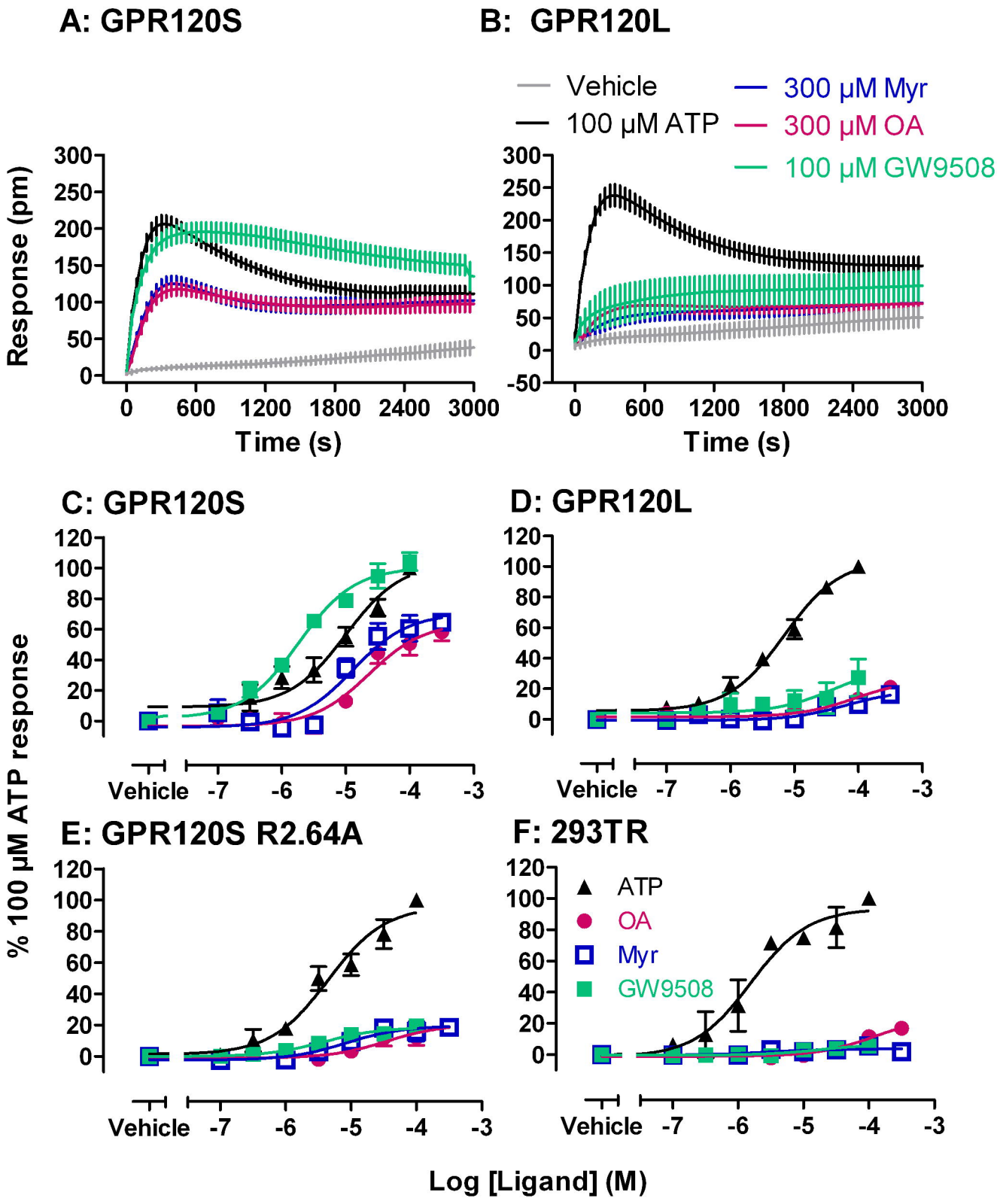
A: GPR120S
B: GPR120L
Vehicle
100 µM ATP
300 µM Myr
300 µM OA
100 µM GW9508
Response (pm)
Time (s)
C: GPR120S
D: GPR120L
% 100 µM ATP response
E: GPR120S R2.64A
F: 293TR
ATP
OA
Myr
GW9508
Log [Ligand] (M)

Figure 4

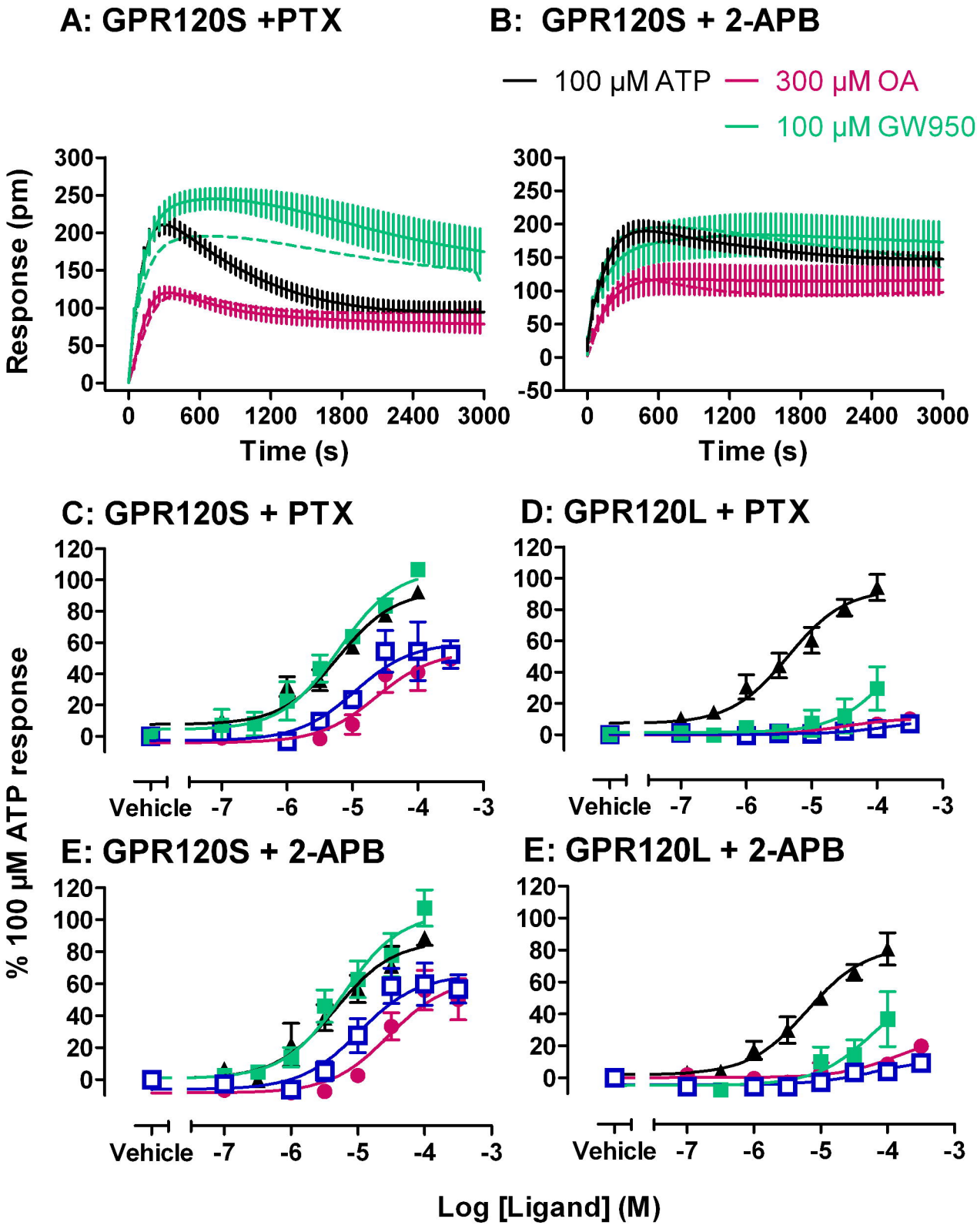
A: GPR120S +PTX
B: GPR120S + 2-APB
100 µM ATP
300 µM OA
100 µM GW9508
Response (pm)
Time (s)
C: GPR120S + PTX
D: GPR120L + PTX
E: GPR120S + 2-APB
E: GPR120L + 2-APB
% 100 µM ATP response
Vehicle
Log [Ligand] (M)

Figure 5

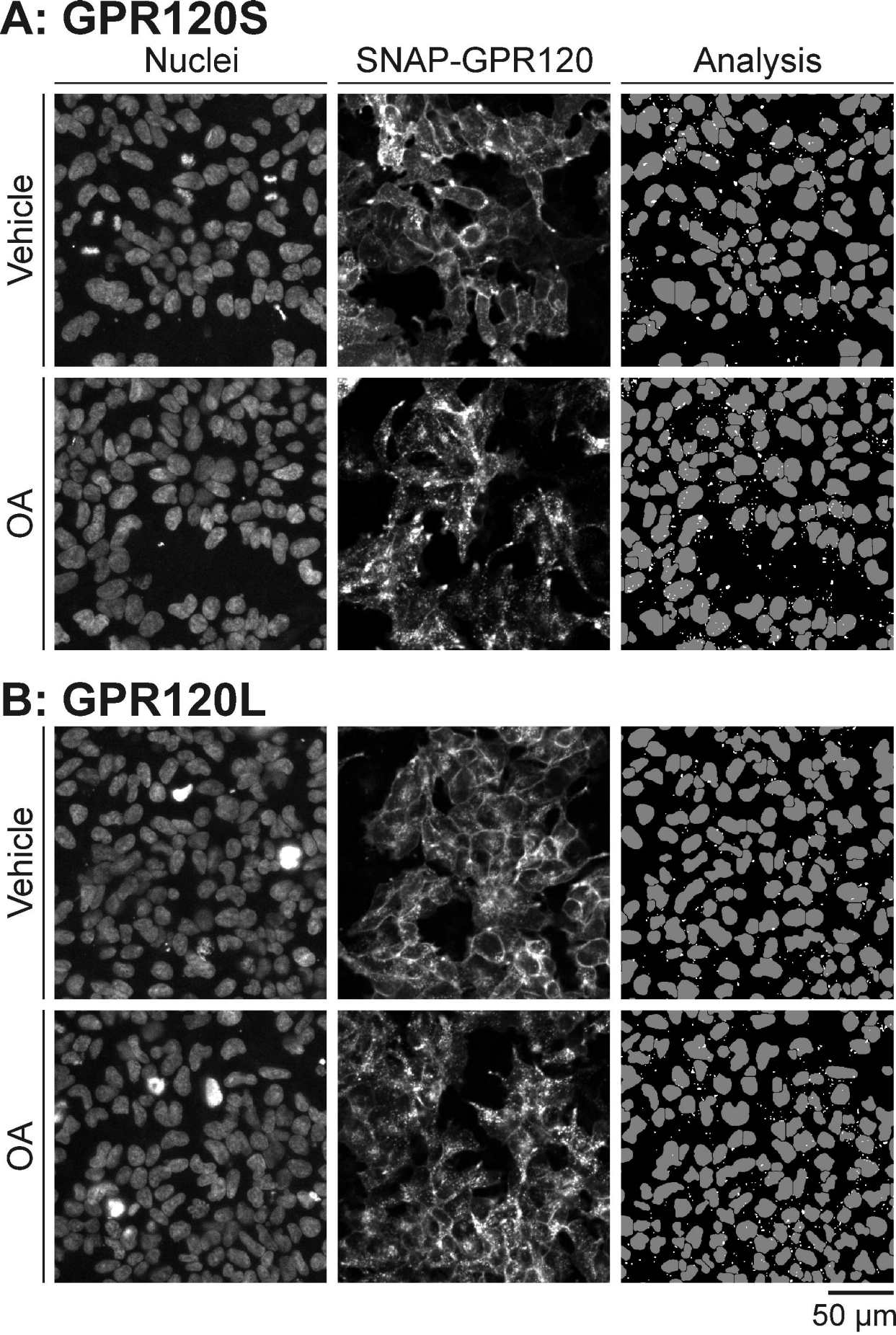
A: GPR120S
Nuclei
SNAP-GPR120
Analysis
Vehicle
OA
B: GPR120L
Vehicle
OA
50 µm

Figure 6

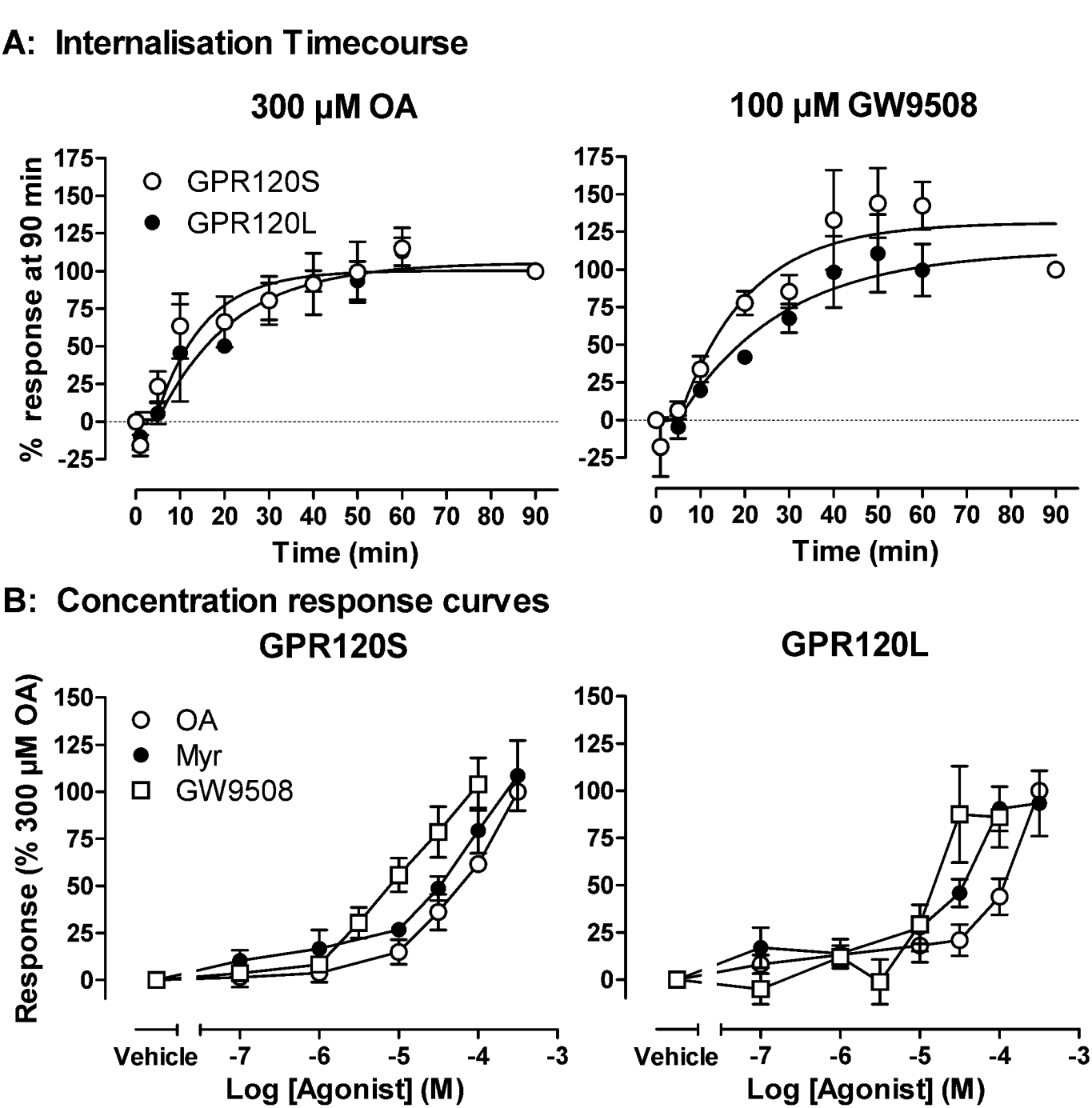
A: Internalisation Timecourse
300 µM OA
100 µM GW9508
% response at 90 min
GPR120S
GPR120L
Time (min)
B: Concentration response curves
GPR120S
GPR120L
Response (% 300 µM OA)
OA
Myr
GW9508
Vehicle
Log [Agonist] (M)

Figure 7

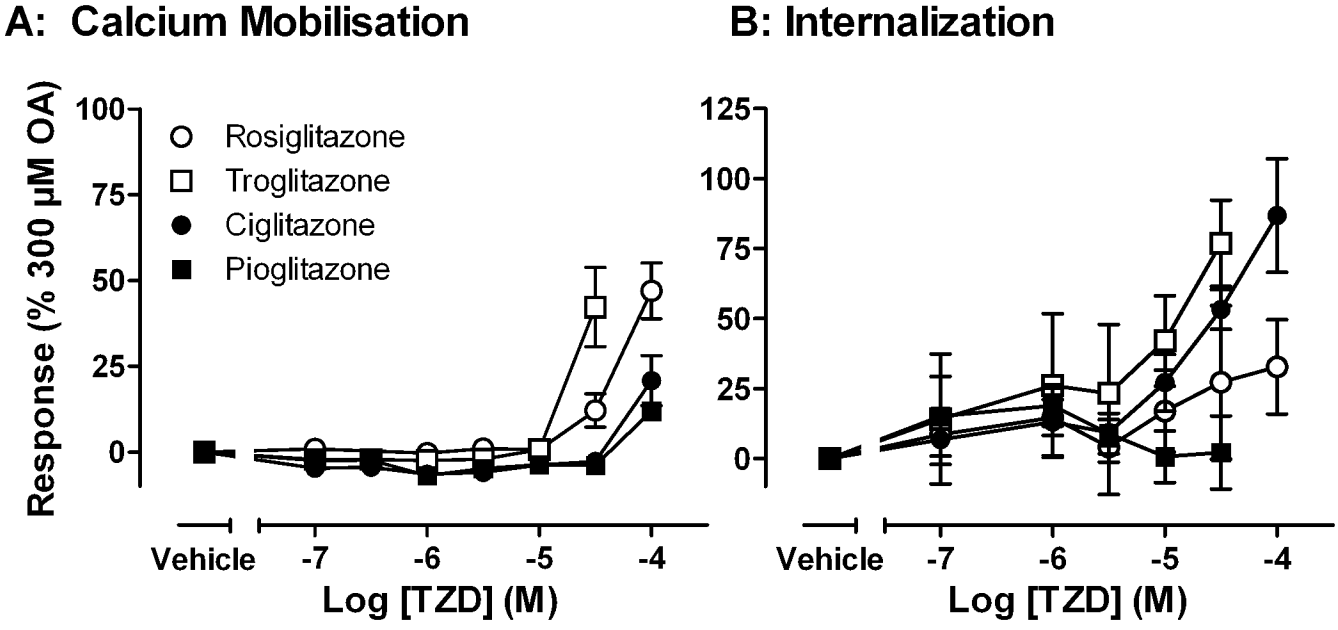
A: Calcium Mobilisation
B: Internalization
Response (% 300 µM OA)
100
75
50
25
0
Rosiglitazone
Troglitazone
Ciglitazone
Pioglitazone
Vehicle
-7
-6
-5
-4
Log [TZD] (M)
125
100
75
50
25
0
Vehicle
-7
-6
-5
-4
Log [TZD] (M)

Figure 8

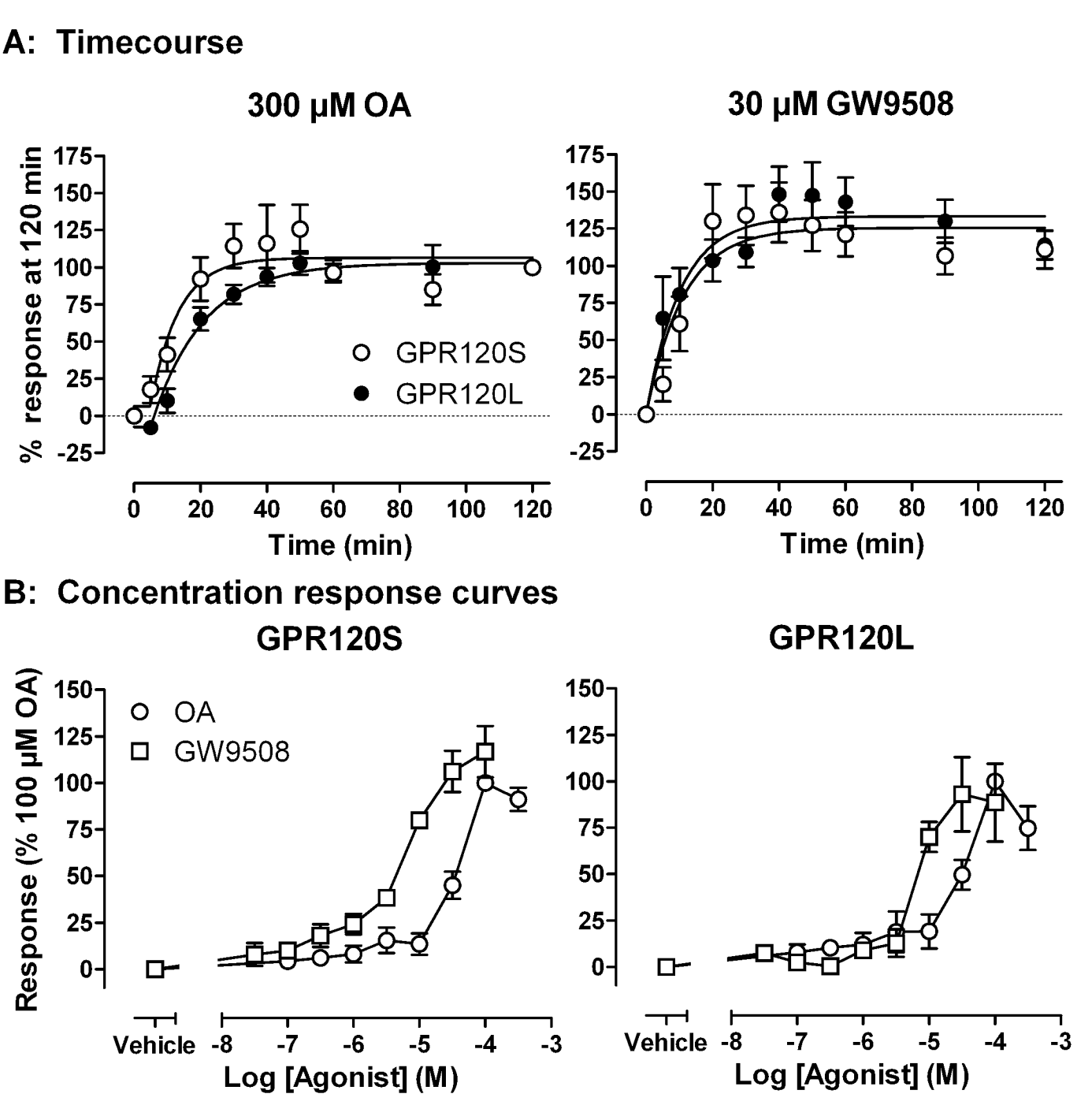
A: Timecourse
300 µM OA
30 µM GW9508
% response at 120 min
GPR120S
GPR120L
Time (min)
B: Concentration response curves
GPR120S
GPR120L
Response (% 100 µM OA)
OA
GW9508
Vehicle
Log [Agonist] (M)

Figure 9

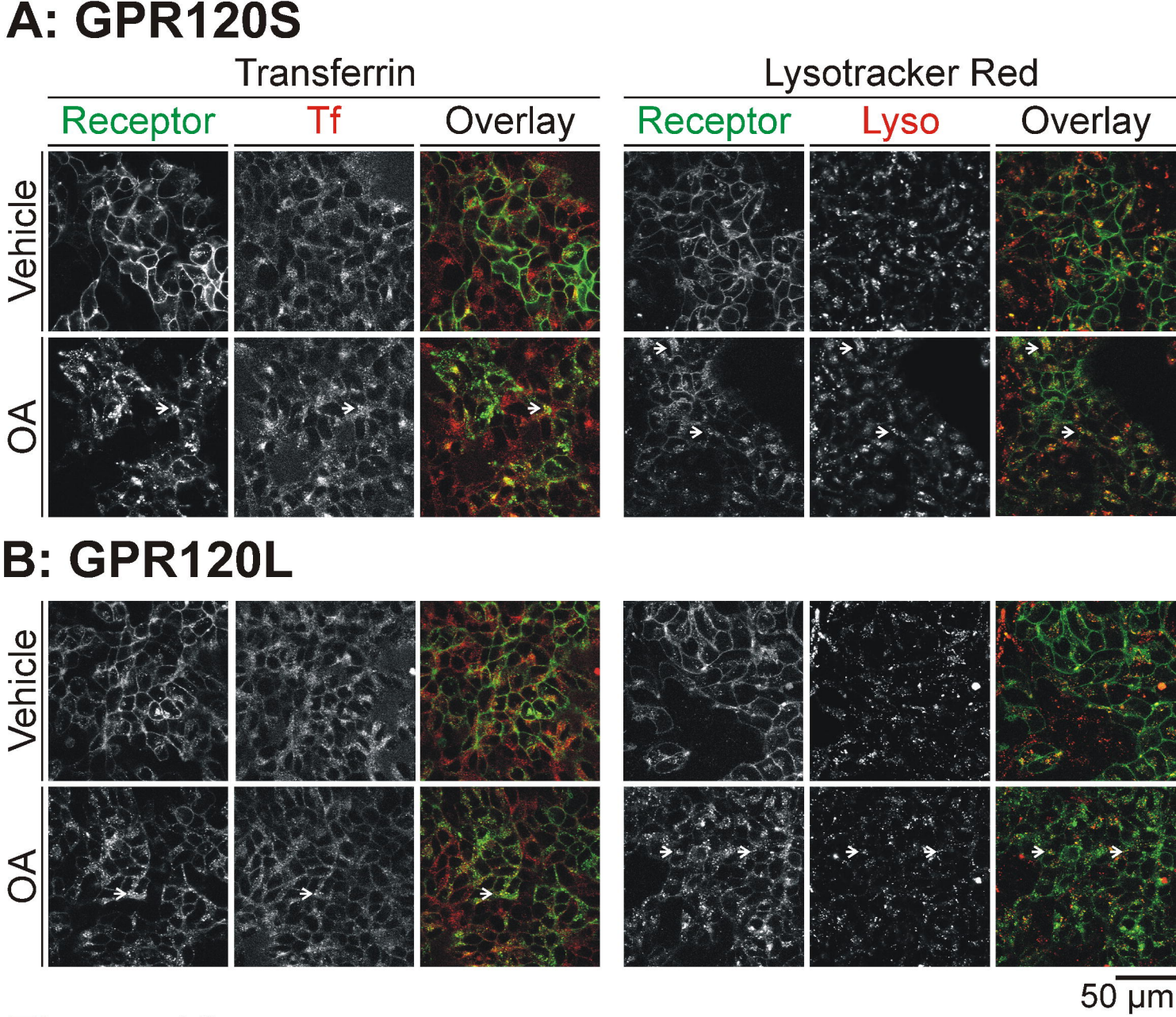
A: GPR120S
Transferrin
Lysotracker Red
Receptor
Tf
Overlay
Receptor
Lyso
Overlay
Vehicle
OA
B: GPR120L
Vehicle
OA
50 μm

Figure 10

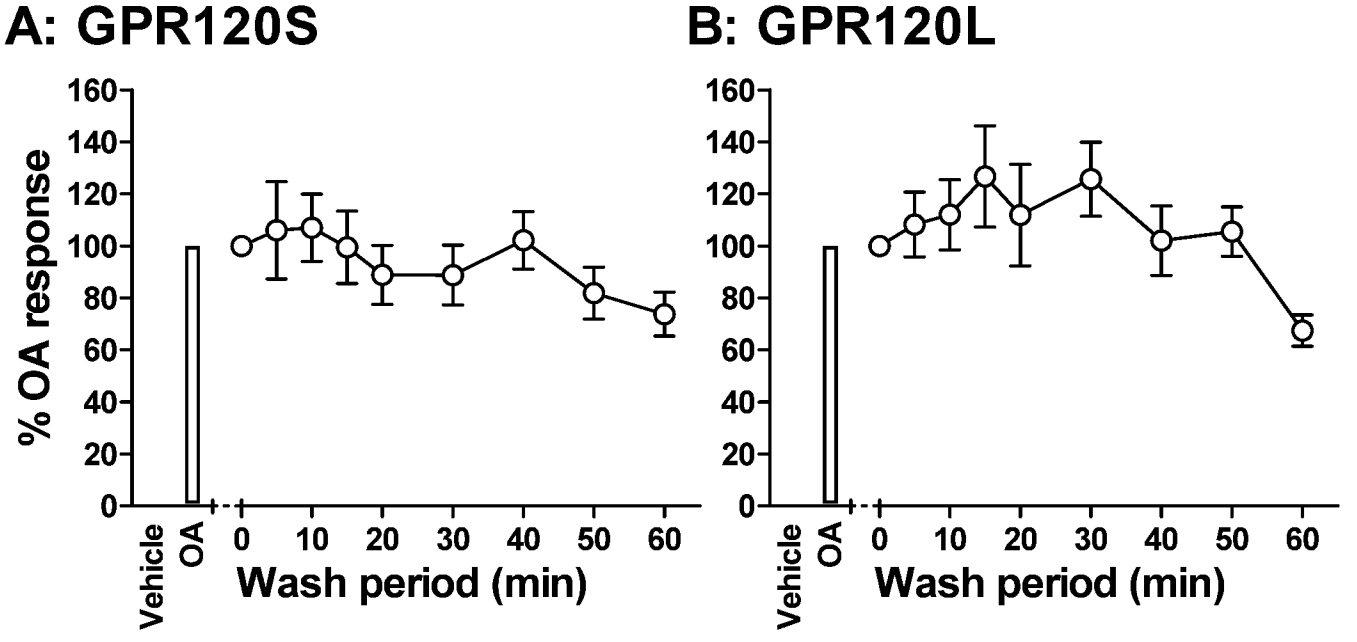
A: GPR120S
B: GPR120L
% OA response
160
140
120
100
80
60
40
20
0
Vehicle
OA
0
10
20
30
40
50
60
Wash period (min)

Figure 11