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Migraine therapeutics differentially modulate the CGRP pathway

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Abstract

Background: The clinical efficacy of migraine therapeutic agents directed towards the calcitonin-gene related peptide (CGRP) pathway has confirmed the key role of this axis in migraine pathogenesis. Three antibodies against CGRP – fremanezumab, galcanezumab and eptinezumab – and one antibody against the CGRP receptor, erenumab, are clinically approved therapeutics for the prevention of migraine. In addition, two small molecule CGRP receptor antagonists, ubrogepant and rimegepant, are approved for acute migraine treatment. Targeting either the CGRP ligand or receptor is efficacious for migraine treatment; however, a comparison of the mechanism of action of these therapeutic agents is lacking in the literature.

Methods: To gain insights into the potential differences between these CGRP pathway therapeutics, we compared the effect of a CGRP ligand antibody (fremanezumab), a CGRP receptor antibody (erenumab) and a CGRP receptor small molecule antagonist (telcagepant) using a combination of binding, functional and imaging assays.

Results: Erenumab and telcagepant antagonized CGRP, adrenomedullin and intermedin cAMP signaling at the canonical human CGRP receptor. In contrast, fremanezumab only antagonized CGRP-induced cAMP signaling at the human CGRP receptor. In addition, erenumab, but not fremanezumab, bound and internalized at the canonical human CGRP receptor. Interestingly, erenumab also bound and internalized at the human AMY_1 receptor, a CGRP receptor family member. Both erenumab and telcagepant antagonized amylin-induced cAMP signaling at the AMY_1 receptor while fremanezumab did not affect amylin responses.

Conclusion: The therapeutic effect of agents targeting the CGRP ligand versus receptor for migraine prevention (antibodies) or acute treatment (gepants) may involve distinct mechanisms of action. These findings suggest that differing mechanisms could affect efficacy, safety, and/or tolerability in migraine patients.

Keywords

AMY1, CGRP, erenumab, fremanezumab, gepant, migraine

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List of abbreviations

Abbreviation	Definition
AM	Adrenomedullin receptor
AMY_1	Amylin subtype 1 receptor
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
CTR	Calcitonin receptor
EEA1	Early endosomal antigen 1
FACS	Fluorescence-activated cell sorting
FSC-A	Forward scatter area
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HEK	Human embryonic kidney
LAMP1	Lysosomal-associated membrane protein 1
MCF-7	Human adenocarcinoma cells
pA_2	Potency of an antagonist
pEC_{50}	Negative log of the half maximal effective concentration

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pIC_{50}	Negative log of the half maximal inhibitory concentration
PDL	Poly-D-lysine
Rab11	Ras-related protein Rab-11
RAMP	Receptor activity-modifying protein
RCU	Red calibrated units
SB	Staining buffer
SK-N-MC	Human neuroepithelioma cells

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that is highly conserved across species. CGRP-expressing sensory nerve fibers and CGRP receptors are widely distributed peripherally and centrally throughout the trigeminovascular system (1). CGRP is a member of the calcitonin (CT) family of structurally related peptides, which also includes amylin, adrenomedullin, and intermedin/adrenomedullin2 (1,2). These peptides have partially overlapping activity on the CGRP-family receptors, which consist of heterodimeric complexes of a class B G protein-coupled receptor (GPCR), the calcitonin receptor (CTR) or the CTR-like receptor (CLR), in association with one of three Receptor Activity Modifying Proteins (RAMP1, 2 or 3) (1). RAMPs are single transmembrane-spanning proteins that alter the pharmacology, ligand binding, functionality and trafficking of CLR, CTR and a few other GPCRs (3).

The association of CLR and RAMP1 forms the canonical CGRP receptor (CLR/RAMP1). The ligand- and gepant-binding domain of the CGRP receptor is located at the interface between the two subunits (4–7). The association of CLR with RAMP2 or RAMP3 form the adrenomedullin receptors, AM_1 and AM_2, respectively (8). Similar to other GPCRs, CGRP receptor activation elicits a myriad of downstream signaling pathways (1). When CGRP binds to the canonical CGRP receptor, rapid phosphorylation of CLR occurs and the receptor undergoes dynamin/clathrin-dependent internalization via recruitment of β-arrestin (9).

The remaining members of this family of receptors include CTR. While CLR alone does not appear to operate as a functional receptor, CTR alone is a receptor for calcitonin (1,10). CTR associates with any of the three RAMPs to form the amylin receptors: AMY_1 (CTR/RAMP1), AMY_2 (CTR/RAMP2) and AMY_3 (CTR/RAMP3) (11). *In vitro*, both CGRP and the related peptide amylin stimulate the AMY_1 receptor (CTR/RAMP1 complex) with equal potency (12); however, the physiological relevance of the AMY_1 receptor in migraine and/or CGRP biology is unclear. The CGRP receptor binding epitopes of erenumab and telcagepant overlap at the CLR/RAMP1 interface and several recognized residues are conserved at the CTR/RAMP1 interface of the AMY_1 receptor (7). Recently the first study to examine AMY_1 internalization reported the occurrence of modest ligand-induced internalization (13). Deciphering the signaling and function of the CGRP family receptors has been challenging for the field, in part due to ligand promiscuity and common receptor components, thus further studies in this area are vital.

The development of therapeutic agents targeting the CGRP pathway has ushered in a new era for migraine therapy. Four CGRP pathway-based monoclonal antibody therapeutic agents have been approved by the US Food and Drug Administration (FDA) for migraine prevention: i) fremanezumab (Teva), ii) galcanezumab (Eli Lilly) and iii) eptinezumab (Lundbeck), all antibodies against CGRP ligand ('CGRP ligand antibody'); and iv) erenumab (Amgen), the sole antibody against the CGRP receptor ('CGRP receptor antibody'). Additionally, ubrogepant (Allergan) and rimegepant (Biohaven), two small molecule CGRP receptor antagonists, are in use for acute migraine therapy. Previous gepants did not receive clinical approval either due to poor pharmacokinetic properties or hepatic safety concerns (14) and newer gepants are currently in clinical trials (15).

A deeper understanding of the mechanism of action of therapeutic agents targeting the CGRP receptor versus ligand is important since this may have implications for their specificity, efficacy and/or side effect profile. Therefore, in this study we have compared binding and signaling of a CGRP ligand antibody (fremanezumab), a CGRP receptor antibody (erenumab) and a small molecule CGRP receptor antagonist (telcagepant) at the canonical human CGRP receptor and AMY_1 receptor. This study revealed several marked differences in targeting the CGRP ligand versus receptor related to receptor binding, signaling and trafficking at both receptors.

Materials and methods

Peptides, antibodies and inhibitors

Human αCGRP, amylin, adrenomedullin, intermedin and calcitonin were purchased from Bachem. Erenumab, a CGRP receptor monoclonal antibody (Amgen; lot 1093104), fremanezumab, a CGRP ligand monoclonal antibody (Teva Pharmaceuticals; lot E15204A001) and isotype control IgG2 antibody (prepared in-house) were used. For flow cytometry, the following antibodies were used: Anti-human IgG Fc APC (Biolegend), anti-human IgG Fc BV421 Biolegend), anti-HA.11 PE (Biolegend), Human c-Myc Alexa Fluor 647-conjugated Antibody (R&D

systems) and anti-myc-FITC (Sigma). For imaging experiments, the following antibodies were used: Early endosomal marker (early endosomal antigen 1 (EEA1), Abcam), lysosomal marker (lysosomal-associated membrane protein 1 (LAMP1), Abcam), late endosome marker (Ras-related protein Rab11, Cell Signaling), goat anti-human 594 and goat anti-rabbit Alexa Fluor 647 (Invitrogen). The small molecule CGRP receptor antagonist telcagepant (MedChemExpress) was used.

Preparation of tagged human RAMPs, CALCR (CTR) and CALCRL (CLR) expression vectors

All reference human gene sequences were obtained from GenBank and used for plasmid constructions after editing to remove non-preferred restriction sites. DNA fragments coding fusion genes of human kappa leader sequence (amino acid sequence: MDMRVPAQLLGLLLLWLRGARC), c-myc tag (EQKLISEEDL) and mature peptide of human RAMP genes (RAMP1, GenBank Sequence ID NM_005855, amino acid 28-148; RAMP2, NM_005854, 36-175; RAMP3, NM_005856, 29-148) were synthesized by Integrated DNA Technologies, then cloned into pCMV6-A-Puro (OriGene). Human influenza hemagglutinin (HA)-tagged human CALCRL plasmid (pCMV3-SP-HA-CALCRL) was purchased from Sino Biological Inc. An expression vector of HA-tagged human CALCR gene was constructed by cloning a corresponding DNA fragment (coding a fusion gene of human kappa leader sequence, HA tag (YPYDVPDYA) and the mature peptide of human CALCR [GenBank ID NM_001742, amino acid 25-474], synthesized by Integrated DNA Technologies into pCMV6-A-Hygro plasmid (OriGene). A plasmid coding eGFP (pSF-CMV-eGFP) and its sequence information was obtained from Oxford Genetics Limited. DNA fragments encoding human kappa leader sequence, eGFP (amino acid 2-239), 6-mer amino acid linker (SGGGGS) and mature peptide of human CALCR (amino acid 25-474) or human CALCRL (GenBank ID NM_005795, amino acid 23-461) were synthesized by Integrated DNA Technologies, then cloned into pSF-CMV-eGFP to replace the existing eGFP with the synthesized fusion genes.

Cell culture, transient transfection and stable cell lines

HEK293S GnTI- cells were obtained from ATCC. HEK293S cells were cultured in 1:1 mixture of DMEM media (Corning) and Ham's F12 media (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 1% penicillin streptomycin L-glutamine (Corning). Cells were maintained in a humidified incubator at 37°C, 5% CO_2. Cells were seeded at a density of 2×10^4 cells per well in 96-well plates. Cells were transfected using the Amaxa nucleofection kit V (Lonza) for HEK293S cells according to the manufacturers' instructions and were grown for an additional 24 to 48 h before further analysis. Equal quantities of CLR/CTR and RAMP plasmid DNA were used.

SK-N-MC cells were obtained from ATCC. Cells were cultured using EMEM media (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 1% penicillin streptomycin L-glutamine (Corning). Cells were maintained in a humidified incubator at 37°C, 5% CO_2. Cells were seeded at 2×10^4 cells per well in 96-well plates for cAMP assays.

N-term GFP-hCALCRL-puro or GFP-hCALCR-puro and N-term myc-hRAMP1-hygro were cloned into LakePharma proprietary expression vectors. HEKS293S were co-transfected with lipofectamine 3000 and plasmids and stable pools were generated after puromycin and hygromycin selection. Pools were FACS sorted by GFP expression and single cell clones were isolated. Intracellular RAMP1 and CALCRL/CALCR expression were monitored by respective c-myc staining and GFP expression via flow cytometry. Stable CGRP receptor (CLR-GFP/RAMP1-myc) HEK293S cells (henceforth referred to as $HEK293S^{CGRP}$) were maintained in DMEM:F12 at 1:1 supplemented with 10% FBS, 1× Penicillin Streptomycin L-glutamine, 0.5 μg/mL puromycin, and 100 μg/mL hygromycin. Stable AMY_1 receptor (CTR-GFP/RAMP1-myc) HEK293S cells (henceforth referred to as $HEK293S^{AMY1}$) were maintained in DMEM:F12 at 1:1 supplemented with 10% FBS, 1× penicillin streptomycin L-glutamine, 0.25 μg/mL puromycin, and 50 μg/mL hygromycin.

cAMP functional assay

Promega cAMP-Glo Max Assay was used to measure cAMP in a cell-based assay according to the manufacturer's instruction. Briefly, adherent cells were plated at $1–2 \times 10^4$ cells per well on 96-well plates. Cells were incubated overnight in a humidified incubator at 37°C, 5% CO_2. On the next day, peptide agonists and antagonist antibodies or small molecule inhibitor were diluted in complete induction buffer (PBS + 500 μM IBMX + 100 μM Ro 20-1724). IBMX (isobutyl-1-methylzanthine) and Ro 20-1724 (4-(3-butoxy-4-methoxy-benzyl) imidazolidone) were purchased from Sigma.

Ligands, antibodies (fremanezumab, erenumab or isotype control) and/or small molecule antagonist (telcagepant) were serially diluted and added to cells.

Agonist peptides were made at 2× fixed concentrations with final concentrations close to their EC_{80}-EC_{90} based on the peptide titration curves to ensure maximal cAMP production. Antibody/small molecule antagonist and peptide were added together at 1:1 ratio to make 1× concentration of each, incubated at room temperature for 15 min then the mixture was added to aspirated cells on the plates. For SK-N-MC cells, fixed concentrations of αCGRP (100 nM) and adrenomedullin/intermedin (1 μM) were used. Luminescence was measured using a Victor V3 plate reader. For pA_2 calculations, which provide a measure of the potency of an antagonist, amylin concentration-response curves were generated in the absence and presence of antagonist concentrations ranging from 100 nM to 1 μM. The pA_2 value is the negative logarithm of the concentration of an antagonist that produces a two-fold shift to the right of the agonist concentration-response curve. Antagonist concentrations were chosen based on titration experiments that determined a window where maximal response was still maintained.

Flow cytometry

All cell staining, washing and antibody dilutions for flow cytometry were done in cold staining buffer (SB): PBS + 2% heat inactivated FBS (Hyclone), 20 mM of HEPES (Lonza, 17–737F), 0.02% NaN_3 (Teknova), and 1 mM EDTA (Sigma). SK-N-MC or $HEK293S^{AMY1}$ cells were used at 1×10^5 cells per well for flow cytometry staining in 96-well plates. Cells were incubated on ice for 20 min in serially diluted antibody preparations either in SB or SB containing ligand. Cells were incubated with secondary anti-human IgG Fc APC (Biolegend) antibody or anti-human IgG Fc BV421 (Biolegend) and Human c-Myc Alexa Fluor 647-conjugated antibody (R&D systems) for 20 min on ice. Cells were washed and resuspended in SB and then plates were assayed using a BD high throughput sampler connected to a BD FACSCelesta flow cytometer.

HEK293S cells transiently transfected with human CTR, CLR and human RAMP1-3 were also used for flow cytometry to determine the specificity of erenumab binding to different receptor combinations. CTR and CLR receptors were HA tagged whereas RAMP1-3 were myc tagged. Cells were transfected using Amaxa nucleofection kit V (Lonza) at 2×10^6 cells per transfection reaction according to the manufacturer's instructions. Transfected cells were cultured in 6-well plates overnight. The following day, cells were harvested and stained with anti-HA.11 PE (Biolegend), anti-myc-FITC (Sigma), and erenumab, fremanezumab or isotype control antibody. All antibodies were used at 1 μg per well except for anti-HA PE, which was used at 0.4 μg per well. Cells were incubated for 20 min on ice and then washed with SB. Anti-human IgG Fc BV421 (Biolegend) was used as secondary antibody to detect antibody staining. Cells were incubated for 20 min on ice and then washed with SB. Sytox Red (Molecular Probes) was used to distinguish live from dead cells. After the final staining step, cells were resuspended in SB and samples were analyzed using a BD FACSCelesta flow cytometer. Healthy cells were identified and gated using the size (forward scatter) and complexity (side scatter) parameters. The violet laser (405 nm), blue laser (488 nm), yellow-green laser (561 nm) and red laser (640 nm) were used to excite and detect BV421, GFP/FITC, PE and AF-647/APC/Sytox Red fluorophores respectively. Samples were analyzed using FlowJo software.

Live cell imaging of antibody internalization

Fremanezumab, erenumab and isotype control antibody were fluorescently labelled with the Lightning-link rapid Fluoprobes647H (Novus Biologicals) labeling kits according to the manufacturers' instructions. HEK293S cells stably expressing human CGRP (CLR-GFP/RAMP1-myc) or AMY_1 (CTR-GFP/RAMP1-myc) receptors were seeded at 1.5×10^4 cells onto PDL coated 96-well imaging plates (Greiner Screenstar) and incubated overnight at 37°C. The following day, cells were incubated with Hoescht (Invitrogen) in Hepes buffered HBSS for 30 min at 37°C to label nuclei. Antibodies (10 μg/ml) in warm Hepes buffered HBSS were added to the cells and imaged immediately on a 37°C pre-heated stage using an IN cell analyzer 6500HS (GE Healthcare) with a Nikon 40×/0.95 microscope objective. Three to four fields of view were captured per well every 2 mins for 30 min for four independent experiments. For presentation, images were colorized and merged in FIJI (National Institutes of Health, Bethesda, MD, USA).

Kinetic internalization assay

HEK293S cells stably expressing human CGRP or AMY_1 receptors were seeded in growth media at 3×10^4 cells per well onto PDL coated 96-well ImageLock plates (Sartorius) and incubated overnight at 37°C. The following day media was replaced with warm imaging media (Fluobrite DMEM with glutamax) and serially diluted antibodies labelled with pH sensitive Zenon™ pHrodo™ iFL red human IgG labeling reagent (Thermo) were tested in duplicate according to manufacturer's instructions. Plates were immediately placed in the Incucyte (Sartorius) at 37°C, 5% CO_2 and fluorescence images were captured for three fields of view per well every 15 min for 11 h.

Subcellular localization using immunocytochemistry

HEK293S^{CGRP} (CLR-GFP/RAMP1-myc) cells were incubated with 10 μg/ml erenumab, fremanezumab or isotype control antibodies, or 1 μM telcagepant or DMSO control for 1 h at 37°C in Hepes buffered HBSS. Cells were then washed, fixed for 5 min with 4% PFA and blocked in blocking buffer (150 mM NaCl, 50 mM Tris Base, 1% BSA, 100 mM lysine; pH 7.4) with 0.2% Triton X-100 for 1 h at room temperature. After PBS washes, subcellular markers anti-LAMP1, anti-EEA1 or anti-Rab11 were added and incubated overnight at 4°C. The next day after several washes, DAPI (ThermoScientific) and the secondary antibodies goat anti-human conjugated to Alexa 594 (to detect human antibodies fremanezumab, erenumab and isotype control) and goat anti-rabbit antibodies conjugated to 647 (to detect subcellular markers) were added in blocking buffer for 2 h at room temperature. Plates were then washed and confocal images were taken using an IN cell analyzer 6500HS (GE Healthcare) with a Nikon 40×/0.95 microscope objective. Images were colorized using the FIJI software (National Institutes of Health, Bethesda, MD, USA).

Data analysis

All statistical analysis and curve fitting were performed using GraphPad Prism 7 or 8 (GraphPad software, San Diego, CA, USA). For flow cytometry and cAMP assays, maximal signaling responses were determined and the data are expressed as a percentage of this maximal response in order to combine data from independent experiments. To define agonist or antagonist potency, pEC_{50} or pIC_{50} values were obtained by fitting a three or four parameter logistic equation to the concentration-response data. The comparison of fits analysis feature in GraphPad Prism 8 was used to determine statistical difference between concentration-response curves. In order to determine antagonist potency at the AMY_1 receptor, pA_2 values were calculated using the Gaddum/Schild EC_{50} shift equation in GraphPad Prism. The hill slopes of the agonist concentration-response curves did not deviate significantly from 1 and were subsequently constrained to 1 for pA_2 value calculations. For statistical analysis, pA_2 values from individual experiments were combined and significant differences determined using one-way ANOVA and Bartlett's *post hoc* test and unpaired two-tailed t tests.

For the kinetic internalization experiments, the integrated intensity of the fluorescent internalized "puncta" for each captured image at every measurement time point was calculated with a custom algorithm using Incucyte software. Puncta were defined as red fluorescent objects that were masked after background subtraction (top-hat method) with criteria for object size $\geq 10\ \mu m^2$ and mean intensity ≥ 0.32 red calibrated units (RCU). Area under the curve calculations were performed in GraphPad Prism 7 (GraphPad software, San Diego, CA, USA).

Statistical significance was defined as $p < 0.05$. Most data points represent mean ± SEM combined from n separate experiments except Figure 1(b), where data points represent mean ± SD. Each independent experiment was performed with duplicate or triplicate wells.

Results

Erenumab, unlike fremanezumab, binds the CGRP receptor

To assess the cell binding capability of fremanezumab and erenumab to the human CGRP receptor, flow cytometry-based binding assays on HEK293S cells transiently expressing both CLR/RAMP1 (Figure 1) were performed. Erenumab bound to ~98% of human CGRP receptor expressing cells while fremanezumab showed no binding (Figure 1(a)), illustrating an obvious difference between these two antibodies. Furthermore, a lack of binding of erenumab to transiently transfected human CLR alone, AM_1 (CLR/RAMP2) and AM_2 (CLR/RAMP3) receptor expressing HEK293S cells was observed (Supplemental Figure 1), confirming that RAMP1 is critical for erenumab binding to the CGRP receptor.

To explore the consequence of CGRP ligand presence to antibody binding on CGRP receptor expressing cells, concentration-response experiments were performed in SK-N-MC cells, which endogenously express the canonical human CGRP receptor. Binding of erenumab to SK-N-MC cells was observed both in the absence (pEC_{50} 8.82 ± 0.06) and presence (pEC_{50} 8.03 ± 0.06) of human αCGRP (100 nM). The observed reduction in binding of erenumab to SK-N-MC cells in the presence of CGRP may be due to competition for receptor binding and/or ligand-induced receptor down-regulation. In comparison, neither fremanezumab nor isotype control antibody bound to SK-N-MC cells in the absence and presence of CGRP (Figure 1(a)) confirming a lack of binding to the CGRP receptor. Taken together, these results confirm that erenumab requires the presence of RAMP1 to bind to the canonical CGRP receptor. Consistent with the fact that fremanezumab targets a ligand and not a cellular target, this antibody does not bind to the CGRP receptor.

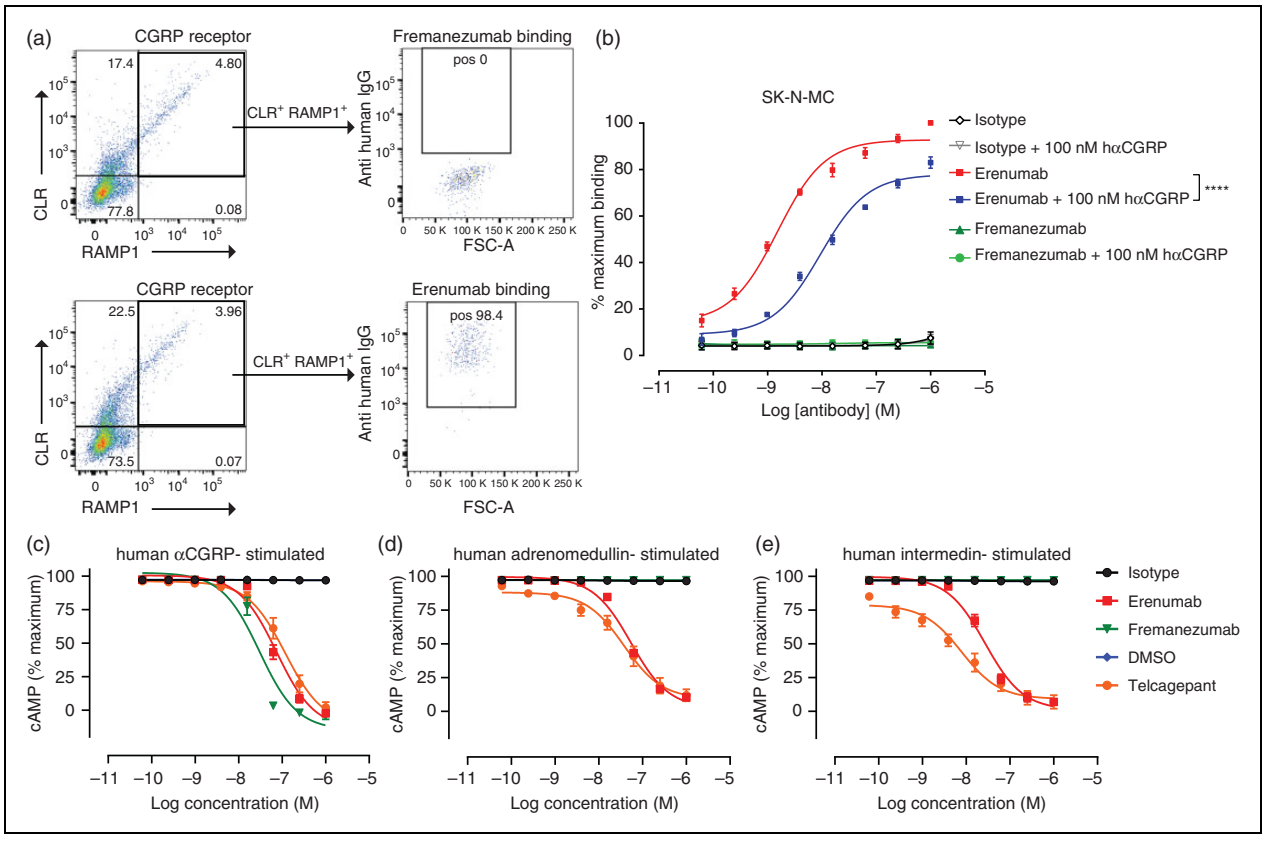

Figure 1. Binding and signaling differences of CGRP pathway therapeutics at the CGRP receptor. (a) Flow cytometry surface binding assay shows that erenumab binds to ~98% of human CGRP receptor (CLR/RAMP1) transiently transfected HEK293S cells while fremanezumab shows no binding to cells. Representative flow cytometry dot plots are shown from at least four independent experiments. (b) Antibody concentration-response curves from flow cytometry binding experiments plotted as a percentage of the maximal binding to SK-N-MC cells (expresses endogenous human CGRP receptor) in the absence and presence of hαCGRP (100 nM). The binding responses of isotype and fremanezumab (in the absence and presence of CGRP) are overlying. Data points represent the mean ± SD (n = 3). Comparison of fits for the erenumab binding curves in the absence and presence of CGRP showed that the shift was significantly different (****$p < 0.0001$). (c) Fremanezumab, erenumab and telcagepant antagonize hαCGRP-induced cAMP signaling in SK-N-MC cells. The binding curves of isotype and DMSO are overlying. Unlike fremanezumab which has no effect, erenumab and telcagepant antagonize human adrenomedullin-induced (d) and human intermedin-induced cAMP signaling (e) in SK-N-MC cells. The responses of fremanezumab, isotype and DMSO are overlying in (d) and (e). Data points represent the mean ± SEM (n = 4).

Erenumab and telcagepant affect multiple ligand signaling at the CGRP receptor

Three human ligands (αCGRP, adrenomedullin and intermedin) were found to increase cAMP accumulation in SK-N-MC cells, which express native human CGRP receptor (CLR/RAMP1) (16). To better define the activity of fremanezumab, erenumab and telcagepant at the CGRP receptor, we examined their ability to antagonize human αCGRP-, adrenomedullin- and intermedin-induced cAMP accumulation (Figure 1(c)–(e)). All three CGRP pathway agents antagonized human αCGRP-induced cAMP accumulation at native CGRP receptors in SK-N-MC cells (Figure 1(c); pIC_{50} values of 7.52 ± 0.07, 7.13 ± 0.05 and 6.93 ± 0.08 for fremanezumab, erenumab and telcagepant, respectively). However, both erenumab and telcagepant antagonized human adrenomedullin-induced (Figure 1(d); pIC_{50} values of 7.27 ± 0.04 and 7.43 ± 0.11, respectively) and human intermedin-induced (Figure 1(e); pIC_{50} values of 7.57 ± 0.05 and 8.10 ± 0.13, respectively) cAMP accumulation in SK-N-MC cells. Fremanezumab had no effect on these ligands. Thus, while both erenumab and telcagepant antagonize CGRP, adrenomedullin and intermedin signaling through the CGRP receptor, fremanezumab only antagonizes CGRP ligand signaling and allows normal adrenomedullin and intermedin signaling to continue at the CGRP receptor.

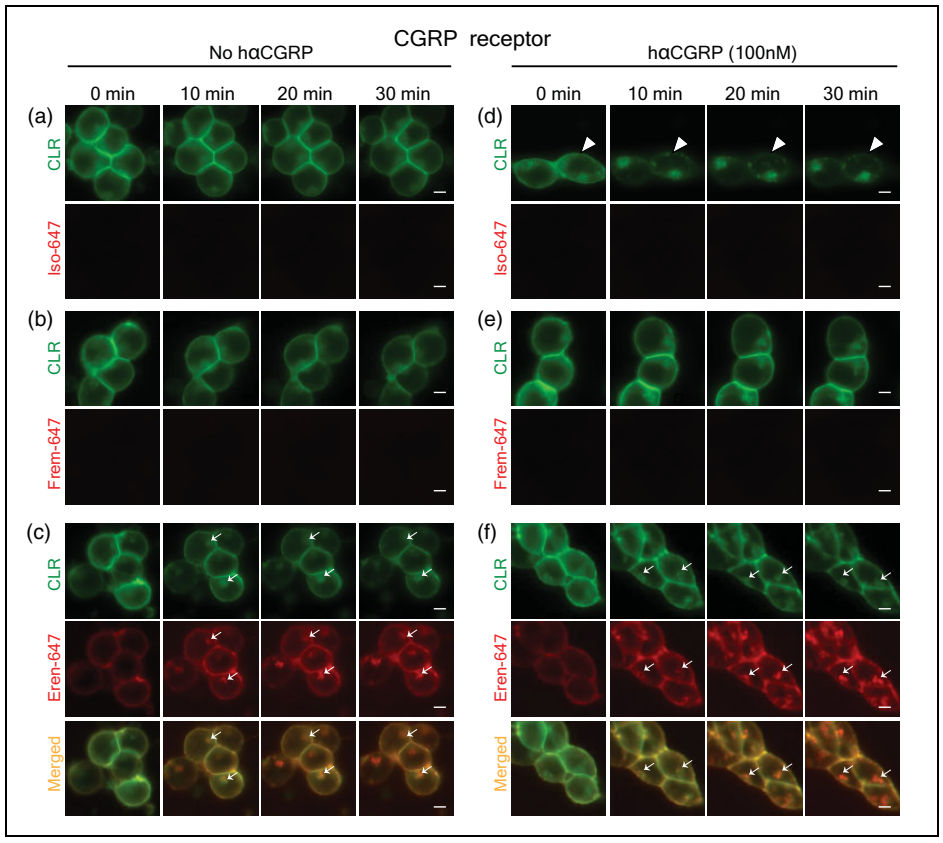

Figure 2. Live imaging of antibody internalization in CGRP receptor expressing cells. HEK293S^{CGRP} (CLR-GFP/RAMP1-myc) cells were imaged in the absence ((a)–(c)) and presence ((d)–(f)) of hαCGRP (100 nM) with Alexa Fluor 647 conjugated antibodies (red): isotype ((a),(d)), fremanezumab ((b),(e)) or erenumab ((c),(f)). As CLR is only expressed on the plasma membrane when co-expressed with RAMP1, surface CLR (green) was used as an indicator of the CGRP receptor on the plasma membrane. At 0 min, both in the absence and presence of hαCGRP, erenumab-647 binds to the plasma membrane. As time progresses, fluorescent intracellular puncta are visualized indicating internalization of erenumab-647 in the absence (c) and presence of CGRP (f). White arrows mark examples of co-localization between CLR and erenumab-647. Fremanezumab-647 is not internalized in the absence (b) and presence of CGRP (e). Both erenumab-647 and fremaneuzumab-647 appear to reduce CGRP-induced internalization of CLR (green panels; (e) and (f)) while decreases in surface CLR with hαCGRP are observed with the isotype-647 ((d), white arrowheads). Representative images are shown from four independent experiments. Scale bar, 5 μm.

Erenumab, unlike fremanezumab, is internalized at the CGRP receptor

To examine whether the CGRP ligand and receptor antibodies undergo internalization, live cell imaging was carried out in HEK293S^{CGRP} cells, which stably express the human CGRP receptor (CLR-GFP/RAMP1-myc). HEK293S^{CGRP} cells show high potency for CGRP and lower potency for intermedin and adrenomedullin (Supplemental Figure 2) as has been previously reported (1). As CLR is only trafficked to the plasma membrane when co-expressed with RAMP1 (10), we utilized plasma membrane CLR-GFP as a surrogate indicator for the surface CGRP receptor. The internalization of Alexa Fluor 647-labelled erenumab, fremanezumab and isotype control antibodies was visualized in the absence and presence of human αCGRP (100 nM). At time 0, CLR was found at the plasma membrane (Figure 2(d); green panel) and a loss of surface CLR with isotype-647 occurred within minutes of exposure to CGRP (Figure 2(d); white arrowheads). This is likely due to ligand-induced internalization and downregulation of the CGRP receptor. Fremanezumab-647 prevented reduction of CGRP receptor surface levels in the presence of CGRP presumably due to prevention of CGRP binding to the receptor (Figure 2(e); green panel) and did not bind or undergo internalization (Figure 2(b) and (e); red panel). In comparison, erenumab-647 bound to the surface of CGRP receptor expressing cells and internalization of erenumab visualized as fluorescent intracellular puncta were seen both in the absence

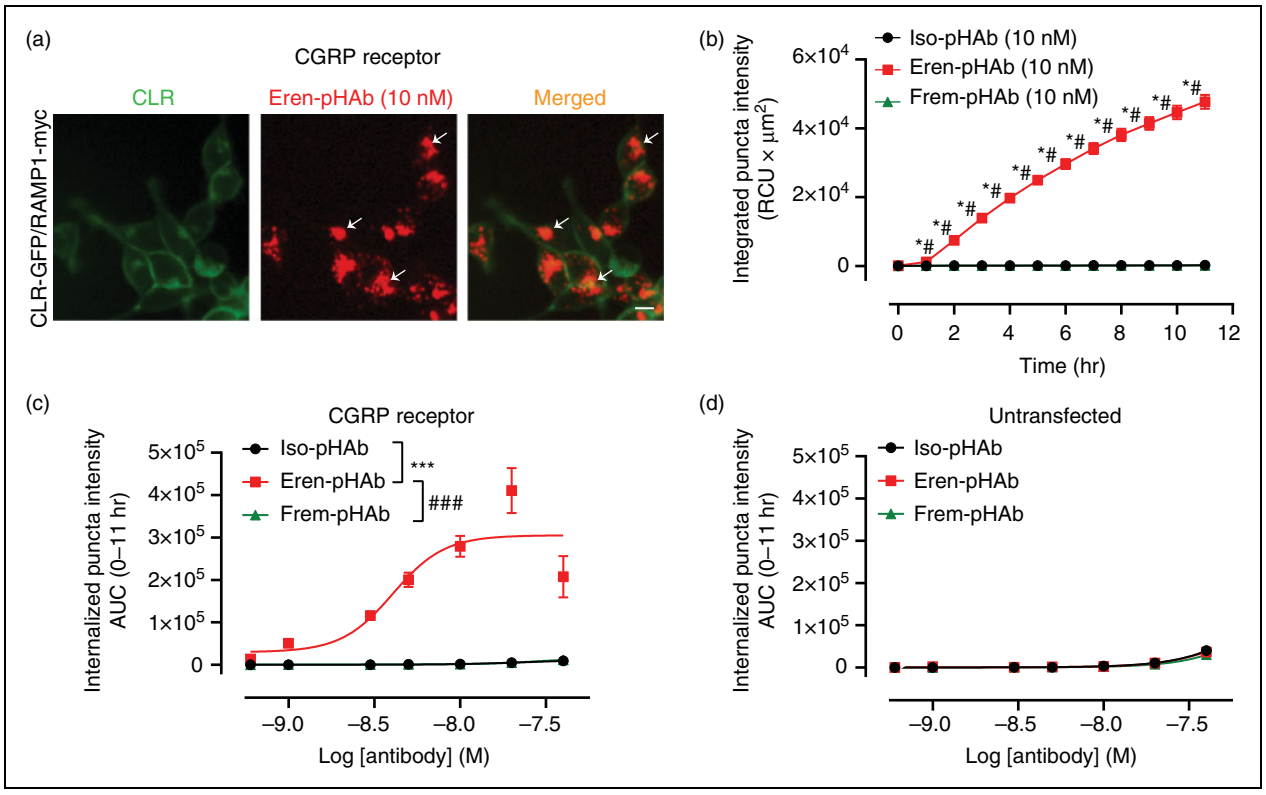

Figure 3. Kinetic quantification of antibody internalization in CGRP receptor expressing cells. Antibody internalization was measured in live cells by labelling antibodies with Zenon pHrodo Red, a pH sensitive reagent that fluoresces in acidic compartments. (a) Representative images show punctate internalized erenumab-pHAb (10 nM) in HEK293S^{CGRP} cells at 11 h. White arrows mark examples of internalized fluorescent erenumab-pHAb puncta. Scale bar, 10 µm. (b) Time course graph of integrated puncta intensity shows robust internalization of erenumab-pHAb (10 nM) and no internalization of fremanezumab-pHAb or isotype-pHAb. $^{*}p < 0.0001$ and $^{\#}p < 0.0001$ for erenumab-pHAb compared to isotype-pHAb and fremanezumab-pHAb, respectively, by repeated measures two-way ANOVA with a *post hoc* Dunnett's test. The responses for isotype-pHAb and fremanezumab-pHAb are overlying. (c) Antibody concentration-response curves plotted as area under the curve graphs of puncta intensity. $^{***}p < 0.001$ and $^{\#\#\#}p < 0.001$ for erenumab-pHAb compared to isotype-pHAb and fremanezumab-pHAb, respectively, by one-way ANOVA with a *post hoc* Dunnett's test. The responses for isotype-pHAb and fremanezumab-pHAb are overlying. (d) No internalization of erenumab-pHAb, fremanezumab-pHAb or isotype-pHAb was observed in untransfected HEK293S cells. The responses for erenumab-pHAb, fremanezumab-pHAb and isotype-pHAb, are overlying. Data points are mean ± SEM (n = 3).

(Figure 2(c); red panel) and presence (Figure 2(f); red panel) of CGRP. Areas of intracellular co-localization between CLR and erenumab during the time course (Figure 2(f); merged panel) suggests that erenumab may undergo internalization through receptor-mediated endocytosis. While a decrease in surface CLR with the isotype control antibody in the presence of CGRP was observed, both erenumab and fremanezumab treated cells showed retention of surface CLR. This suggests that both antibodies may reduce CGRP-induced receptor internalization albeit with different mechanisms.

To quantify internalized antibodies, live kinetic internalization assays were performed by conjugating erenumab, fremanezumab or isotype control to Zenon pHrodo Red, a fluorescently labelled Fab fragment that specifically recognizes the Fc fragment of human origin. The fluorescence intensity of pHrodo Red is very low at neutral or basic pH (outside the cell or on the cell surface), but increases in the acidic milieu of the endosomal and lysosomal lumen (when labelled "antibody-pHAb" is internalized). In HEK293S^{CGRP} cells, incubation with erenumab-pHAb resulted in visualization of fluorescent intracellular puncta (Figure 3(a)). The accumulation of erenumab-pHAb intracellular puncta increased over time (Figure 3(b)) and was concentration-dependent (Figure 3(c); pEC_{50} value of 8.64 ± 0.36). Consistent with the live imaging results, no internalization with fremanezumab-pHAb or isotype-pHAb was observed either with time (Figure 3(b)) or antibody concentration (Figure 3(c)). Importantly, erenumab-pHAb was not internalized in control untransfected HEK293S cells that do not express the CGRP receptor (Figure 3(d)). Taken together with our

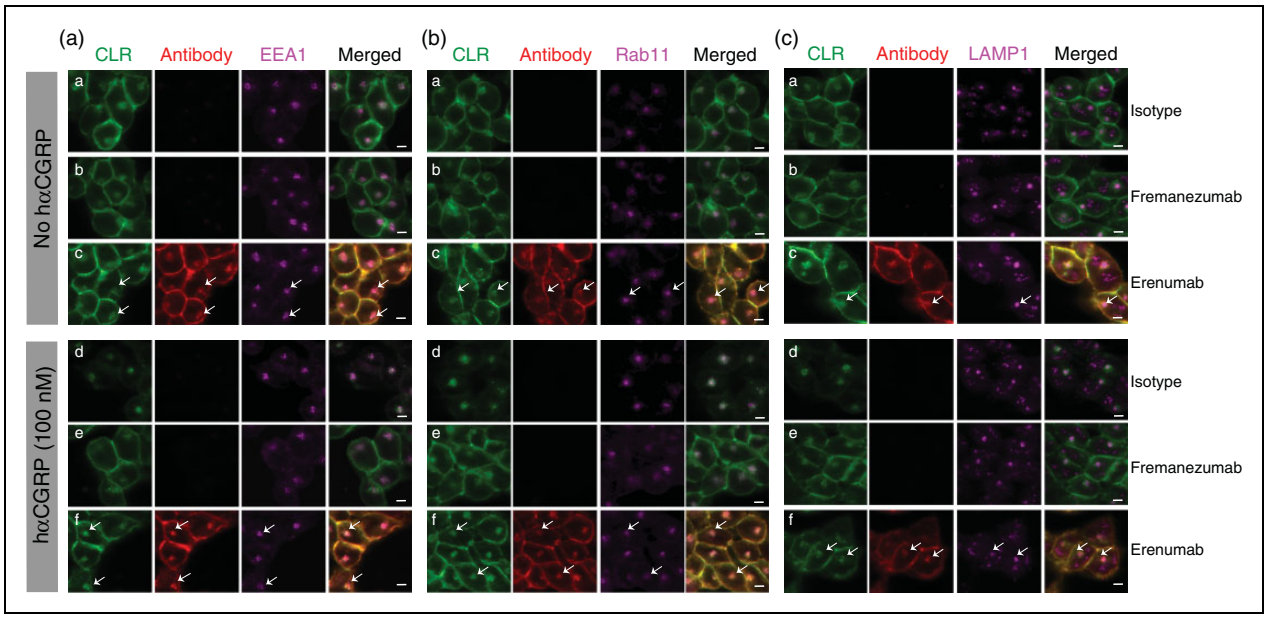

Figure 4. Subcellular localization of internalized antibodies and effect on CGRP receptor localization. HEK293S^{CGRP} (CLR-GFP/RAMP1-myc) cells were incubated with isotype control antibody, fremanezumab or erenumab for 1 hr at 37°C (in the absence and presence of 100 nM hαCGRP) stained with markers for the early endosome – EEA1 (a), recycling endosome – Rab11 (b) or lysosome – LAMP1 (c). Erenumab was internalized both in the absence (panel c) and presence of hαCGRP (panel f) and co-localized predominately in early endosomes ((a)c, f) and recycling endosomes ((b)c, f) with some lysosomal localization ((c)c, f). White arrows mark examples of co-localization between CLR, erenumab and the subcellular marker. No internalization of fremanezumab and isotype control was observed. Representative images from at least three independent experiments. Scale bar, 5 μm.

prior data, this result strongly supports the finding that erenumab, unlike fremanezumab, undergoes internalization at the human CGRP receptor.

Subcellular localization of the CGRP receptor and therapeutic agents

To compare the subcellular localization of therapeutic antibodies, HEK293S^{CGRP} (CLR-GFP/RAMP1-myc) cells were incubated for 1 h with 10 μg/ml unconjugated isotype control antibody, fremanezumab or erenumab in the absence and presence of CGRP and stained with markers of the early endosome (EEA1), recycling endosome (Rab11) or lysosome (LAMP1). Consistent with our previous findings, no internalization either in the absence or presence of CGRP was detected with the isotype control (Figure 4(a)–(c); panels a, d) or fremanezumab (Figure 4(a)–(c); panels b, e). Moderate co-localization between erenumab, CLR and putative early endosomes (Figure 4(a); panels c, f) and recycling endosomes (Figure 4(b); panels c, f) was observed, suggesting that the receptor antibody was trafficked to these organelles. This finding is consistent with previous observations that indicate that the CGRP receptor is trafficked to the early endosome (13,17,18). Some co-localization between erenumab, CLR and the lysosomal marker LAMP1 was also observed, suggesting that a proportion may also be targeted for lysosomal degradation (Figure 4(c); panels c, f).

In addition, similar to previous findings, in the absence of CGRP, CLR was localized at the plasma membrane with isotype control antibody (Figure 4(a)–(c); panel a). However, in the presence of CGRP, downregulation of surface CLR is observed in cells incubated with the isotype control (Figure 4(a)–(c); panel d). This decrease in surface CLR with CGRP was not observed with fremanezumab (Figure 4(a)–(c); panel e), erenumab (Figure 4(a)–(c); panel f) and telcagepant (Figure S3(a)–(c); panel c). We speculate that through receptor binding, erenumab and telcagepant may reduce ligand-induced CGRP receptor downregulation. The effect with fremanezumab is likely due to preventing exogenous CGRP from binding the receptor. Taken together, these findings suggest that differing mechanisms underlie the ability of all three CGRP pathway therapeutic agents to decrease ligand-induced receptor downregulation.

Erenumab, unlike fremanezumab, binds the AMY$_1$ receptor

Due to the structural similarities between the cleft of the CGRP (CLR/RAMP1) and AMY$_1$ (CTR/RAMP1)

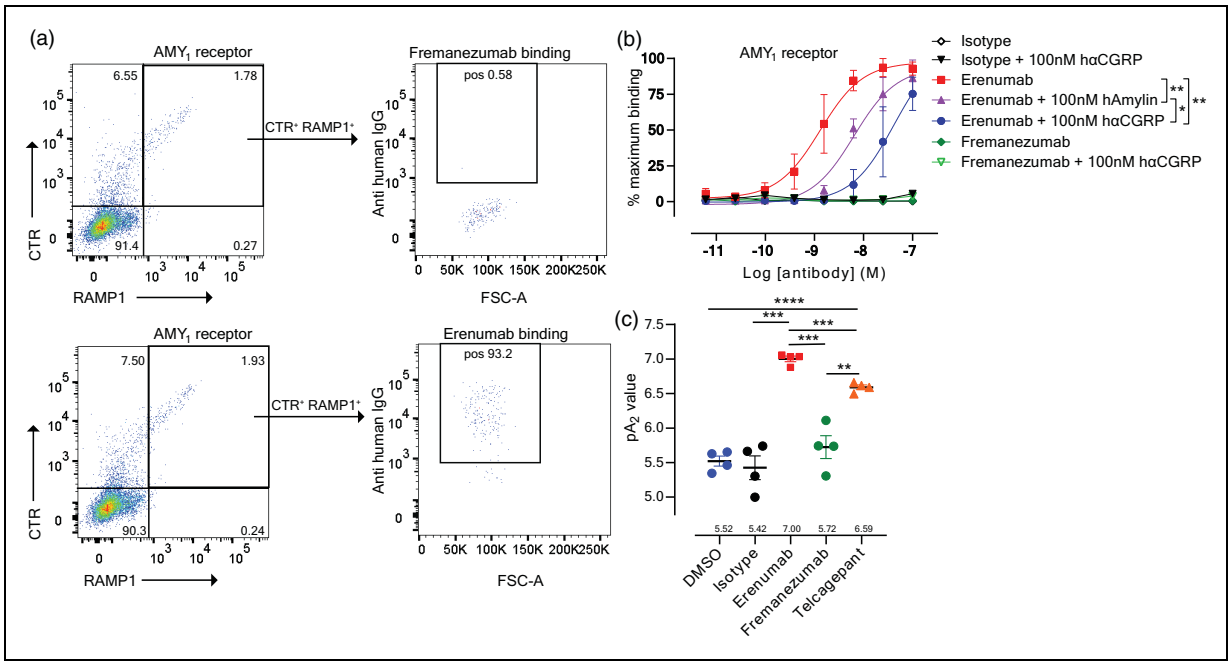

Figure 5. Erenumab binds the AMY_1 (CTR/RAMP1) receptor and both erenumab and telcagepant affect amylin-induced signaling. (a) Erenumab binds to ~93% of transiently transfected human AMY_1 receptor HEK293S cells while fremanezumab shows no binding. Representative flow cytometry dot plots are shown from at least four independent experiments. (b) Flow cytometry binding assays in $HEK293S^{AMY1}$ cells suggest competitive binding between erenumab and receptor ligands hαCGRP and amylin. The binding responses of isotype and fremanezumab (in the absence and presence of CGRP) are overlying. Data points represent the mean ± SEM (n = 3). Statistical analysis of curves by comparison of fits (*$p < 0.05$, **$p < 0.01$). (c) pA_2 values indicating antagonist potency of the test agents in amylin-induced cAMP signaling assays in $HEK293S^{AMY1}$ cells. Data points represent the mean ± SEM and the mean value is noted (n = 4). **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by unpaired two-tailed t test.

receptors and the cross reactivity of gepants to both receptors (12,18–20), we hypothesized that erenumab may bind the AMY_1 receptor. Antibody binding to the AMY_1 receptor was measured in transiently transfected HEK293S (CTR-HA/RAMP1-myc) using a flow cytometry-based assay. Interestingly, erenumab bound ~93% of human AMY_1 receptor cells (Figure 5(a)). Fremanezumab did not bind the AMY_1 receptor cells. We next tested whether erenumab bound to other CTR-based receptors. Erenumab did not bind to transiently transfected HEK293S cells expressing the human CTR, AMY_2 (CTR/RAMP2) or AMY_3 (CTR/RAMP3) receptors (Supplemental Figure 4). These results indicate that erenumab binds the human AMY_1 receptor and that RAMP1 is critical for its binding.

To confirm erenumab binding to the AMY_1 receptor we generated a stable human AMY_1 receptor (CTR-GFP/RAMP1-myc) cell line ($HEK293S^{AMY1}$), which showed equipotent CGRP and amylin cAMP responses (Supplemental Figure 5). In line with the postulation that co-transfection of CTR and RAMP1 may result in two receptor populations being expressed on the plasma membrane, the CTR alone and CTR/RAMP1 (AMY_1) receptor (1), we observed potent calcitonin functional responses in these cells that suggests the presence of dual receptors (Supplemental Figure 5). The erenumab concentration curve in a flow cytometry binding assay with $HEK293S^{AMY1}$ cells clearly showed that erenumab bound to the AMY_1 receptor (Figure 5(b); pEC_{50} value of 8.88 ± 0.17). A rightward shift in the erenumab binding curves was observed in the presence of saturating concentrations of either CGRP (pEC_{50} value of 7.38 ± 0.31; **$p < 0.01$ vs. erenumab alone) or amylin (pEC_{50} value of 8.19 ± 0.14; **$p < 0.01$ vs. erenumab alone). This suggests decreased binding of erenumab to the AMY_1 receptor in the presence of these ligands. Since the AMY_1 receptor does not show robust ligand-induced receptor downregulation (13), it is possible that erenumab competes with the ligand for receptor binding. Fremanezumab and isotype control did not bind to AMY_1 receptor cells either in the absence or presence of CGRP.

Erenumab and telcagepant affect amylin signaling at the AMY_1 receptor

To examine the activity of fremanezumab, erenumab and telcagepant at the AMY_1 receptor, we investigated

their ability to antagonize amylin-induced cAMP signaling. From cAMP experiments, pA_2 values were determined from amylin concentration-response curves in the absence and presence of various antagonist concentrations. Both receptor binders, erenumab and telcagepant, were found to affect amylin signaling at the AMY_1 receptor (pA_2 values of 7.00 ± 0.04 and 6.59 ± 0.03, respectively). The effect of fremanezumab (pA_2 value of 5.72 ± 0.16) was similar to isotype and DMSO controls (pA_2 values of 5.42 ± 0.17 and 5.52 ± 0.07, respectively). This result indicates that amylin signaling at the AMY_1 receptor is only antagonized by the receptor binders. Although the $HEK293S^{AMY1}$ cells likely express both amylin-responsive calcitonin and AMY_1 receptors, the lack of binding of erenumab to calcitonin receptors (Supplemental Figure 4) suggests that the antagonism of amylin signaling occurs at the AMY_1 receptor. If this is the case, then the observed antagonism of amylin-signaling at the AMY_1 receptor by erenumab is possibly underestimated.

Erenumab, unlike fremanezumab, is internalized at the AMY_1 receptor

Similar to the imaging and trafficking studies performed with $HEK293S^{CGRP}$ cells, the internalization of erenumab in $HEK293S^{AMY1}$ cells was also investigated. Live imaging experiments were carried out in $HEK293S^{AMY1}$ cells in the absence and presence of CGRP using fluorescently conjugated receptor/ligand antibodies. Human αCGRP did not reduce surface CTR with isotype-647 (Figure 6(d)) in line with findings of low levels of ligand-induced AMY_1 receptor internalization (13). Isotype-647 (Figure 6(a),(d)) and fremanezumab-647 (Figure 6(b),(e)) did not undergo internalization in AMY_1 receptor expressing cells in the absence or presence of CGRP; however, erenumab-647 was internalized under both conditions (Figure 6(c),(f)). The CTR is a functional receptor by itself and can be expressed on the cell surface without a requirement for RAMPs (21). Thus surface CTR potentially indicates the presence of both CTR and AMY_1 receptors.

Using kinetic quantification experiments of internalization in $HEK293S^{AMY1}$ cells, fluorescent intracellular puncta were observed in erenumab-pHAb treated wells (Figure 7(a)). Erenumab-pHAb internalization increased over time (Figure 7(b)), was concentration dependent (Figure 7(c); pEC_{50} value of 8.05 ± 0.02) and did not occur in control untransfected HEK293S cells (Figure 3(d)). No internalization was detected with fremaneuzunab-pHAb or isotype-pHAb in $HEK293S^{AMY1}$ cells (Figure 7(b)–(c)). Taken together, these results confirm that erenumab not only binds the canonical CGRP receptor, but also binds and undergoes internalization at the related AMY_1 receptor. In contrast, fremanezumab does not bind to the AMY_1 receptor.

Subcellular localization of the AMY_1 receptor and therapeutic antibodies

To determine the subcellular localization of internalized erenumab, we performed confocal imaging experiments in $HEK293S^{AMY1}$ cells. The localization of 10 μg/ml unconjugated erenumab, fremanezumab or isotype control antibody was investigated in the absence and presence of CGRP with markers of the early endosome (EEA1) or lysosome (LAMP1). Consistent with our live cell imaging experiments and a previous report (13), no change in surface CTR was observed with CGRP and isotype control antibody (Figure 8; compare green in panel a vs. panel d). No internalization in the absence and presence of CGRP was observed with either isotype control antibody (Figure 8 (a)–(b) a, d) or fremanezumab (Figure 8 (a)–(b) b, e). Both in the absence and presence of human αCGRP (100 nM), some areas of co-localization were observed between erenumab and putative early endosomes (Figure 8(a); panels c, f) and lysosomes (Figure 8(b); panels c, f). Taken together, our data indicate that erenumab binds and undergoes internalization at the AMY_1 receptor, as shown by its presence in putative early endosomes and lysosomes.

Discussion

Three classes of therapeutic agents that target the CGRP pathway have been approved for migraine treatment: a) Monoclonal antibodies against the CGRP ligand (fremanezumab, galcanezumab, eptinezumab) for migraine prevention; b) a CGRP receptor monoclonal antibody (erenumab) for migraine prevention; and c) small molecule CGRP receptor antagonists (ubrogepant and rimegepant) for acute migraine treatment. Although all these therapeutic agents target the CGRP pathway, they have different mechanisms of action due to effects on the CGRP ligand versus the receptor. Herein, we report differences in the action of ligand binding therapeutics compared to receptor binding therapeutics in relation to receptor binding, signaling and intracellular trafficking. Importantly, we also report that erenumab binds not only to the canonical human CGRP receptor (CLR/RAMP1), but also to the human amylin AMY_1 receptor (CTR/RAMP1) and affects signaling and undergoes internalization at both receptors.

An obvious key difference in the mechanism of action of a receptor binding therapeutic (antibody or

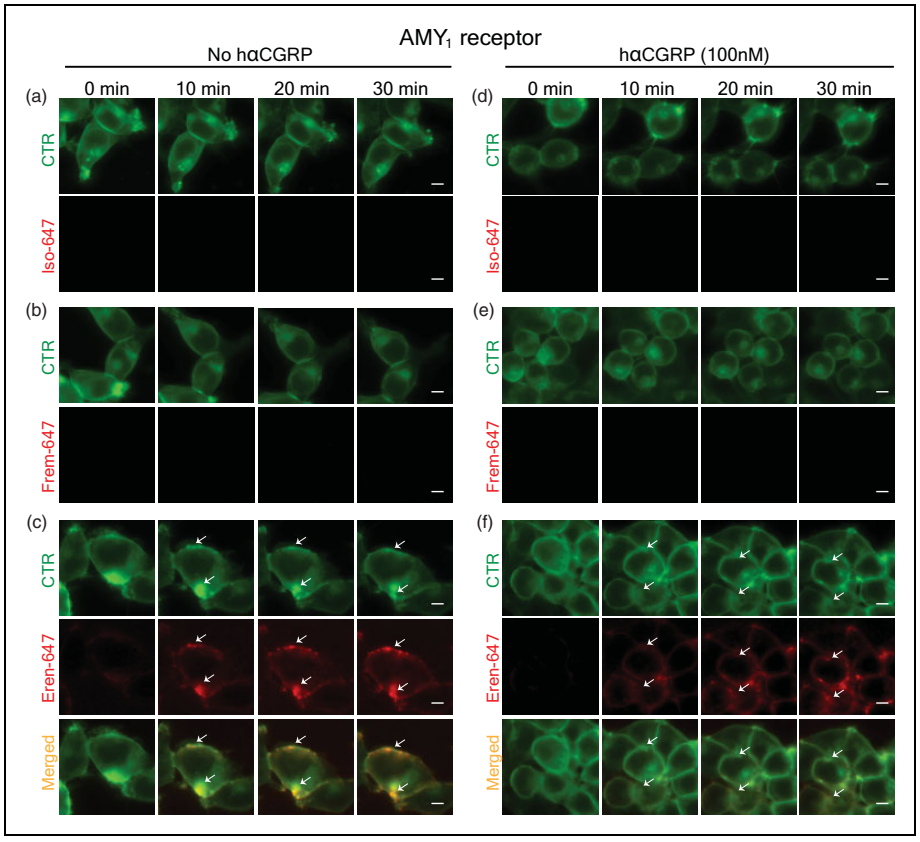

Figure 6. Live imaging of antibody internalization in AMY_1 receptor expressing cells. HEK293S^{AMY1} cells (CTR-GFP/RAMP1-myc) were imaged immediately in the absence ((a)–(c)) and presence ((d)–(f)) of 100 nM hαCGRP with Alexa Fluor 647 conjugated antibody (red): Isotype ((a),(d)), fremanezumab ((b),(e)) or erenumab ((c),(f)). Both in the absence and presence of hαCGRP, erenumab-647 binds to the plasma membrane and is internalized ((c),(f)). White arrows mark examples of co-localization between CTR and erenumab-647. Neither isotype-647 ((a),(d)) nor fremanezumab-647 is internalized ((b),(e)). Representative images from four independent experiments. Scale bar, 5 μm.

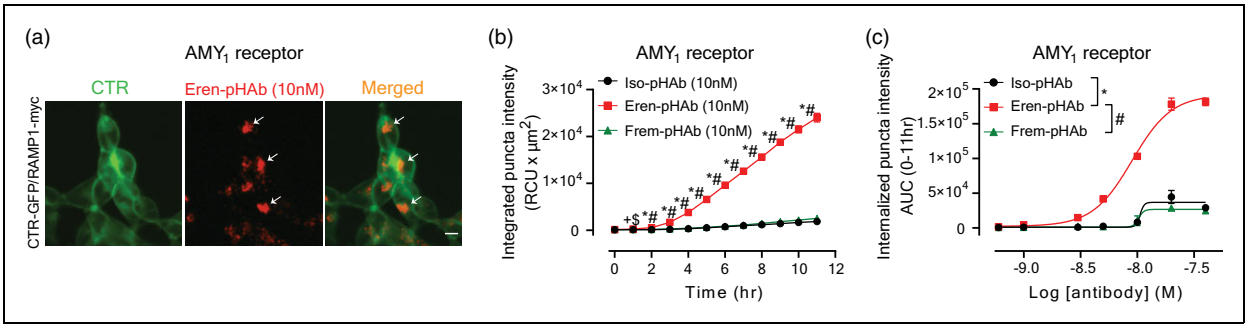

Figure 7. Erenumab is internalized in AMY_1 receptor expressing cells. (a) Representative images show punctate internalized erenumab-pHAb in HEK293S^{AMY1} cells. Scale bar, 10 μm. (b) Time course graph of integrated puncta intensity showed robust internalization of erenumab-pHAb (10 nM) in AMY_1 receptor cells compared to no internalization of fremanezumab-pHAb or isotype-pHAb. $+p < 0.01$, $*p < 0.0001$ compared to isotype-pHAb and $^{\$}p < 0.05$ and $^{\#}p < 0.0001$ compared to fremanezumab-pHAb by repeated measures two-way ANOVA with a *post hoc* Dunnett's test. The responses for isotype-pHAb and fremanezumab-pHAb are overlying. (c) Antibody concentration-response curves plotted against area under the curve of puncta intensity. The responses for isotype-pHAb and fremanezumab-pHAb are overlying. Data points are mean ± SEM (n = 3); $*p < 0.05$ and $^{\#}p < 0.05$ erenumab-pHAb compared to isotype-pHAb and fremanezumab-pHAb, respectively, by one-way ANOVA with a *post hoc* Dunnett's test.

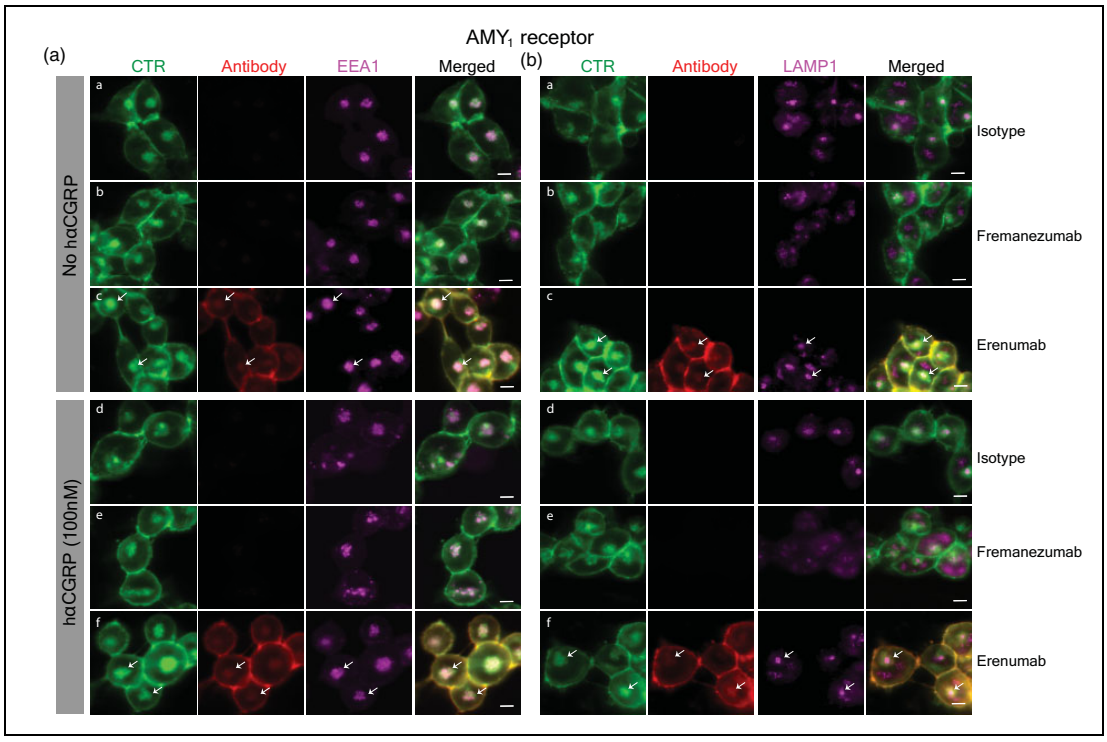

Figure 8. Subcellular localization of erenumab in AMY_1 receptor cells. Representative images of $HEK293S^{AMY1}$ (CLR-GFP/RAMP1-myc) cells incubated with isotype control antibody, fremanezumab or erenumab for 1 h at 37°C (in the absence and presence of 100 nM hαCGRP) stained with early endosome marker EEA1 (a) or lysosomal marker LAMP1 (b). Erenumab was internalized both in the absence ((a)c, (b)c) and presence of hαCGRP ((a)e, (b)e) and co-localized in early endosomes ((a)c, (a)f) with some lysosomal localization ((b)c, (b)f). Fremanezumab and isotype control were not internalized. White arrows mark examples of co-localization between CTR, erenumab and the subcellular marker. Representative images from three independent experiments. Scale bar, 5 μm.

small molecule) compared to a ligand binding antibody is binding to cells that express the receptor. Fremanezumab binds a non-cellular target and did not bind CGRP receptor expressing cells. Erenumab binds the CGRP receptor and our results confirmed that RAMP1 is critical for the interaction. Consistent with this finding, a recent crystallography study reports that erenumab binds to residues on both CLR and RAMP1 (7). Erenumab and telcagepant share epitope residues but the epitope of erenumab is larger (7).

Receptor activation by CGRP facilitates conformational changes and leads to diverse downstream signaling pathways (22), the major event being cAMP accumulation (23–24) although additional downstream signaling events have been reported (20). It has been proposed that erenumab and gepants act by blocking access to the peptide-binding cleft at the interface of CLR and RAMP1 (4–7). In corroboration, we observed that both receptor binding agents, erenumab and telcagepant, inhibited CGRP-, adrenomedullin- and intermedin- induced cAMP signaling at the CGRP receptor. Since the *in vivo* physiological significance of adrenomedullin and intermedin signaling at the CGRP receptor is unclear, the consequences, if any, of antagonism of their signaling through the CGRP receptor is unknown. Both adrenomedullin and intermedin are expressed in peripheral tissues and regulate vasodilation and cardiac function (25–26). In comparison, fremanezumab inhibited only CGRP ligand signaling at the CGRP receptor while allowing adrenomedullin and intermedin signaling to proceed unperturbed. It is likely that CGRP ligand antibodies target CGRP activity at CGRP-family receptors that are responsive to CGRP, such as the AMY_1 receptor.

Receptor internalization triggered by agonist stimulation occurs for several cell surface GPCRs and removes receptors from the cell surface including CLR-based receptors (27–28). Co-localization of labelled receptor complexes (CLR and RAMP1) suggest that both receptor components co-internalize (18,29). Using multiple assays, we confirmed that erenumab was internalized at the CGRP receptor, unlike fremanezumab. Internalization has implications for an antibody's pharmacokinetic properties, degradation and/or recycling. At the 1 h time point, internalized

erenumab co-localized predominantly to putative early and recycling endosomes with some co-localization to lysosomes in CGRP receptor expressing cells. The significance of internalized CLR complexes has not been extensively explored, although one study suggested that endosomal CGRP receptor signaling mediates nociceptive transmission (17). The data suggest that erenumab undergoes trafficking in the absence and presence of CGRP at the CGRP receptor, while CGRP-induced decreases in surface CLR levels are not observed with erenumab. Although the mechanism for this is unclear and requires further study, a possible explanation for these observations is that in the absence and presence of CGRP, erenumab may undergo internalization by constitutive receptor recycling, where receptors are internalized from the cell surface and recycled in an agonist-independent manner. The physiological relevance of constitutive receptor internalization of GPCRs is unclear and understudied. However, emerging evidence suggests that some GPCRs, including the CGRP receptor, signal from intracellular compartments (17) and thus constitutive receptor internalization may permit sustained cellular responses following transient ligand stimulation. Erenumab targets the same region on the CGRP receptor as the CGRP ligand (7), which suggests that erenumab may prevent CGRP-induced decreases in surface CLR by directly competing with the ligand for receptor binding. As this study utilized tools and techniques specific to antibody internalization, it is unknown whether telcagepant undergoes internalization at the CGRP receptor. The observation with the receptor antibody suggests that this effect could extend to other CGRP receptor-binding agents, like gepants, but this requires future exploration. Additionally, different mechanisms related to the CGRP pathway may possibly be involved in the effectiveness of the gepants in treating migraine acutely versus the CGRP pathway antibodies, used as preventatives.

Importantly, we have determined that erenumab, apart from binding the canonical CGRP receptor, also binds the AMY_1 receptor with a critical requirement for RAMP1, the common subunit in the CGRP and AMY_1 receptor. At the CLR/RAMP1 interface, erenumab recognizes five residues in RAMP1 and 18 residues in CLR (7). Out of 18 CLR residues recognized by erenumab, 10 residues are conserved between CLR and CTR (7). Thus, these conserved residues between CLR and CTR may provide a mechanistic explanation for the interaction between erenumab and the AMY_1 receptor (CTR/RAMP1). The mechanism of antibody internalization needs further investigation since modest ligand-induced internalization of the AMY_1 receptor has been reported (13). A possible explanation for erenumab internalization at the AMY_1 receptor is agonist-independent constitutive receptor endocytosis. Subcellular imaging experiments confirmed internalization of erenumab in $HEK293S^{AMY1}$ cells with localization to early endosomes and some localization to lysosomes an hour after antibody incubation. The absence of interaction between fremanezumab and the AMY_1 receptor highlights another key difference between the antibodies that target the CGRP ligand versus the receptor itself.

With respect to AMY_1 receptor signaling, both erenumab and telcagepant antagonized human amylin-induced signaling at the AMY_1 receptor. It has been long known that many small molecule antagonists designed to target the CGRP receptor also block activity at the AMY_1 receptor (19,20,30–32). Antagonism at both the CGRP and AMY_1 receptors has been shown for rimegepant (32) and ubrogepant (33). Downstream signaling pathways and in turn pharmacological responses of all GPCRs, including the CGRP-family of receptors, are very highly dependent on the cellular context of differing recombinant cell lines and expression systems (20,34). Thus, further studies of multiple signaling pathways in different model cell lines/native tissues are needed to characterize the cross-receptor pharmacology actions of erenumab and clinical gepants.

Amylin and its receptors are important for controlling food intake and obesity (35). Pramlintide, an amylin analogue, is approved for use in insulin-requiring diabetes (36). Thus, the potential for possible metabolism-related side effects caused by the chronic blockade of the AMY_1 receptor exists. It has been suggested that the AMY_1 receptor acts as a dual receptor for CGRP and amylin (12). However, the biological relevance of the AMY_1 receptor in CGRP and/or amylin biology, or with respect to migraine and/or metabolism, is unclear. Thus, further understanding of the *in vivo* function of the AMY_1 receptor is urgently needed. One other study has examined erenumab function at the AMY_1 receptor (37). Based on the evidence that erenumab did not block calcitonin activity in the amylin-responsive MCF-7 cells, erenumab was suggested to lack an effect on the AMY_1 receptor. However, the interpretation of these experiments is complicated. The presence of a functional AMY_1 receptor in MCF-7 cells is unclear (11,38) and these cells likely express multiple endogenous receptors, including amylin, CGRP and calcitonin receptors (39). Thus, their use as a model system for this peptide family has been dissuaded (1). Additionally, calcitonin (not CGRP or amylin) was used as the agonist in that study (37), further complicating interpretation. A recent human tissue cross-reactivity study concluded that there was no off-target binding of erenumab (40); however, since AMY_1 and CGRP receptor

expression may occur in similar regions (12) these observations do not preclude erenumab's binding to the AMY_1 receptor.

In conclusion, our data show that therapeutic agents targeting the CGRP ligand versus the receptor have diverse mechanisms of action. Taken together with previous reports on gepants, our results highlight that there are three distinct mechanistic classes of drugs based on receptor binding, signaling and drug internalization: i) Monoclonal antibodies against the CGRP ligand, ii) erenumab (the first FDA approved GPCR monoclonal antibody against the CGRP receptor); and iii) "gepants", small molecule inhibitors of the CGRP receptor. This study provides important insights into the mechanisms by which CGRP-pathway directed therapeutics function, and suggests that these differing mechanisms could affect efficacy, safety, and/or tolerability in migraine patients.

Key findings

- The CGRP ligand versus receptor agents differentially affect CGRP receptor signaling.
- Erenumab binds to both the canonical CGRP receptor and the related AMY_1 receptor and affects amylin signaling at the AMY_1 receptor.
- Erenumab, unlike fremanezumab, undergoes internalization in CGRP and AMY_1 receptor expressing cells.
- The diverse mechanisms of action of CGRP ligand versus receptor agents may differentially affect efficacy, safety, and/or tolerability in migraine patients.

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Declaration of conflicting interests

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