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## Pharmacological characterisation of mouse calcitonin and calcitonin receptor-like receptors reveals differences compared to human receptors

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### Abstract

**Background and Purpose:** The calcitonin (CT) receptor family is complex, comprising two receptors (the CT receptor [CTR], and the CTR-like receptor [CLR]), three accessory proteins (RAMPs), and multiple endogenous peptides. This family contains several important drug targets, including CGRP which is targeted by migraine therapeutics. The pharmacology of this receptor family is poorly characterised in species other than rats and humans. To facilitate understanding of translational and pre-clinical data we need to know the receptor pharmacology of this family in mice.

**Experimental Approach:** Plasmids encoding mouse CLR/CTR and RAMPs were transiently transfected into Cos7 cells. cAMP production was measured in response to agonists in the absence or presence of antagonists.

**Key Results:** We report the first synthesis and characterisation of mouse adrenomedullin, adrenomedullin 2 and  $\beta$ CGRP, and of mouse CTR without or with mouse RAMPs. Receptors containing m-CTR had subtly different pharmacology than human receptors; they were promiscuous in their pharmacology, both in absence and presence of RAMPs. Several peptides, including mouse  $\alpha$ CGRP and mouse adrenomedullin 2 were potent agonists of the m-CTR:m-RAMP3 complex. The pharmacological profile of receptors comprising m-CLR:m-RAMPs were generally similar to the profile of their human counterparts, albeit with reduced specificity.

**Conclusion and Implications:** Mouse receptor pharmacology differed compared to human, with mouse receptors displaying reduced discrimination between ligands. This creates challenges

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Author Contributions

MLG, CSW, and DLH designed experiments. MAB, PWRH, and AS performed peptide chemistry. MLG, RLB, SC, MAJ, and JP performed experiments and analysed data. MLG, SC, and DLH wrote the manuscript. All authors approved this manuscript.

for interpreting which receptor may underlie an effect in pre-clinical models, and thus translation of findings from mice to humans. It also highlights the need for new ligands to differentiate between these complexes.

## Keywords

Adrenomedullin; calcitonin; amylin; CGRP; receptor activity-modifying protein; calcitonin receptor; calcitonin receptor-like receptor

## Introduction

Migraine is a debilitating condition characterised by moderate to severe headache and other symptoms such as visual aura, nausea, and sensitivity to light/sound (Edvinsson et al., 2018). Migraine is a leading cause of disability worldwide and is the leading cause of neurological disability (Steiner et al., 2013). Recently, six drugs which are designed to reduce the signalling of **calcitonin gene-related peptide** (CGRP) have been approved to treat this disorder. Two drugs are small molecule receptors antagonists (**ubrogepant** and **rimegepant**), the remaining four are monoclonal antibodies of which three target CGRP itself, while the fourth (**erenumab**) targets a receptor for CGRP (Ray et al., 2021).

CGRP is part of a broader peptide family which includes **calcitonin** (CT), **amylin** (Amy), **adrenomedullin** (AM), and **adrenomedullin 2/intermedin** (herein referred to as AM2). These peptides have low amino acid sequence identity. However they are united by key structural features, namely, an amidated C-terminus, and an N-terminal disulfide loop between two cysteine residues (Figure 1). Although CGRP is the most prominently linked to migraine, other peptides from this family are being investigated for their role in the condition. Recent studies have reported that an Amy analogue (**pramlintide** [Pram]) and AM can provoke migraine-like headache (Ghanizada et al., 2021a; Ghanizada et al., 2021b). These investigations are supported by recent reports showing that Amy-like immunoreactivity in plasma was elevated in chronic migraineurs (Irimia et al., 2021), and the finding that repeated administration of AM can cause headaches in humans (Kita et al., 2020). Thus, it may be therapeutically worthwhile to extend beyond CGRP.

The receptors for these peptides are formed from different protein subunits which combine to form different receptor subtypes. The **CGRP receptor** comprises a class B G protein-coupled receptor (GPCR), the **calcitonin receptor-like receptor** (CLR) in complex with the accessory protein **receptor activity-modifying protein (RAMP) 1**. However, when CLR combines with **RAMP2** and **RAMP3**, it forms the **AM1** and **AM2** receptors, which have high affinity for AM and AM2. The human (h-) AM<sub>1</sub> and AM<sub>2</sub> receptors have relatively low affinity for CGRP. The **calcitonin receptor** (CTR) also forms complexes with the three RAMPs. These complexes are all activated by Amy, hence **AMY1**, **AMY2** and **AMY3** receptors. However, CGRP is also able to effectively activate the h-AMY<sub>1</sub> receptor (Hay et al., 2018). The receptor nomenclature for these receptors implies that there is a clear distinction in ligand preference between the receptors, but the expanded experimental data now show that several of the receptor complexes are potently activated by several endogenous ligands. This appears to be more pronounced at rat (r-) CGRP family receptors.

For example, CGRP gains potency compared to AM and Amy at the r-AM<sub>2</sub> and r-AMY<sub>3</sub> receptors, in addition to being a potent agonist of the r-CGRP and r-AMY<sub>1</sub> receptors (Bailey et al., 2012; Hay et al., 2003).

Rodent models are commonly used to explore the physiological function of peptides, and to perform pre-clinical validation of drugs. Thus, knowledge of differences in receptor pharmacology between species is vital. Current migraine research includes a variety of mouse (m-) models in experiments (Vuralli et al., 2019), and includes models where h-RAMP1 overexpression in the central nervous system can be linked to sensitisation of migraine-like behaviours (Recober et al., 2009) in addition to metabolic effects (Zhang et al., 2011). The latter, then adds a mixed-species complexity. There are also ongoing drug discovery programmes for other peptides in this family, especially Amy for diabetes and obesity, which use rat and mouse models (Enebo et al., 2021; Henriksen et al., 2021; Zakariassen et al., 2020). Despite their common usage as models, we have only a rudimentary understanding of the pharmacology of CLR/CTR family receptors in mice. This is in contrast to the pharmacological profiles of h/r-CLR-based and h/r-CTR-based receptors which are better defined (Figure S 1, S 2 and S 3). This is an important gap in our knowledge because there are differences in the amino acid sequences of both the peptides and receptor components between species (Figure 1 a, Figure S 4 and S 5).

Early investigations into complexes of m-CLR with m-RAMP1, m-RAMP2, and m-RAMP3 showed that the profiles of agonists at mouse receptors were similar to their profile of human receptors, albeit with reduced selectivity. These initial reports did not investigate all endogenous agonists of the CT family, such as CT, AM<sub>2</sub>, Amy and  $\beta$ CGRP. Work with antagonists showed subtle differences between human and mouse receptors (Bailey et al., 2012; Husmann et al., 2003; Koller et al., 2004). m-CTR or mCTR:m-RAMP complexes have barely been studied and therefore it is not known whether these form receptors for Amy or CGRP in mice (Bohn et al., 2017). Without characterisation we are essentially blind when trying to interpret data from mouse models. Species such as mice, rats, and canines are vital for drug discovery, and thus information regarding how drug targets function across species is of high importance. In order to develop a framework for interpreting data for these receptors and peptides in mice, we set out to determine the pharmacology of m-CTR, m-CLR, and m-RAMPs in transfected cells.

## Methods

### DNA constructs

CTR can exist as one of multiple splice variants, with both tissue and species dependencies (Hay et al., 2018). Here, unless otherwise specified, CTR will refer to the mouse CT<sub>(a)</sub> receptor which we have used in all experiments. This splice variant is conserved between humans, rats, and mice, as opposed to some other variants which display differences between species. m-CT<sub>(a)</sub>, m-CLR, m-RAMP1, m-RAMP2, and m-RAMP3 were purchased from Origene (Catalogue numbers MC216948, MC216307, MC206240, MC207508, and MC202180, respectively). These constructs were encoded in the pcmv6 expression vector and are *Mus musculus* sequences. All were cloned from C57BL6 sequences, except for m-RAMP1 which was originally cloned from the FVB/N strain. This DNA sequence is 99%

identical to the m-RAMP1 sequence cloned from C57BL6, and the protein sequences are 100% identical. The m-RAMP2 used in this study has a threonine at position 10 within the signal sequence. This corresponds with GenBank Accession #AAH69992.1, while the sequence reported by UniProt has a proline in this position. This is likely to reflect a natural variation in the population, of which there may be more than one (Figure S 6). pcDNA3.1(+) was also used as an empty vector to explore the pharmacology of m-CLR and m-CTR in the absence of m-RAMPs. For full amino acid sequences, and comparison between human, rat and mouse sequences, see Figure S 4 and S 5. Constructs were confirmed by DNA sequencing, through the DNA Sequencing facility at the University of Auckland.

## Peptides

The amino acid sequences for Amy and  $\alpha$ CGRP are strictly conserved between rats and mice; this is indicated throughout by using the prefix m/r-. m/r- $\alpha$ CGRP was purchased from Bachem Pharmaceuticals (Cat# 4025897, Bubendorf, Switzerland), American peptide (Sunnyvale, CA, USA, supplier no longer available) or synthesised in-house; the three sources showed bioequivalence in cAMP assays (data not shown). Salmon [s]-CT and s-CT8-32, were purchased from American Peptide, m/r-Amy was purchased from American peptide. m/r- $\alpha$ CGRP8-37 and h-AM22-52 were purchased from Bachem (Cat# 4034544 and 4028071, respectively). m- $\beta$ CGRP, h- $\alpha$ CGRP, Pram, AC187, m-AM, m-AM240, m-AM2<sub>47</sub>, m-CT, and h-AM were synthesised in-house. Peptides were synthesised in-house using an Fmoc solid-phase synthesis approach. The synthesis of Pram, h- $\alpha$ CGRP, and h-AM has been reported previously (Bower et al., 2018; Garelja et al., 2020; Yule et al., 2016); detailed information regarding the synthesis of other peptides can be found in the supplemental information. Peptide sequences were in accordance with data from UniProt.

Where the peptide content was unknown, a peptide content of 80% was assumed. All peptides were reconstituted in UltraPure™ DNase/RNase-Free Distilled Water (Cat# 10977015, ThermoFisher Scientific, New Zealand) and working stocks of either 10 mM or 1 mM were aliquoted into protein lo-bind tubes (Cat# EP0030108116, Eppendorf, Hamburg, Germany) and stored at  $-30^{\circ}$  C. Peptides were further diluted as needed for cAMP assays.

## Cell culture and transfection

Cell culture, plating, and transfection were conducted as previously described (Bailey & Hay, 2006; Bower et al., 2018). Cos-7 cells (originally from the American Type Culture Collection, RRID CVCL\_0224) were used throughout this study. These cells were chosen as they do not endogenously express CTR, CLR, or RAMPs (Bailey & Hay, 2006), thus avoiding the confounding factor of background CT-family receptor expression. It would have been preferable to use a rodent-derived cell line but we are not aware of any that do not endogenously express the receptor components. Cells were tested for mycoplasma contamination using a MycoAlert kit according to the manufacturers protocol (Lonza, Basel, Switzerland; Cat# LT07-118) and found to be free from infection.

Cells were cultured in complete media, comprising Dulbecco's modified Eagle media (DMEM; Cat# 11995065, ThermoFisher) supplemented with New Zealand origin heat-inactivated foetal bovine serum (Cat# 10372019, Gibco, MA, USA) to a final concentration

of 8% v/v. Cells were grown in a 37 °C humidified incubator at 95% room air/5% CO<sub>2</sub> to a maximum passage number of 30.

Cells were sub-cultured in either T-75 flasks (Cat#FAL353136, Corning, New York, USA) or T-175 cell culture flasks (Cat # CORN431080, Corning) until 80-90% confluent. On the day of passaging, complete media and TrypLE Express (Cat#12605-010, Gibco) were warmed to 37°C. For T-75 flasks, culture media was removed, and cells washed once with 5 mL room temperature Dulbecco's phosphate-buffered saline (DPBS, Cat# 14190250, Gibco). DPBS was then removed and replaced with 5 mL TrypLE Express. Cells were then incubated at 37 °C for approximately five minutes. Cells were resuspended by agitation, then TrypLE was neutralised by adding 5 mL complete media. Either 1 or 2 mL of the resulting cell suspension was then transferred to a new T-75 flask containing 12 mL of complete media. These flasks would typically be ready for passaging after four or three days, respectively. For T-175 flasks, volumes were doubled.

Cells were seeded into 96 well SpectraPlates (Cat# 6005658, PerkinElmer, MA, USA) at a density range of 15,000-20,000 cells per well. Cell density was determined using a Countess II Cell Counter (ThermoFisher). Following seeding, cells were returned to the incubator for 18-24 hours before being transfected with receptor components. Transfection was performed using polyethylenimine (Cat# 408727, Sigma-Aldrich, St. Louis, MO, US), as described previously (Bailey & Hay, 2006; Bower et al., 2018). Briefly, a transfection mix comprising 5% filter sterilised glucose (10% final volume; Cat# AJA783, ThermoFisher), 0.25 µg total DNA per well, polyethylenimine (ratio 10:1 to total DNA), and complete media was created. Media from plates were aspirated and replaced with 100 µL transfection mix per well. Constructs were transfected at a ratio of 1:1 (receptor:RAMP). When investigating the ability of m-CTR or m-CLR to signal in the absence of RAMPs, cells were transfected with m-CTR/m-CLR and pcDNA3.1(+) at a ratio of 1:1. Following transfection, cells were returned to the incubator for approximately 48 hours before being used in cAMP assays.

### **cAMP cell signalling assay**

cAMP stimulation was performed using a method previously described (Woolley et al., 2017). All cAMP assays were performed using stimulation media comprising DMEM supplemented with 1 mM 3-isobutyl-1-methylxanthine (Cat# 15879, Sigma-Aldrich) and 0.1% w/v bovine serum albumin (Cat# ABRE-100g, MP Biomedicals, New Zealand). Forskolin, at a final concentration of 100 µM, was used as a positive control.

Prior to stimulation of Cos-7 cells, the transfection mix was aspirated and replaced with 50 µL of stimulation media and cells left to incubate for 30 minutes at 37 °C. Peptides were serially diluted in stimulation media to the appropriate concentrations. Following serum starvation, cells were stimulated with peptides or cAMP media alone for 15 minutes at 37 °C. For antagonism assays, the antagonist was added to cells followed immediately by the agonist. No pre-incubation was used. In these antagonist assays, we generally used the cognate agonist of each receptor, based on the known human pharmacology. For m-CTR:m-RAMPs we used both m-CT and m/r-Amy as agonists. Additionally, m/r-αCGRP was used as an agonist of m-CTR:m-RAMP1/3 (Bailey et al., 2012).

All media was then removed via aspiration and replaced with 50  $\mu$ L of ice-cold absolute ethanol to stop cAMP production. Plates were then stored at  $-30^{\circ}$  C for a minimum of 15 minutes and a maximum of two weeks before processing.

### **cAMP quantification using a LANCE protocol**

cAMP production was quantified using a LANCE cAMP kit (Cat# AD0263, PerkinElmer) as previously described (Bower et al., 2018). Ethanol was evaporated in a fume hood. Cells were lysed using 50  $\mu$ L lysis buffer (10 mM  $\text{CaCl}_2$ , 50 mM HEPES, 0.35% TritonX-100, all in  $\text{ddH}_2\text{O}$ , pH 7.4). After addition of lysis buffer, cells were gently shaken on an orbital shaker for 10-15 min to produce cell lysates.

Cell lysate (5  $\mu$ L) was then transferred to a 384 well white OptiPlate (Cat# 6007290, PerkinElmer). In parallel, a standard curve was created according to kit instructions by serially diluting a stock standard of cAMP in lysis buffer. All lysate transfers, antibody and detection mix additions were performed manually or on a JANUS Automated Liquid Handling Station (PerkinElmer). The LANCE anti-cAMP antibody was diluted 1:200 in lysis buffer, and 5  $\mu$ L added to each well. Following this, plates were pulse-centrifuged for 10-15 seconds, reaching 400 x *g*, then incubated for 30 minutes at room temperature. Detection mix (Europium-W8044 labelled streptavidin and biotin-cAMP diluted 1:4500 and 1:1500 in lysis buffer, respectively) was added to all wells (10  $\mu$ L per well), and plates were pulse centrifuged for 10-15 seconds, reaching 400 x *g*. The plates were then incubated at room temperature for between 4 and 20 hrs. Plates were read on an Envision plate reader (PerkinElmer), with excitation at 340 nm and emissions detected at 615 and 665 nm. Molar quantities of cAMP were calculated using the standard curve created in each experiment.

### **Experimental design**

For each transfection day, the order in which transfection mixes were added to plates was randomised. In cases where multiple transfections were being included on a single plate, these positions were randomised between days. On each stimulation day, the tested peptides were randomly assigned to positions on the plate. This was for both agonist assays (agonists randomly assigned positions), and antagonism assays (antagonists and media control randomly assigned to plate positions) to remove potential bias from plate positions.

In all cases, duplicate or triplicate technical replicates were performed within each biological replicate. A biological replicate consisted of plating cells from a distinct passage flask, transfection with separate transient transfection mixes, and stimulation with separate dilutions of peptides. Each biological replicate is referred to as an independent experiment and counted as one *n*. Blinding was not conducted in this study. This manuscript complies with the British Journal of Pharmacology's recommendations and requirements relating to experimental design and analysis (Curtis et al., 2018).

### **Data analysis – Agonist assays**

Experiments were biologically replicated a minimum of three or five times. Where three experiments did not produce quantifiable data sets, they were curtailed at  $n = 3$  and no statistical analysis conducted. In all other cases, a minimum of five independent experiments

were performed. There is variation in the sample sizes between some receptor/ligand combinations (range of 5-14 biological replicates). This is due to certain agonists being used as controls in early experiments. To avoid arbitrarily excluding data, these experiments were included in our analyses. Control curves from antagonist experiments were not included in agonist analyses because we did not want to further unbalance the size of data-sets.

Data were analysed using GraphPad PRISM 9 (GraphPad Software, La Jolla, CA). Curves were only fit to data when at least two data-points were above the basal cAMP production; otherwise the response was deemed unquantifiable and defined as not a curve. If a data set had sufficient points for a fit but did not reach a plateau, the curve-fit max was constrained using the mean response at the highest concentration of peptide (Garelja et al., 2020). When fitting agonist curves to individual experiments, F-tests were used to determine whether the Hill slope significantly differed from one (i.e. whether the data were better fit by three-parameter or four-parameter nonlinear regression). From each biological replicate we derived a pEC<sub>50</sub> and E<sub>max</sub> value; these values were then combined to generate mean data for analysis. pEC<sub>50</sub> values were used in order to enable EC<sub>50</sub> values to fit a Gaussian distribution. On rare occasions, there were some experiments for peptide:receptor combinations in which the Hill slope was significantly different from one, and others in which the Hill slope did not differ from one. In these cases, a “majority rules” approach was taken when presenting the combined data, however the individual values used in statistical analyses were derived from the curve of best-fit for each replicate.

Combined concentration-response curves were generated for presentation by combining the mean of data points from individual experiments. These data were then fit with either a three-parameter or four-parameter non-linear regression according to the “majority rules” approach dictated by the results of the individual experiments. pEC<sub>50</sub> and E<sub>max</sub> values were analysed using unpaired one-way analysis of variance (ANOVA). When this ANOVA reached a statistical threshold ( $p < 0.05$ ) and there was no variance inhomogeneity, a post-hoc Tukey’s test was employed, comparing each peptide to every other peptide. All data are presented as the mean  $\pm$  s.e.m. of  $n$  independent experiments. This unpaired, non-normalised approach was taken because the results from peptides were not linked in any way (unpaired analysis across peptides), nor was there a reference ligand on each plate to normalise to (non-normalised analysis). In all cases, a difference was considered significant when  $p < 0.05$ .

The purpose of this study was to develop parameters for receptor activation that can then be translated to *in vivo* mouse work. Thus, we have opted to focus on direct measures of receptor activation such as pEC<sub>50</sub> in molar concentrations rather than binding.

### Data analysis – Antagonist assays

For m-CLR:m-RAMP1, the agonist used was m/r- $\alpha$ CGRP and for m-CLR:m-RAMP2/3 this was m-AM. At m-CTR based receptors, we tested antagonists against m-CT and m/r-Amy. In select other cases we also used m/r- $\alpha$ CGRP as the agonist at m-CTR based receptors because we were interested in investigating agonist-dependent antagonism.

In order to determine whether antagonism was present in a given assay, we used a sum-of-squares F-test to compare whether the two datasets (agonist in the absence and presence of antagonist) could be fit with a shared  $EC_{50}$ . In cases where a single  $EC_{50}$  could be fit to the two datasets, it was concluded that there was no detectable antagonism in the assay.

When no antagonism was detectable, we did not attempt to derive  $pA_2$  values, nor to perform statistical tests. A single experiment was performed using h-AM<sub>22-52</sub> at m-CLR:m-RAMP1; this experiment detected no antagonism and was consistent with multiple previous reports. We did not repeat this experiment, and thus refer to this result as exploratory in text. In all other cases where antagonism was undetectable, we curtailed experiments after a minimum of three independent experiments. When this test showed that antagonism was detectable, a minimum of five independent experiments were performed. From each individual experiment we derived  $pA_2$  values using the Gaddum-Schild equation supplied in GraphPad PRISM 9. When examining the results of antagonist assays, parallelism of the curves was assessed by comparing the fits where the  $n_H$  parameter was shared across the curve in the absence and presence of antagonist to fits in which each curve was allowed its own Hill slope factor. In the majority of tested data sets the Hill slope factor was equal to one in the absence and presence of antagonist, thus for all  $pA_2$  analyses the Hill slope was constrained to one. The antagonists used in this paper are fragments of the agonists, thus we assumed that antagonism would be competitive and surmountable. This assumption is consistent with results from previous studies using these antagonists (Bailey et al., 2012; Hay et al., 2003). Accordingly, the Schild slope was constrained to one in all cases.

$pA_2$  values derived from these equations were then compared using either unpaired Student's *t*-test or unpaired one-way ANOVA as dictated by the number of groups being compared. When this ANOVA reached a statistical threshold ( $p < 0.05$ ) and there was no variance inhomogeneity, a post-hoc Tukey's test was employed comparing the mean  $pA_2$  of each antagonist to all other antagonists. In all cases significance was accepted when  $p < 0.05$ .

## Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

## Results

### Agonist pharmacology at m-CLR:m-RAMP complexes

We characterised mouse receptors using species-matched mouse peptides. In addition, we have also characterised receptor activation in response to other agonists that are commonly used in the study of this peptide family (h-AM and h- $\alpha$ CGRP, s-CT, and Pram, Figure 1). Two forms of m-AM2 were examined; a 47 amino acid peptide and a 40 amino acid peptide (which lacks the first seven amino acids of the 47 amino acid peptide). This approach was taken because both peptide lengths have been reported in humans, and there is evidence to



suggest that these different lengths have different physiological effects in mice (Hong et al., 2012).

We first confirmed that m-CLR was unable to signal alone. Cells transfected with m-CLR:pcDNA did not respond to stimulation with m-AM or m/r- $\alpha$ CGRP at concentrations as high as 10  $\mu$ M (Figure S 7). This is consistent with results from other mammalian species and suggests that without RAMP interaction, m-CLR does not translocate to the cell surface, nor bind peptide, though we did not test this (Hay et al., 2018).

We then characterised receptor complexes comprising m-CLR:m-RAMPs. For m-CLR:m-RAMP1, the most potent peptides were m/r- $\alpha$ CGRP, h- $\alpha$ CGRP, and m- $\beta$ CGRP, which were all equipotent. The next most potent was m-AM<sub>240</sub>, which was approximately 14-fold less potent than m/r- $\alpha$ CGRP (Figure 2, Figure S 1). There were no significant differences in  $E_{max}$  between any peptides at this receptor.

In cells transfected with m-CLR:m-RAMP2, the most potent peptides were m-AM, h-AM and m-AM<sub>240</sub>. These were followed by m-AM<sub>247</sub>, which was ~seven-fold less potent than m-AM (Figure 2, Figure S 1). There were no significant differences in  $E_{max}$  between any peptides at this receptor.

Receptors formed through the interaction of m-CLR and m-RAMP3 had less than a 10-fold difference in potency between m-AM (the most potent agonist) and the tested forms of CGRP, AM, and AM2 (Figure 2,). m/r-Amy and Pram were 32-fold and 55-fold less potent than m-AM, respectively. There were no significant differences in  $E_{max}$  between any peptides at this receptor. In all experiments using m-CLR, m-CT and s-CT were unable to stimulate quantifiable cAMP production within the tested concentration range (Figure 2).

### Agonist pharmacology at m-CTR and m-CTR:m-RAMP complexes

In cells transfected with m-CTR:pcDNA (i.e. m-CTR alone) s-CT was the most potent peptide, m- $\beta$ CGRP was the most potent non-CT peptide being ~10-fold more potent than m/r-Amy (Figure 3, Figure S 2). There were no significant differences in  $E_{max}$  between any peptides at this receptor.

Broadly speaking, most non-CT peptides were more potent in cells transfected with m-CTR:m-RAMP1 than cells with m-CTR alone (Figure 3, Table 1). This was most pronounced for Pram (14-fold increase), m-AM<sub>247</sub> (eight-fold increase), m/r-Amy and m/r- $\alpha$ CGRP (both five-fold increase; Figure S 3).  $\beta$ CGRP was also potent at this receptor (Figure S 2). There were no significant differences in  $E_{max}$  between any peptides at this receptor.

Co-transfection of m-CTR with m-RAMP2 induced only minor changes in peptide potency relative to m-CTR alone, and did not affect the potency of m/r-Amy (Figure 3, Figure S 3). There were no significant differences in  $E_{max}$  between any peptides at this receptor.

Co-transfection of m-CTR with m-RAMP3 induced substantial increases in the potency of several peptides, relative to cells transfected with m-CTR alone. m/r-Amy was seven-fold more potent at m-CTR:m-RAMP3 over m-CTR (Figure 3, Figure S 3) but the increases were

largest for m-AM<sub>247</sub> (26-fold increase), h- $\alpha$ CGRP (12-fold increase) and m-AM (11-fold increase). The majority of tested peptides had a pEC<sub>50</sub> within one log unit of m/r-Amy; the only exceptions were s-CT and h-AM (28-fold more potent, and 14-fold less potent than m/r-Amy, respectively; Figure S 2). There were no significant differences in E<sub>max</sub> between any peptides at this receptor.

Note that m-CT responses could be acting through m-CTR alone in cells co-transfected with RAMPs. This has been discussed previously for human receptors and is a complication of CTR being able to traffic to the cell surface without RAMP (Hay et al., 2018). It is important to be aware of this when interpreting the data with m-CT.

### Antagonist pharmacology

We additionally characterised these receptors using commonly cited antagonists (Figure 1 b). Our data above show that there is limited scope to differentiate receptors *in vitro* based on agonism alone, especially for m-CTR-based receptors which displayed little selectivity between agonists. Antagonists are useful tools when used in conjunction with agonists to better define receptors responsible for effects. Thus, we reasoned it will be important to combine agonists and antagonists in model systems to attribute discrete outcomes to defined receptors. We focused on peptide antagonists because the major class of small molecules targeting this receptor family (the -gepantans) often have low affinity at receptors from non-primate species (Doods et al., 2000; Moore et al., 2020; Moore et al., 2010; Salvatore et al., 2008).

$\alpha$ CGRP<sub>8-37</sub> is a commonly used antagonist of CGRP receptors. m/r- $\alpha$ CGRP<sub>8-37</sub> was an effective antagonist of m-CLR:m-RAMP1 (pA<sub>2</sub> of 8.48  $\pm$  0.18, n = 5; Figure 4) and a significantly weaker antagonist of m-CLR:m-RAMP3 (pA<sub>2</sub> of 6.20  $\pm$  0.20, n = 5; Figure 4); m/r- $\alpha$ CGRP<sub>8-37</sub> was unable to antagonise cAMP production in cells transfected with m-CLR:m-RAMP2 in four exploratory experiments (Figure 4). We compared this profile to the commonly used AM-receptor antagonist, h-AM<sub>22-52</sub>. In an initial exploratory experiment, 10  $\mu$ M hAM<sub>22-52</sub> was unable to cause a rightward shift at m-CLR:m-RAMP1, consistent with results from humans, rats, and previous mouse work (Booe et al., 2018; Husmann et al., 2003; Oliver et al., 2001). Concentrations higher than 10  $\mu$ M were not practical to test, and therefore this experiment was not repeated (data not shown). In contrast, hAM<sub>22-52</sub> was a weak antagonist at both m-CLR:m-RAMP2 and m-CLR:m-RAMP3 and (Figure 4). AM<sub>22-52</sub> was significantly more effective at antagonising receptors comprising m-CLR:m-RAMP2 (pA<sub>2</sub> of 5.71  $\pm$  0.03, n = 5) over m-CLR:m-RAMP3 (pA<sub>2</sub> of 5.19  $\pm$  0.21, n = 5), although the difference was small (three-fold). Interestingly, at receptors comprising m-CLR:m-RAMP3, m/r- $\alpha$ CGRP<sub>8-37</sub> was 10-fold more effective than h-AM<sub>22-52</sub> at antagonising cAMP production; this difference was statistically significant.

Previous reports show that  $\alpha$ CGRP<sub>8-37</sub> can also act as an antagonist at the AMY<sub>1</sub> receptor (Bailey et al., 2012; Hay et al., 2005; Walker et al., 2015). To explore the selectivity of this antagonist at mouse receptors, we characterised its ability to antagonise m/r- $\alpha$ CGRP stimulated cAMP production in cells transfected with m-CTR:m-RAMP1. m/r- $\alpha$ CGRP<sub>8-37</sub> was 50-fold less effective at receptors comprising m-CTR:m-RAMP1 relative to m-CLR:m-RAMP1 (Table 2), this difference was statistically significant. Our work showed that m/r-

$\alpha$ CGRP was also an effective agonist of m-CTR:m-RAMP3 (Figure 2), thus we included this receptor in our characterisation and found that m/r- $\alpha$ CGRP<sub>8-37</sub> could antagonise m/r- $\alpha$ CGRP with a pA<sub>2</sub> ~100-fold lower than its pA<sub>2</sub> at m-CLR:m-RAMP1 (Table 2, Figure 4). We noted a partial agonist effect of m/r- $\alpha$ CGRP<sub>8-37</sub> at m-CTR:m-RAMP3. This effect was statistically significant as determined by a paired Student's *t*-test comparing the E<sub>min</sub> of curves in the absence and presence of m/r- $\alpha$ CGRP<sub>8-37</sub> (mean cAMP production of 1.78 ± 0.54 nM and 4.52 ± 0.57 nM, respectively, both n = 5; Figure 4), this is consistent with previous publications which have used high concentrations of this peptide (Walker et al., 2018). Small molecule antagonists (olcegepant and rimegepant) show differences in their ability to antagonise CGRP and Amy at the h-AMY<sub>1</sub> receptor (Pan et al., 2020; Walker et al., 2018). We therefore investigated the ability of m/r- $\alpha$ CGRP<sub>8-37</sub> to antagonise m/r-Amy at receptors comprising m-CTR:m-RAMP1 and found that m/r- $\alpha$ CGRP<sub>8-37</sub> was equally effective against both agonists (Table 2, Figure 4).

The N-terminally truncated analogue of sCT (sCT<sub>8-32</sub>), and the acetylated peptide AC187, are widely available and commonly cited peptide antagonists of CTR-based receptors. Thus, we sought to define the pharmacology of these peptides at m-CTR-based receptors. When using m/r-Amy as an agonist, sCT<sub>8-32</sub> and AC187 were equivalent in all cases, except for receptors comprising m-CTR:m-RAMP1, where AC187 was 10-fold more effective than s-CT<sub>8-32</sub>. Additionally, AC187 was slightly more effective against m/r-Amy at receptors comprising m-CTR:m-RAMPs over m-CTR alone (Figure 4, Table 2).

We also investigated the selectivity of AC187 for m-CTR-based receptors over m-CLR:m-RAMP1 (Figure 4), where it was ~300-fold more effective at antagonising m/r- $\alpha$ CGRP at m-CTR:m-RAMP1 and m-CTR:m-RAMP3 (Table 2) compared to m-CLR:m-RAMP1 (pA<sub>2</sub> of 5.84 ± 0.05, n = 5; Figure 4), these differences were statistically significant as determined by a one-way ANOVA with Dunnett's post-hoc test comparing the pA<sub>2</sub> of AC187 at m-CLR:m-RAMP1 to its pA<sub>2</sub> at m-CLR:m-RAMP1 and m-CLR:m-RAMP3.

## Discussion

Drugs which build upon the successes of CGRP-targeting therapeutics will be important in further reducing the burden of migraine. To facilitate the development of drugs targeting the CGRP/CT family, we need to understand their pharmacology in mice. We report here a reference framework which will be useful to investigators researching migraine, and other conditions such as cardiovascular and metabolic disorders (Hay et al., 2018).

To facilitate comparisons to human receptors, we have created two figures showing the relative potency of ligands at each receptor in humans and mice (Figure 5 and 6). Human data are the mean of values reported throughout the literature (see figure legends of Figure 5 and 6 for sources). The mouse receptor pharmacology for CLR-based receptors is a composite of our results in this study and previously published literature on these receptors. These figures are useful for comparing a single receptor complex between species; however they were not designed to compare across receptors. This is most relevant for CTR-based receptors, for which the fold-induction of peptide potency at CTR:RAMP complexes relative to CTR alone is important. For this comparison, we refer readers to Figure S 3.

Consistent with receptors cloned from humans, our data show that m-CLR:m-RAMP1 is most potently activated by CGRP peptides and more weakly by AM peptides (Figure 5 A); this is congruous with rat receptors (Bailey et al., 2012). Likewise, our work with antagonists showed a consistent pharmacology across humans and mice, with  $\alpha$ -CGRP<sub>8-37</sub> being much more potent than AM-based antagonists (Figure 5 B), which is again congruous with rat receptors (Walker et al., 2015). Thus, the pharmacology of this receptor complex appears broadly consistent in all tested species.

m-CLR:m-RAMP2 had a strong preference for m-AM over m/r- $\alpha$ CGRP, which is consistent with human receptors (Figure 5 A) and rat receptors (Kuwasako et al., 2002). However, we report that m-AM and m-AM<sub>240</sub> had similar pEC<sub>50</sub> and E<sub>max</sub> values at this receptor, while at receptors cloned from humans h-AM<sub>2</sub> is a partial agonist that is approximately 10-fold less potent than h-AM (Garelja et al., 2020; Musa et al., 2019; Weston et al., 2016). The difference in potency between AM and AM<sub>2</sub> is one of the hallmarks of a h-AM<sub>1</sub> receptor; we now show that this does not appear to translate between species. We saw very weak antagonism with h-AM<sub>22-52</sub> and no antagonism with m/r- $\alpha$ CGRP<sub>8-37</sub>. Previously, m/r- $\alpha$ CGRP<sub>8-37</sub> has been reported as a very weak antagonist of m-CLR:m-RAMP2 (Husmann et al., 2003), thus it is likely that a higher concentration of antagonist would have induced measurable antagonism in our hands. Nevertheless, our antagonist findings seem to agree with human and rat receptors, where AM based antagonists are between two and 10-fold more effective than h- $\alpha$ CGRP<sub>8-37</sub> and m/r- $\alpha$ CGRP<sub>8-37</sub> (Figure 5 B; Hay et al., 2003).

m-CLR:m-RAMP3 showed only five-fold discrimination between m-AM and m/r- $\alpha$ CGRP (Figure 5 A). This aligns with rat receptors and previous data from mouse receptors (Bailey et al., 2012; Hay et al., 2003; Husmann et al., 2003). In contrast, human AM<sub>2</sub> receptors have a stronger preference for h-AM over CGRP peptides (Figure 5 A), highlighting a difference in pharmacology across species. However, both forms of m-AM<sub>2</sub> were ~eight-fold more potent at m-CLR:m-RAMP3 compared to m-CLR:m-RAMP2 (Table 1), which is consistent with humans (Musa et al., 2019). Like rat receptors, our data showed that m/r- $\alpha$ CGRP<sub>8-37</sub> was ~10-fold more effective than h-AM<sub>22-52</sub> at m-CLR:m-RAMP3; in humans h- $\alpha$ CGRP<sub>8-37</sub> and h-AM<sub>22-52</sub> are equivalent (Figure 5 B), which again highlights a difference in receptor pharmacology across species. As with m-CLR:m-RAMP2, the differences in pharmacology between mice and humans preclude simple translation between a human AM<sub>2</sub> receptor and a “mouse AM<sub>2</sub> receptor”.

Consistent with cloned human, rat, and porcine receptors, m-CTR displayed a strong preference for m-CT and s-CT over m/r-Amy (Bailey et al., 2012; Kikumoto et al., 2003; Udawela et al., 2006) (Figure 6 A). However, while the human CTR has a preference for h-Amy over h- $\alpha$ CGRP and h-AM<sub>240</sub>, this was less apparent at the mouse CTR (Figure 6 A). We report m- $\beta$ CGRP as a relatively potent agonist of m-CTR; this is unique to mice.  $\beta$ CGRP has previously been highlighted as a peptide which does not have conserved trends in pharmacology across species (Bailey et al., 2012); our data adds to this and highlights that further investigations into this underexplored peptide are warranted.

The “AMY receptor” nomenclature is defined by a significant enhancement of Amy binding and signalling upon co-expression of CTR with a RAMP (Poyner et al., 2002).

Co-expression of m-CTR with m-RAMP1 or m-RAMP3 induced a five-fold and seven-fold increase in m/r-Amy potency, while co-expression with m-RAMP2 had no effect on m/r-Amy potency (Figure S 3, Table 1). On average, co-expression of h-CTR with h-RAMP1, h-RAMP2, or h-RAMP3 causes a 11-, two-, and six-fold increase in the potency of h-Amy (Figure S 3). Rat receptors have been defined only with r-RAMP1 and r-RAMP3; co-expression of r-CTR with r-RAMP1 or r-RAMP3 causes a nine-fold or 72-fold increase in the potency of m/r-Amy, respectively (Figure S 3). Our data suggest that the induction of m-AMY<sub>1/3</sub> receptor phenotype is modest when compared to rat receptors, and slightly weaker than human receptors. It is worth highlighting that the pharmacology of h-CTR:h-RAMP2 is dependent on cell background, and this may also be the case for rat and mouse (Morfis et al., 2008; Tilakaratne et al., 2000).

AMY receptor nomenclature is also defined by Amy being more potent than other non-CT peptides. h-Amy is equipotent to h- $\alpha$ CGRP at the h-AMY<sub>1</sub> receptor, and 10-fold more potent than h- $\alpha$ CGRP at the h-AMY<sub>3</sub> receptor. Our data show that m/r-Amy and m/r- $\alpha$ CGRP are equipotent at receptors comprising m-CTR and either m-RAMP1 or m-RAMP3; this is consistent with rat receptors (Bailey et al., 2012). Additionally, our data show there is less discrimination between m/r-Amy and other peptides at m-CTR-based receptors relative to the human receptors. Thus, while there are some similarities across species, the pharmacology is clearly not conserved. These mixed profiles create significant challenges for nomenclature as some receptors do not have a clear endogenous agonist (Vanhoutte et al., 1996), especially when the most potent agonist of the “m-AMY receptors” appears to be m- $\beta$ CGRP (Figure 6 A).

Despite these differences, we report a relatively conserved phenotype with regards to antagonist pharmacology (Figure 6 B). From these data, AC187 emerges as a potentially useful tool as it shows discrimination (>100-fold) between CTR and CLR-based receptors. CGRP<sub>8-37</sub> had the opposite pharmacology, being more potent at CLR than CTR-based receptors, though the fold difference was lower than for AC187 (~50-fold discrimination). However, because both were antagonists of multiple receptors, it is important to use these peptides in conjunction with other pharmacological tools to accurately identify receptors.

These findings have implications for our understanding of physiological data. For instance, we show that m/r- $\alpha$ CGRP has nano-molar potency at four of the seven tested receptors. Thus, inferences drawn about receptors responsible for a given effect are complicated by the fact that effects could be occurring via m-CLR:m-RAMP1/3, or m-CTR:mRAMP1/3, or a combination of these receptors. m- $\beta$ CGRP has an additional complication in that it is also a potent agonist of m-CTR alone. h-AM<sub>22-52</sub> was a very weak antagonist of m-CLR:m-RAMP2/3, thus studies that use this to ascertain the contributions AM receptors make to a defined physiological outcome must use very high concentrations to see antagonism (Owji et al., 2008). Until now it has been unclear why the different lengths of human AM2 have distinct physiological effects in mice given the two lengths are equivalent in humans (Hong et al., 2012; Musa et al., 2019). Our data shows these two fragments have distinct pharmacology at mouse receptors, which could influence their physiological function. To the author's knowledge, this is the first synthesis and characterisation of mouse AM2 in the

literature, and given its apparently broad spectrum activity across several mouse receptors, it warrants further investigation.

After removing the signal sequences, h-CLR and m-CLR are 95% similar (BLOSUM90 matrix, threshold of 1). Using the same analysis technique, h-CTR and m-CTR are 88% similar. This could explain why the pharmacology of CLR-based receptors was more conserved across these two species. We explored this further using recently published structures of receptors from this family (Liang et al., 2020; Liang et al., 2018; Liang et al., 2017), and found that in addition to there being more differences across for species for CTR relative to CLR, the differences were also more distributed across the receptor. Differences for CLR were clustered in the extreme ECD, the linker region (which joins the ECD to transmembrane domain 1), and the intracellular portions of transmembrane domains 1/2. Conversely, for CTR the differences were found across the receptor including in the peptide binding pocket, the intracellular G protein binding pocket, and the extreme C-terminal tail (Figure 7). Cumulatively, these mutations may have a large impact on the ability of m-CTR to bind RAMP, peptides and interact with intracellular signalling proteins.

We used a similar approach to explore why m-RAMP3 might produce receptors with promiscuous profiles that differ from the defined profiles of h-RAMP3. This difference is surprising given the relative conservation of amino acid sequences between human and mouse RAMP3 (Figure S 5, 87% similarity between m-RAMP3 and h-RAMP3). RAMP1, which has a lower level of similarity between species (81% similarity between m-RAMP1 and h-RAMP1), has a more conserved effect on CTR and CLR when comparing across species. The differences between m-RAMP3 were not near the peptide binding pocket (Figure 7), suggesting that pharmacological differences were unlikely due to differential peptide contacts between species. The linker region of the RAMP has a strong influence on receptor phenotype by influencing conformational dynamics of the receptor complex (Liang et al., 2020). Examining the linker in m-RAMP3, there are two substantial differences between humans and mice, V109T, and L111W (changes presented relative to the human sequence). A structure captures these residues in the proximity of extracellular loop 2, specifically within 4 angstroms of CLR Y278/D280 (CTR F285/D287). These residues are known to influence the signalling of CLR and CTR (Pham et al., 2019; Watkins et al., 2016). Introducing side chains which change the polarity (V109T) and aromaticity (L111W) of the RAMP could influence receptor dynamics and therefore receptor pharmacology. While m-RAMP1 also has a change in the linker region, this change is less drastic, replacing a small hydrophobic residue with a slightly larger hydrophobic residue (V111L). This may explain why the m-RAMP1 pharmacology is more similar to h-RAMP1 than the m-RAMP3 is to h-RAMP3 however, this is speculative.

A limitation with this work is that the receptor sequences were all derived from the C57BL6 strain of mice. There are noted strain specific differences in CGRP induced migraine-like behaviours (Mason et al., 2017; Wattiez et al., 2019). There are numerous single nucleotide polymorphisms, insertions, and deletions in CLR, CTR, and RAMP genes between strains (Keane et al., 2011), which could manifest as differences in the sequence or expression of CGRP-responsive receptors. Future studies which explore the pharmacological effects of these genetic differences could shed light on the cause of physiological differences. Given

the established use of mice genetically modified to over-express h-RAMP1 (Recober et al., 2009; Zhang et al., 2011), it would be interesting to further investigate receptors formed between mouse receptors and human RAMPs (Bohn et al., 2017). Previous work indicates that mixed species receptors do not necessarily replicate the pharmacology of receptors comprising subunits from individual species (Bohn et al., 2017; Hay et al., 2003; Husmann et al., 2000), thus further investigation would be invaluable to understanding these models. Mice which incorporate fully humanised receptors under endogenous promoters may also be useful for increasing the translatability of physiological findings, though issues relating to the differing splice variants across species must be considered. These mice would have the additional benefit of responding to small molecule antagonists such as olcegepant, which are less effective at rodent receptors than human receptors (Moore et al., 2020; Salvatore et al., 2008).

A further limitation to this work is that we have only measured cAMP production. There are reported differences in the ability of human receptors to stimulate alternative signalling pathways, for instance, the h-CGRP receptor can stimulate measurable IP<sub>1</sub> production, while the h-AM<sub>1</sub> receptor cannot (Garelja et al., 2020). Additionally, antagonists of these receptors can act in a pathway specific manner (Walker et al., 2018). Thus, future investigations into alternative signalling pathways regulated by mouse receptors may allow greater distinction between this family. Binding studies would also be valuable.

In conclusion, calcitonin family receptors cloned from mice are similar, but not directly comparable to, their human counterparts. Given the complicated pharmacology of this receptor family, a future research avenue might be to use genetically modified mice that express humanised receptors in place of mouse receptors to try improve the translatability of findings, thereby facilitating the discovery of novel therapeutics. In light of our findings, a cautious approach should be taken to assigning a particular calcitonin family receptor to a given function in mice.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Conflict of interest

D.L.H. has received research support from Living Cell Technologies and has acted as an advisor or consultant for Amgen, Merck and Intarcia.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design and Analysis](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

## Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Abbreviations

<b>AM</b>	adrenomedullin
<b>Amy</b>	Amylin
<b>CGRP</b>	calcitonin gene-related peptide
<b>CLR</b>	calcitonin receptor-like receptor
<b>CT</b>	calcitonin
<b>CTR</b>	calcitonin receptor
<b>DMEM</b>	Dulbecco's modified Eagle media
<b>DPBS</b>	Dulbecco's phosphate buffered saline
<b>GPCR</b>	G protein-coupled receptor
<b>HEK293S</b>	Human Embryonic Kidney cells
<b>Pram</b>	Pramlintide
<b>RAMP</b>	receptor activity-modifying proteins

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**What is already known**

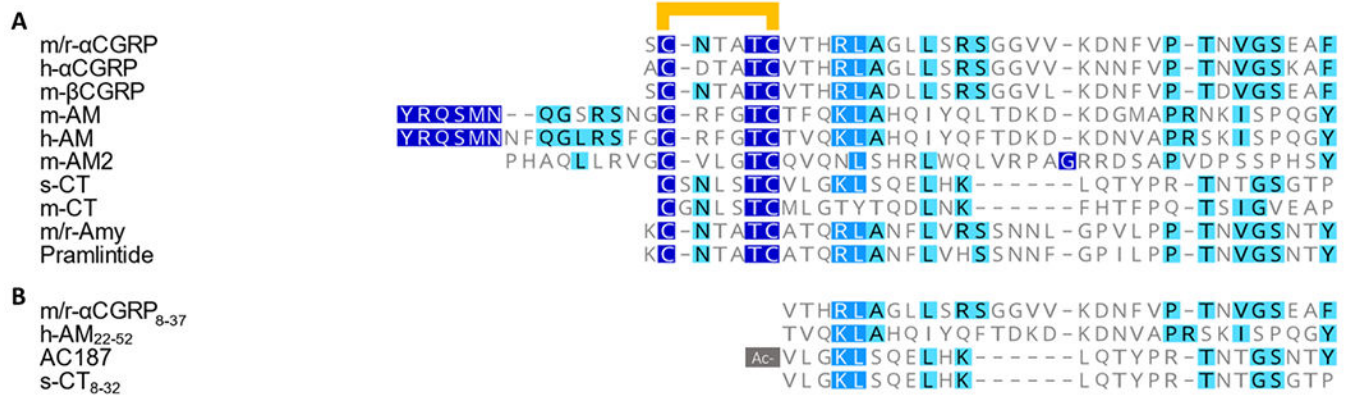
- CGRP and related peptides are implicated in migraine pathophysiology
- The pharmacology and physiology of this family is complex, involving multiple receptors and accessory proteins

**What this study adds**

- Pharmacological characterisation of mouse receptors with endogenous ligands and commonly used pharmacological tools
- Mouse receptors displayed reduced ligand specificity relative to their human counterparts

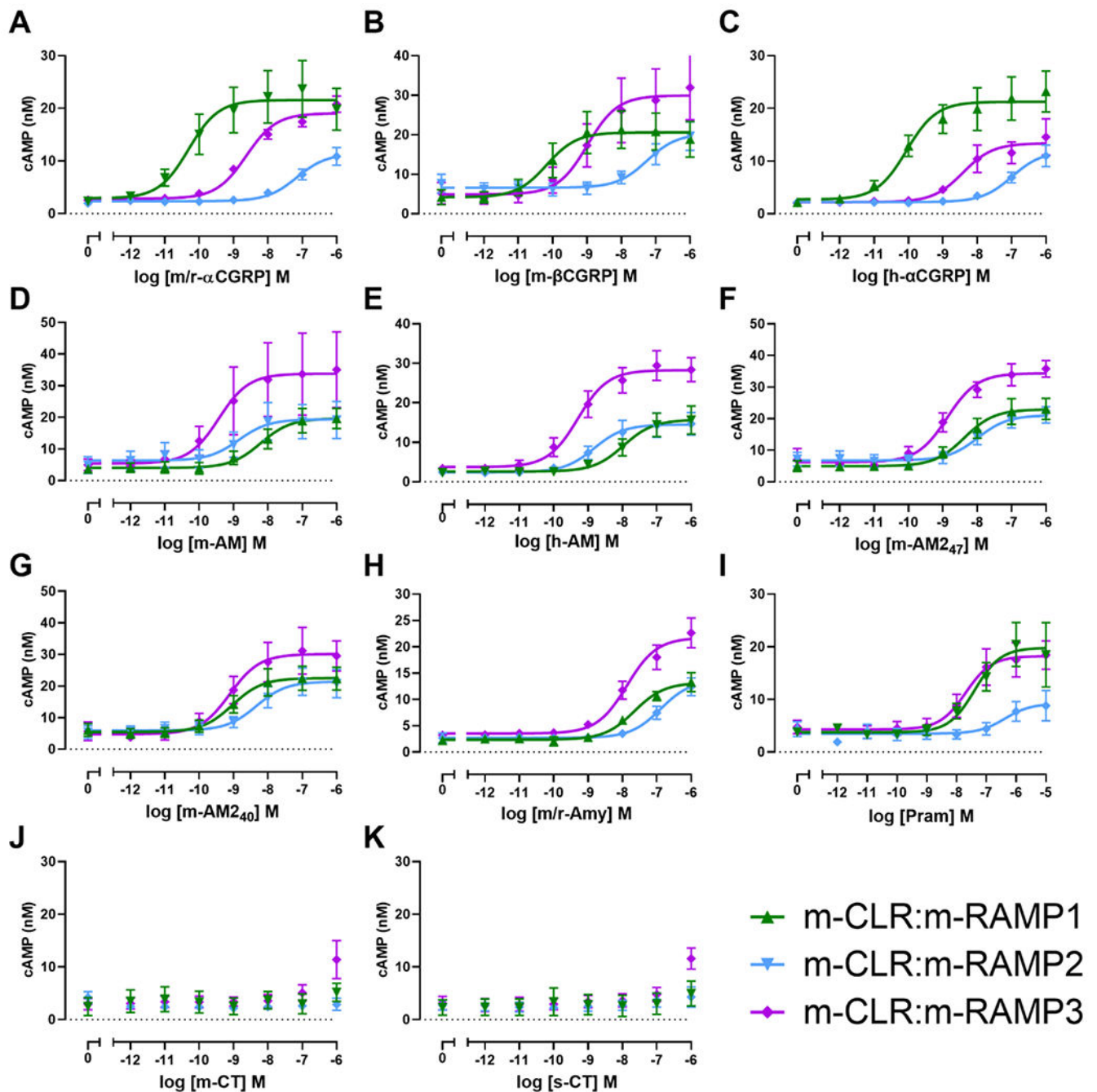
**Clinical significance**

- Our findings are a framework for interpreting pre-clinical studies of this receptor family in mice

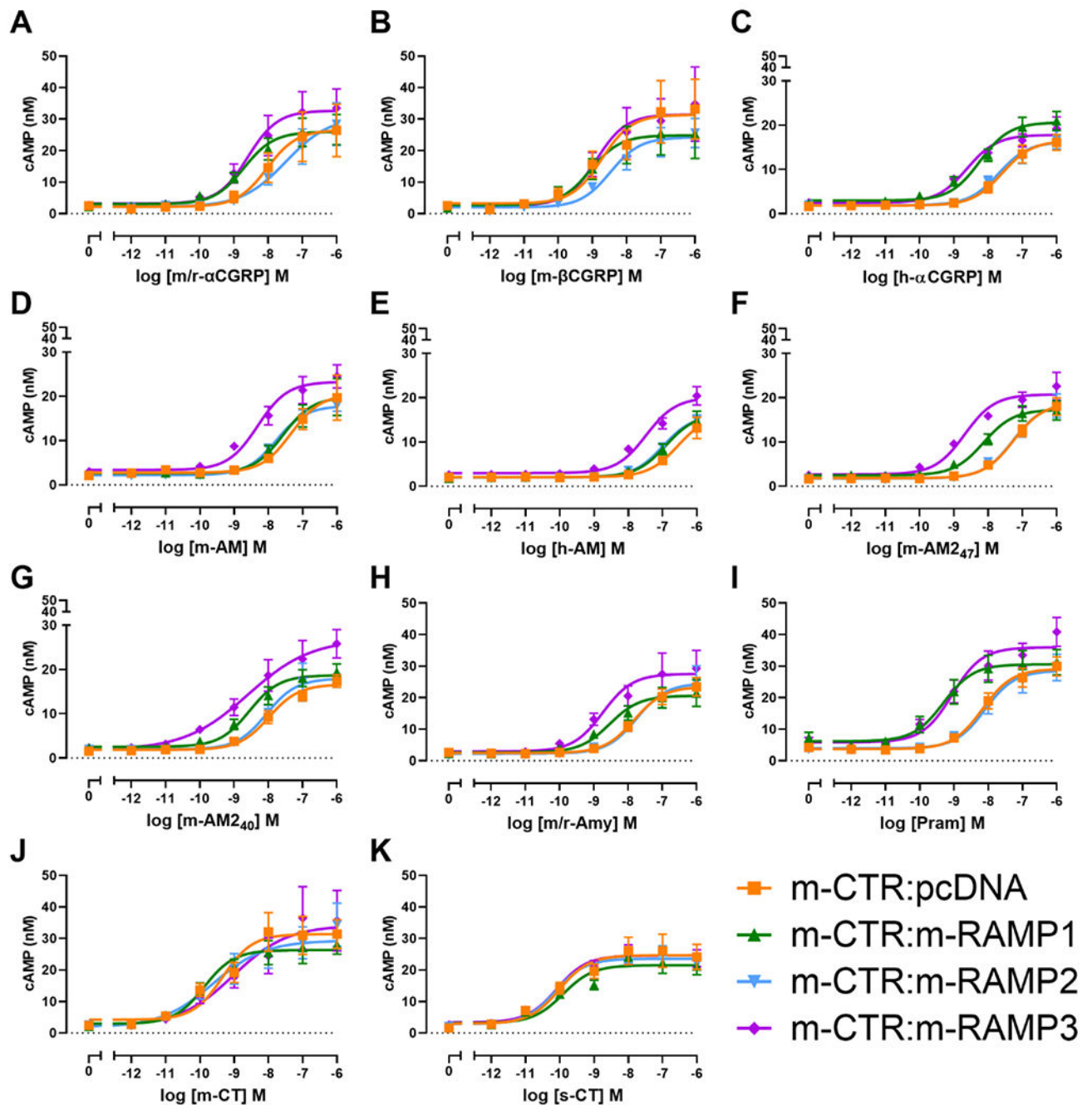


**Figure 1.**

(a) Amino acid sequence alignment of agonists used in this study. In all cases there is a disulfide bond between the two conserved cysteine residues in the N-terminus (shown as a yellow bar). (b) Amino acid sequence alignment of antagonists used in this study. The N-terminal valine of AC187 is acetylated as indicated by the grey box. All agonists and antagonists are amidated on the C-terminal residue. All alignments were performed in Geneious Prime using the inbuilt ClustalW sequence alignment. A dark blue box with white text indicates an exact match, a medium blue box with white text indicates amino acids with >80% similarity, and a light blue box with black text indicates between 60% and 80% similarity as scored by Geneious.

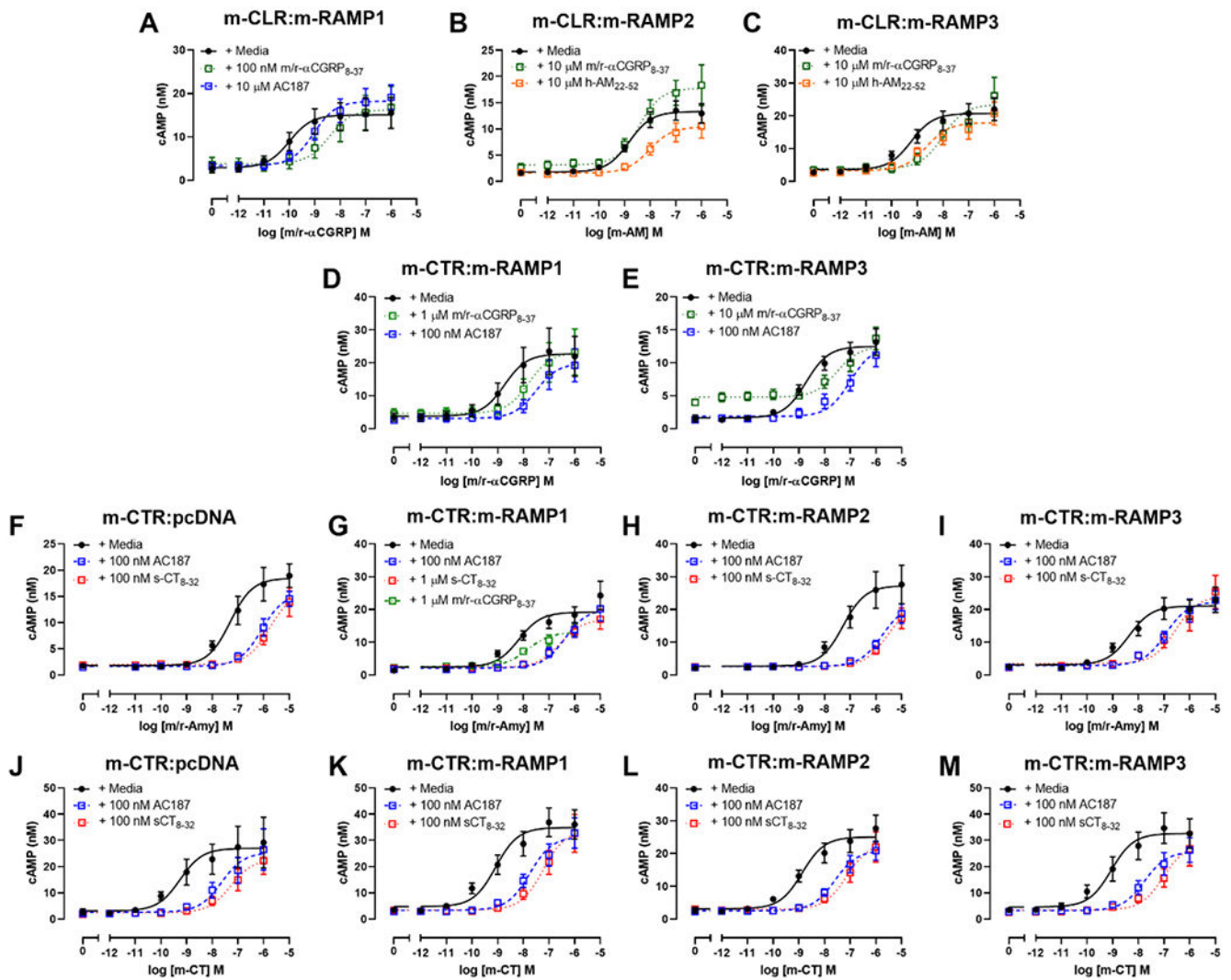


**Figure 2.** Concentration-response curves for m/r- $\alpha$ -CGRP (A), m- $\beta$ CGRP (B), h- $\alpha$ CGRP (C), m-AM (D), h-AM (E), m-AM<sub>247</sub> (F), m-AM<sub>240</sub> (G), m/r-Amy (H), Pram (I), m-CT (J), and s-CT (K) stimulating cAMP production in Cos7 cells transfected with m-CLR in the presence of m-RAMPs. Each point is the mean  $\pm$  s.e.m. of at least three (flat-lines) or five (curves) independent experiments performed in duplicate or triplicate (see Table 1 for individual *n* numbers).



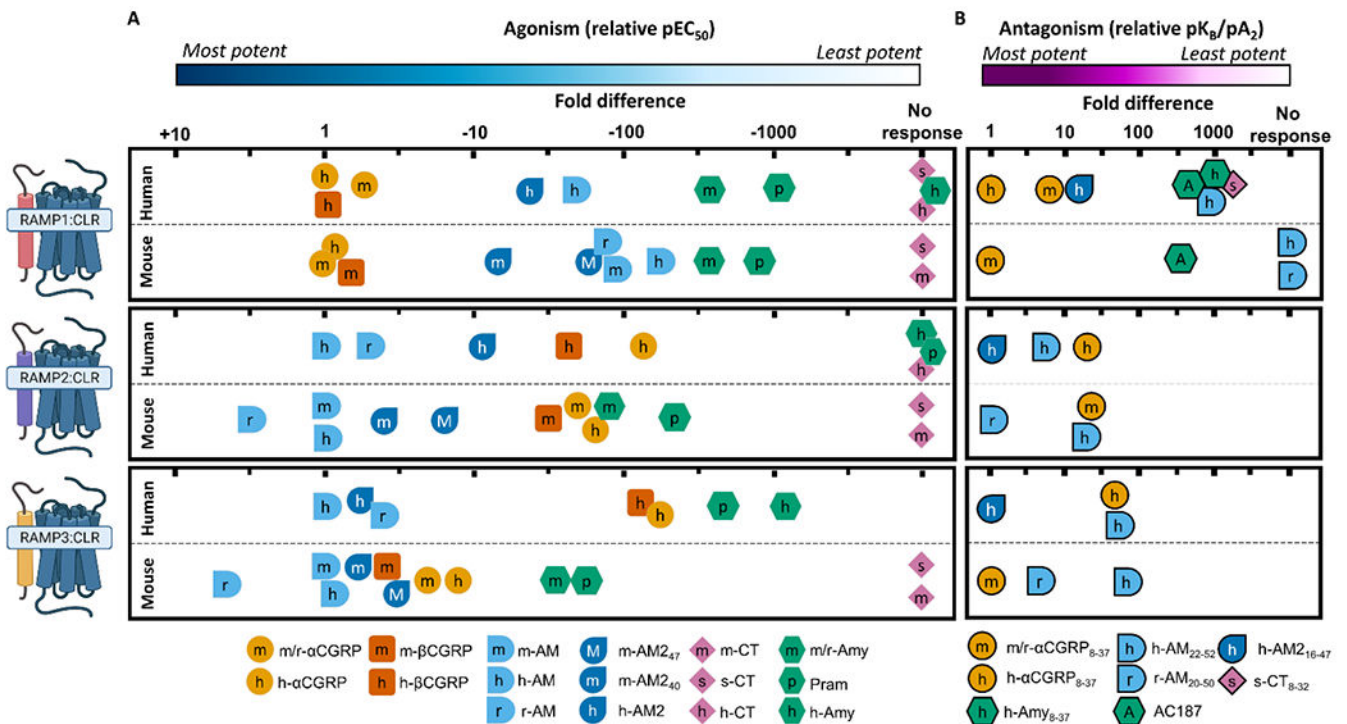
**Figure 3.** Concentration-response curves for m/r- $\alpha$ CGRP (A), m- $\beta$ CGRP (B), h- $\alpha$ CGRP (C), m-AM (D), h-AM (E), m-AM<sub>247</sub> (F), m-AM<sub>240</sub> (G), m/r-Amy (H), Pram (I), m-CT (J), or s-CT (K) stimulating cAMP production in Cos7 cells transfected with m-CTR in the absence or presence of m-RAMPs as indicated. Each point is the mean  $\pm$  s.e.m. of at least five independent experiments performed in duplicate or triplicate (see Table 1 for individual  $n$  numbers).





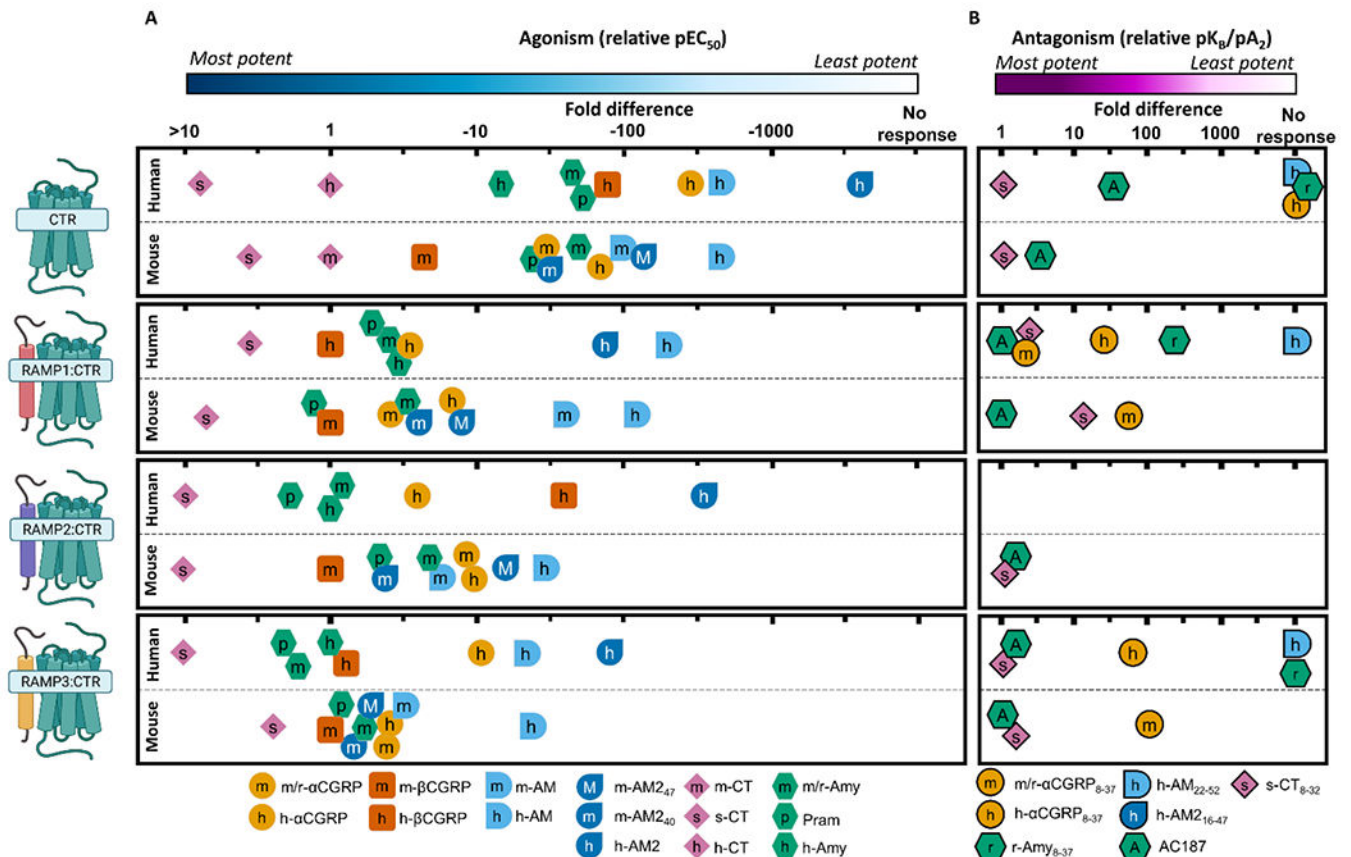
**Figure 4.**

Concentration-response curves showing antagonism of m/r- $\alpha$ CGRP (A, D, E), m-AM (B and C), m/r-Amy (F, G, H, I), and m-CT (J, K, L, M) by various peptide antagonists in Cos7 cells. Cells were transfected with m-CLR:m-RAMP1 (A), m-CLR:m-RAMP2 (B), m-CLR:m-RAMP3 (C), m-CTR:m-RAMP1 (D, G, K), m-CTR:m-RAMP3 (E, I, M), m-CTR:pcDNA (F, J), or m-CTR:m-RAMP2 (H, L). Data are the mean  $\pm$  s.e.m. of at least five independent experiments, except for m/r- $\alpha$ CGRP<sub>8-37</sub> in panel B which is  $n = 4$  (for individual  $n$  numbers, see Table 2).



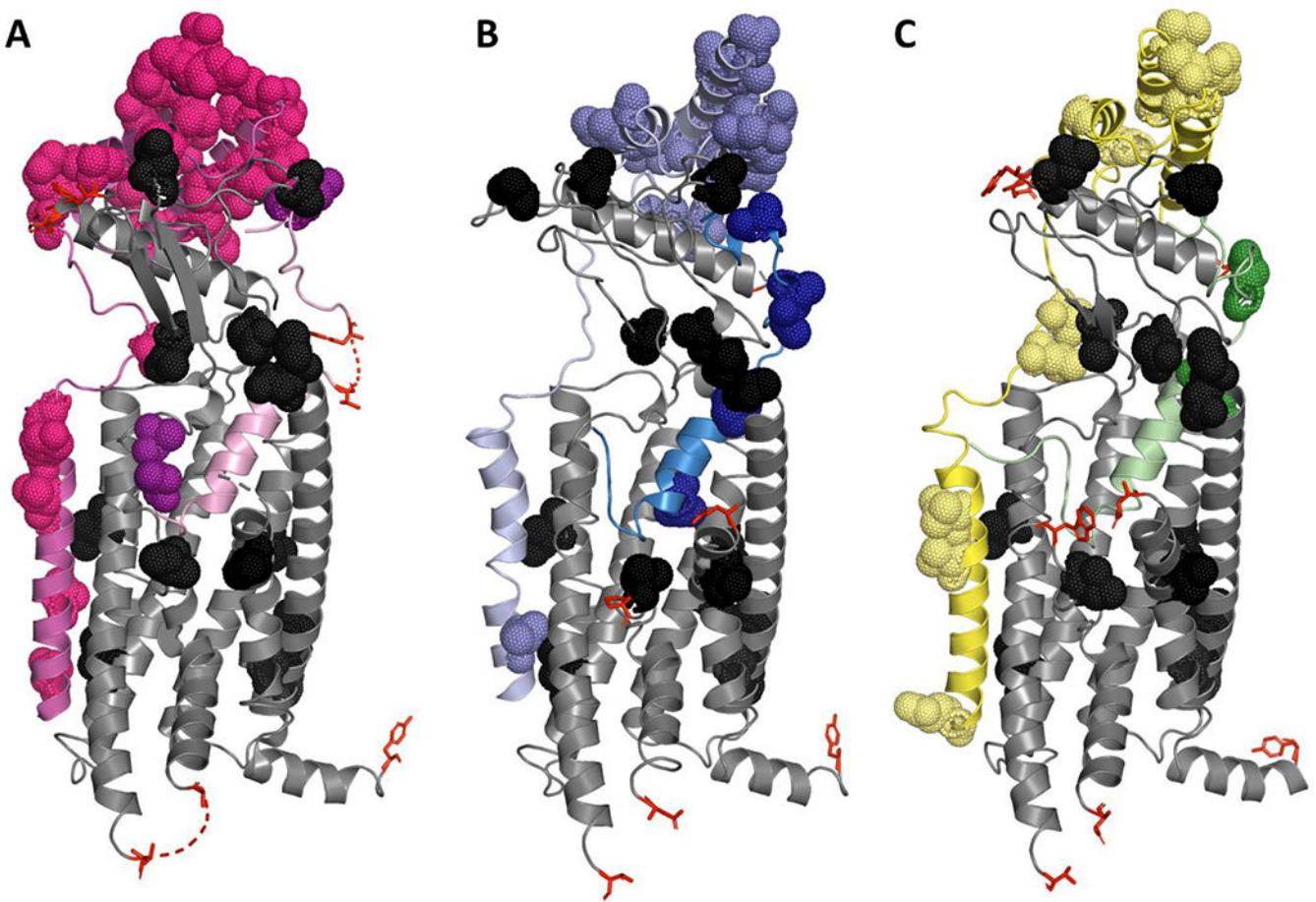
**Figure 5.**

Relative potency of peptide agonists (A) or antagonists (B) at CLR-based receptors from human and mouse, to enable comparison of relative ligand profiles at each receptor between species. Agonist potency is pEC<sub>50</sub> and antagonism is pK<sub>B</sub>/pA<sub>2</sub> values. For agonism, the potency of each peptide is displayed relative to the most potent species matched peptide at each receptor, for antagonism the potency is displayed relative to the most potent antagonist. Each tick represents half a log unit. It is important to note that the absolute potencies are not captured in this figure. This presentation approach was taken because absolute agonist potency is system-dependent, and studies are more easily compared when using relative potency. Data for mouse receptors is from this paper, Husmann et al. (2003), and Koller et al. (2004); data for human receptors is collated from Hay et al. (2018) and Garelja et al. (2020), and updated to include Gingell et al. (2020), Ghanizada et al. (2021b), and Hendrikse et al. (2020). Receptor figures created with [BioRender.com](https://www.biorender.com).



**Figure 6.**

Relative potency of peptide agonists (A) or antagonists (B) at CTR-based receptors from human and mouse, to enable comparison of relative ligand profiles at each receptor between species. Agonist potency is pEC<sub>50</sub> and antagonism is pK<sub>B</sub>/pA<sub>2</sub>. For agonism, the potency of each peptide is displayed relative to the most potent species matched peptide at each receptor, for antagonism the potency is displayed relative to the most potent antagonist. The antagonist data for CTR alone is antagonism of species matched CT, while the antagonist data for CTR:RAMP complexes is derived from antagonism of Amy or CGRP. Species matched CT was omitted from receptors comprising CTR in complex with RAMPs because these peptides exert their effect through free CTR, rather than RAMP complexed CTR (Hay et al., 2018). Each tick represents half a log unit. It is important to note that the absolute potencies are not captured in this figure. This presentation approach was taken because absolute agonist potency is system-dependent, and studies are more easily compared when using relative potency. Data for mouse receptors is from this paper; data from human receptors is collated from Hay et al., (2018), and updated to include Ghanizada et al., (2021b), Gingell et al., (2020), Hendrikse et al., (2020), and Yule et al. (2019). Receptor figures created with [BioRender.com](https://www.biorender.com).



**Figure 7.** Structural models of (A) h-αCGRP (pink) bound to h-CLR:h-RAMP1 (grey:magenta, respectively), (B) h-AM (blue) bound to h-CLR:h-RAMP2 (grey:pale blue, respectively), and (C) h-AM2 (green) bound to h-CLR:h-RAMP3 (grey:yellow, respectively). Amino acids that differ between the human and mouse sequence are shown as black dots. Where there is an amino acid difference that is not captured in the deposited structure, the amino acids that flank the difference are shown as red sticks. The pdb codes for (A), (B), and (C) are 6E3Y, 6UUN, and 6UVA, respectively.

pEC<sub>50</sub> and E<sub>max</sub> values for CT-family peptides stimulating cAMP production in Cos7 cells transfected with combinations of m-CTR, m-CLR, m-CLR, and m-RAMPs.

Table 1.

Agonist	m-CLR:m-RAMP1		m-CLR:m-RAMP2		m-CLR:m-RAMP3		m-CTR:pcDNA		m-CTR:m-RAMP1		m-CTR:m-RAMP2		m-CTR:m-RAMP3	
	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>	pEC <sub>50</sub>	E <sub>max</sub>
m-CT	NC	NC	NC	NC	NC	NC	9.42 ± 0.16	32.63 ± 5.25	9.83 ± 0.14	27.14 ± 3.40	9.45 ± 0.30	31.28 ± 6.56	9.04 ± 0.21	36.98 ± 9.91
s-CT	NC	NC	NC	NC	NC	NC	10.05 ± 0.14	24.80 ± 4.21	10.02 ± 0.18	22.45 ± 2.96	10.13 ± 0.11	24.59 ± 1.95	10.14 ± 0.11	25.07 ± 2.11
m/r-Amy	7.61 ± 0.19	22.17 ± 2.76	6.88 ± 0.05	13.71 ± 1.80	7.83 ± 0.15	22.17 ± 2.76	7.83 ± 0.10	23.77 ± 2.83	8.56 ± 0.11	21.56 ± 3.88	7.76 ± 0.10	28.81 ± 4.99	8.70 ± 0.17	29.89 ± 5.98
Pram	7.27 ± 0.13	18.95 ± 2.80	6.49 ± 0.13	8.18 ± 1.92	7.59 ± 0.18	18.95 ± 2.79	8.11 ± 0.08	29.57 ± 3.03	9.25 ± 0.12	30.83 ± 4.24	8.11 ± 0.09	28.71 ± 4.11	8.91 ± 0.20	37.3 ± 4.29
m/r-αCGRP	10.16 ± 0.12	19.11 ± 1.35	7.15 ± 0.20	11.69 ± 1.81	8.66 ± 0.10	19.11 ± 1.34	7.98 ± 0.07	29.66 ± 7.24	8.64 ± 0.06	26.39 ± 4.34	7.53 ± 0.13	27.85 ± 6.30	8.59 ± 0.15	34.17 ± 6.51
m-βCGRP	10.05 ± 0.17	30.29 ± 8.14	7.33 ± 0.12	20.89 ± 4.58	8.91 ± 0.16	30.29 ± 8.13	8.77 ± 0.10	35.60 ± 10.90	9.07 ± 0.07	25.47 ± 7.17	8.46 ± 0.13	25.18 ± 4.31	8.95 ± 0.11	32.52 ± 9.70
h-αCGRP	10.08 ± 0.11	13.46 ± 2.94	7.03 ± 0.06	11.89 ± 2.29	8.39 ± 0.08	13.46 ± 2.93	7.54 ± 0.11	16.69 ± 1.84	8.22 ± 0.08	20.58 ± 2.02	7.50 ± 0.08	17.26 ± 2.33	8.61 ± 0.20	18.21 ± 2.24
m-AM	8.18 ± 0.11	30.86 ± 7.96	8.81 ± 0.07	19.43 ± 5.82	9.33 ± 0.16	30.86 ± 7.95	7.43 ± 0.12	20.94 ± 5.71	7.56 ± 0.07	16.65 ± 1.14	7.71 ± 0.14	18.65 ± 1.87	8.45 ± 0.14	24.55 ± 2.82
h-AM	7.90 ± 0.13	28.88 ± 3.08	8.80 ± 0.16	14.70 ± 3.02	9.26 ± 0.14	28.88 ± 3.07	6.72 ± 0.08	14.67 ± 2.91	7.01 ± 0.12	15.88 ± 2.79	7.00 ± 0.10	15.87 ± 1.54	7.56 ± 0.10	19.92 ± 2.24
m-AMZ47	8.35 ± 0.10	34.42 ± 2.84	7.98 ± 0.04	21.10 ± 2.49	8.88 ± 0.10	34.42 ± 2.83	7.25 ± 0.07	18.95 ± 2.11	8.16 ± 0.14	17.5 ± 2.07	7.37 ± 0.10	18.78 ± 3.13	8.67 ± 0.12	21.17 ± 1.95
m-AMZ40	9.02 ± 0.06	30.30 ± 6.09	8.43 ± 0.13	21.47 ± 4.55	9.11 ± 0.18	30.29 ± 6.08	7.91 ± 0.17	17.37 ± 0.92	8.52 ± 0.17	19.31 ± 1.88	8.09 ± 0.13	17.71 ± 2.77	8.78 ± 0.06	25.8 ± 3.19

Data are the mean ± s.e.m. of *n* independent experiments. E<sub>max</sub> values are presented as nM values of cAMP. pEC<sub>50</sub> and E<sub>max</sub> values of individual peptides were compared across receptors using unpaired one-way ANOVA with Tukey's post-hoc test comparing the results at a receptor to the results at each other receptor. In this table significance is shown only relative to a reference receptor (m-CLR:m-RAMP1 for m/r-αCGRP, m-βCGRP, and h-αCGRP; m-CLR:m-RAMP2 for m-AM and h-AM, and m-CLR:m-RAMP3 for m-AMZ40 and m-AMZ47; m-CTR:pcDNA for s-CT and m-CT, and m-CTR:m-RAMP1 for m/r-Amy and Pram), significance is indicated by an asterisk (\*). pEC<sub>50</sub> and E<sub>max</sub> values were also compared within receptors using unpaired one-way ANOVA with Tukey's post-hoc test, comparing the mean value of an individual peptide at a given receptor to each other peptide at the same receptor. In this table significance is shown only relative to a reference peptide at each

receptor (m/r- $\alpha$ CGRP at m-CLR:m-RAMP1, m-AM at m-CLR:m-RAMP2/3, m-CT at m-CTR:pcDNA, and m/r-Amy at m-CTR:m-RAMP1/2/3), significance is indicated by a hash symbol (#). To view this data in graphical format, see Figure S 8.

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**Table 2.**

pA<sub>2</sub> values for antagonism at m-CTR-based receptors in transfected Cos7 cells.

Antagonist	Agonist	m-CTR:pcDNA		m-CTR:m-RAMP1		m-CTR:m-RAMP2		m-CTR:m-RAMP3	
		pA <sub>2</sub>	<i>n</i>	pA <sub>2</sub>	<i>n</i>	pA <sub>2</sub>	<i>n</i>	pA <sub>2</sub>	<i>n</i>
sCT <sub>8-32</sub>	m-CT	9.17 ± 0.17	5	8.74 ± 0.27	5	8.79 ± 0.17	5	8.89 ± 0.27	5
	m/r-Amy	8.44 ± 0.18 <sup>^</sup>	5	7.47 ± 0.19 <sup>^*</sup>	5	8.75 ± 0.05	5	8.41 ± 0.12	5
AC187	m-CT	8.59 ± 0.13	5	8.37 ± 0.19	6	8.34 ± 0.11	5	8.35 ± 0.26	5
	m/r-Amy	8.07 ± 0.12 <sup>^*</sup>	6	8.76 ± 0.12	6	8.57 ± 0.09	6	8.34 ± 0.10	6
	m/r-αCGRP	-	-	8.26 ± 0.14	5	-	-	8.65 ± 0.08 <sup>*</sup>	5
m/r-αCGRP <sub>8-37</sub>	m/r-Amy	-	-	6.88 ± 0.17	5	-	-	-	-
	m/r-αCGRP	-	-	6.80 ± 0.08	5	-	-	6.49 ± 0.16	6

Data are the mean ± s.e.m. of *n* independent experiments. An \* (asterisk) indicates a significant difference between the pA<sub>2</sub> of an antagonist across receptors (i.e. across a row). This explores whether a single antagonist is more effective at antagonising an individual agonist across different receptors. Analyses were performed using one-way ANOVA with Tukey's post-hoc test, comparing the pA<sub>2</sub> at each receptor to every other receptor, the exception being those with only two receptors, which were analysed by unpaired Student's *t*-test. In this table, differences are shown relative to a reference receptor. The reference receptor for sCT<sub>8-32</sub> was m-CTR:pcDNA, for AC187 and m/r-αCGRP<sub>8-37</sub> the reference was m-CTR:m-RAMP1. A ^ (caret) indicates a significant difference between the pA<sub>2</sub> of an antagonist between agonists (columns, within antagonists). Analyses were performed using unpaired Student's *t*-tests, except for AC187 at m-CTR:m-RAMP1 and m-CTR:m-RAMP3, which were analysed by one-way ANOVA with Tukey's post-hoc test comparing the pA<sub>2</sub> for an agonist to each other agonist. For sCT<sub>8-32</sub> and AC187, differences are shown compared to m-CT. There was no significant difference between the ability of m/r-αCGRP<sub>8-37</sub> to antagonise m/r-Amy and m/r-αCGRP.