Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review:"X"

Running title: Pharmacology of the calcitonin/CGRP family

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Abbreviations

AM; adrenomedullin; AM2/IMD; adrenomedullin 2/intermedin; AMY; amylin receptor; CGRP; calcitonin gene-related peptide; CRSP; calcitonin receptor stimulating peptide; CT; calcitonin; calcitonin receptor; CTR; calcitonin receptor-like receptor; CLR; ECD; extracellular domain; ECL; extracellular loop; GPCR; G protein-coupled receptor; GRK; GPCR kinase; h; human; r; rat; receptor activity-modifying protein; RAMP; TMD; transmembrane domain

Keywords

Adrenomedullin, amylin, calcitonin, CGRP, migraine, diabetes

Abstract

The calcitonin/calcitonin gene-related peptide (CGRP) family of peptides includes calcitonin, α and β CGRP, amylin, adrenomedullin (AM) and adrenomedullin 2/intermedin (AM2/IMD). Their receptors consist of one of two G protein-coupled receptors (GPCRs), the calcitonin receptor (CTR) or the calcitonin receptor-like receptor (CLR). Further diversity arises from heterodimerisation of these GPCRs with one of three receptor activity-modifying proteins (RAMPs). This gives the CGRP receptor (CLR/RAMP1), the AM_1 and AM_2 receptors (CLR/RAMP2 or RAMP3) and the AMY_1, AMY_2 and AMY_3 receptors (CTR/RAMPs1-3 complexes, respectively). Apart from the CGRP receptor, there are only peptide antagonists widely available for these receptors and these have limited selectivity, thus defining the function of each receptor *in vivo* remains challenging. Further challenges arise from the probable co-expression of CTR with the CTR/RAMP complexes and species-dependent splice variants of the CTR ($CT_{(a)}$ and $CT_{(b)}$). Furthermore, the $AMY_{1(a)}$ receptor is activated equally well by both amylin and CGRP and the preferred receptor for AM2/IMD has been unclear. However, there are clear therapeutic rationales for developing agents against the various receptors for these peptides. For example many agents targeting the CGRP system are in clinical trials and pramlintide, an amylin analogue, is an approved therapy for insulin-requiring diabetes. This review provides an update on the pharmacology of the calcitonin family of peptides by members of the corresponding subcommittee of the International Union of Basic and Clinical Pharmacology and colleagues.

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1. Introduction to the family, their receptors and current classification

The peptides calcitonin (CT), calcitonin gene-related peptide (CGRP), amylin, adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2/IMD) form a family of related peptides (Figure 1). CGRP exists in two forms, αCGRP and βCGRP; in some species, βCGRP is not found but another peptide, CT receptor stimulating peptide (CRSP) is found instead (Katafuchi *et al*., 2009). There has been considerable expansion of the family in fish, such that there are two forms of CT and 5 forms of AM (Watkins *et al*., 2013).

The peptides themselves, whilst showing only limited sequence homology, are related structurally by possession of a disulphide-bonded N-terminus, a region with strong alpha-helical tendencies and a C-terminus structured around a beta-turn and a C-terminal amide. The peptides range in length from 32 (CT) to 52/53 amino acids (AM, AM2/IMD). In the latter two peptides, the first residues N-terminal to the disulphide bond do not appear to be necessary for biological activity and the AM and AM2/IMD peptides can be considered as functional ~40 amino acid peptides (Bower and Hay., 2016, Watkins *et al.*, 2013, Hong *et al.*, 2012, Bailey and Hay 2006).

The peptides have a range of biological activities. CT, the first to be discovered, is a hormone produced by C cells of the thyroid, whose role is to reduce plasma calcium and promote bone formation (Findlay and Sexton, 2004), although CT-deficient mice show a paradoxical inhibition of bone formation due to enhanced sphingosine-1-phosphate production (Keller *et al*., 2014). Amylin is produced by the pancreas and functions as a satiety hormone, regulating nutrient intake but may also have other roles as recently reviewed (Hay *et al*., 2015). CGRP and AM are both potent vasodilators (Russell *et al*., 2014, Hinson *et al*., 2000). CGRP is a neuromodulator found in sensory neurons; it plays an important role in neurogenic inflammation (i.e where sensory nerves release mediators that promote inflammation); in this case CGRP causes vasodilation and promotes fluid exudation from blood vessels. AM is chiefly found in endothelial cells; it is important both in vascular homeostasis and also angiogenesis and lymphangiogenesis. AM2/IMD is also found in vascular endothelial cells and probably has complementary roles to AM, although much about this peptide remains unclear. Each peptide appears to have both peripheral and central actions, though due to the complexity of this peptide-receptor system, it is not yet clear which effects are physiological versus pharmacological or which receptors are responsible for many effects.

The peptides all act at class B G protein coupled receptors (GPCRs). There are seven distinct receptors for the peptides in mammals (excluding splice variants), but only two GPCRs; the CT receptor (CTR) and CT receptor-like receptor (CLR, known as CL or CRLR in older literature). The additional functional receptors arise from the association of CTR or CLR with receptor activity-modifying proteins (RAMPs) (McLatchie *et al*., 1998). There are three RAMPs. These each have an N-terminus of around 100-120 amino acids, a single transmembrane domain and a C-terminus of around 10 residues (Hay and Pioszak, 2016). The receptors are shown in Figure 2.

Like other class B GPCRs, activation of CLR and CTR follows the two-domain model, where this is achieved by binding of the C-terminus of the peptide to the extracellular domain (ECD) of the receptor, contributing to the overall affinity of the peptide. The peptide N-terminus binds to the transmembrane domain (TMD) of the receptor. The receptors for the CT/CGRP family preferentially signal through Gs and cAMP production, although other signal transduction pathways may be activated (Walker *et al*., 2010). As further work characterising the signalling of these receptors emerges, it will be important to consider how the pharmacology of these receptors compares at different signalling pathways.

The current scheme for receptor classification may be found on the IUPHAR/BPS Guide to Pharmacology website (http://www.guidetopharmacology.org/) at http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=11, and is shown in Figure 2. This is reviewed each year and is fully annotated with current references (Hay and Poyner 2017, Alexander *et al.,* 2015). Readers are referred to this for details of classification and also for information on receptor distribution. These pages mainly consider human receptors and so this information may not automatically apply to other species, where there are frequently differences in pharmacology. The structure-function relationships of RAMPs, CGRP and amylin have been recently considered elsewhere (Bower and Hay, 2016, Watkins *et al*., 2013, Hay and Pioszak, 2016), as has the clinical pharmacology of CGRP antagonists and antibodies (Karsan and Goadsby, 2015, Hou *et al*., 2017, Tso and Goadsby, 2017). In this review, the intention is to explore areas where there are significant gaps in our understanding, to guide research in this field.

2. Pharmacology

The current classification of the seven receptors is based on work done shortly after the discovery of the RAMP family (McLatchie *et al*., 1998, Christopoulos *et al*., 1999, Muff *et al*., 1999). The CLR by itself will not reach the cell surface in any significant amount and does not respond to any known ligand. With RAMP1 it becomes the CGRP receptor (i.e. CLR/RAMP1). Association with the other two RAMPs gives AM receptors; the AM_1 receptor with RAMP2 (CLR/RAMP2) and the AM_2 receptor with RAMP3 (CLR/RAMP3) (Figure 2, Figure 3). AM2/IMD shows a preference for the AM_2 receptor; this is discussed further below.

The CTR, by itself, preferentially responds to CT. The CTR can also associate with the three RAMPs to give AMY_1, AMY_2 and AMY_3 receptors (Figure 2). As their names suggest, these respond to amylin (Hay *et al*., 2015; Poyner *et al*., 2002). There are however a number of important extra considerations. The CTR exists as a number of splice variants and these are species dependent. The most significant of these for the human receptor are the absence ($CT_{(a)}$) or presence ($CT_{(b)}$) of a 16 amino acid insert in the first intracellular loop; this impairs coupling of the CTR to Gq whilst making little difference to Gs coupling. Thus in turn gives (a) and (b) subtypes of each of the AMY receptors (Moore *et al.*, 1995, Poyner *et al.*, 2002). Secondly, the CTR can express at the cell surface on its own so in transient expression systems, it is highly likely that there will be mixed populations of CTR/RAMP complexes and CTR alone. This makes it very difficult to interpret the action of CT at AMY receptors in functional assays, as CT will produce a strong cAMP response via the CTR that is inevitably present. This is illustrated in Figure 4 at the $AMY_{1(a)}$ and $AMY_{3(a)}$ receptor transfected into Cos7 cells (Hay *et al.*, 2005). At both of these receptors CT fails to displace ^{125}I-amylin indicating that CT and amylin do not share a common receptor (Figure 4A and B). However, at both receptors CT stimulates a potent cAMP response (Figure 4C and D). This disconnect between binding and function could be explained by the presence of free CTR in these cells. The complex between CTR and RAMP2 is particularly difficult to observe, and depends on the cell type used, and so the pharmacology of this receptor is poorly explored.

Many class B GPCRs form heterodimers. This does not seem to have been addressed in any published study for CTR or CLR. For CLR the requirement for a RAMP may mitigate against this. For CTR, homodimerisation is well described (Harikumar *et al*., 2010); the main ligand responsive species may be a dimer, with G protein binding causing monomer formation (Furness *et al.*, 2016).

For most receptors, the main pharmacological tools for their characterisation are the peptide agonists themselves and N-terminally truncated peptides that usually act as antagonists. For some combinations of peptides and receptors there is reasonable selectivity; thus at the AM receptors there is a preference for AM over CGRP for ligand binding and cAMP production (Figure 3). However, over the entire family it is difficult to use these agents to fully distinguish between receptors (Bailey and Hay, 2006, Hay *et al*., 2005). For CLR-based receptors, non-peptide antagonists are also available (Salvatore *et al*., 2006); those of the "gepant" class bind to the receptor ECD at the interface between RAMP1 and CLR and have better selectivity than peptide antagonists (Moore and Salvatore, 2012), although they still need to be used with care as they can also block AMY_1 receptors (Walker *et al*., 2017, Hay and Walker, 2017).

2.1 Heterogeneity in CGRP-responsive receptors

The early literature on CGRP receptors was dominated by discussion of heterogeneity. Many responses could be antagonised by $CGRP_{8-37}$, with a pA_2 of about 8 on human and rat cells. By contrast, in a number of model systems, typified by the rat vas deferens, CGRP agonism is relatively resistant to $CGRP_{8-37}$. It was suggested that CGRP was acting via another receptor; the $CGRP_2$ receptor. Molecular cloning demonstrated that the "$CGRP_1$" receptor corresponds to the CLR/RAMP1 complex and it has been suggested that the "$CGRP_2$" receptor represented the action of CGRP at the various AM and AMY receptors (Hay *et al*., 2008). The high potency of CGRP in functional (cAMP) and binding assays at the $AMY_{1(a)}$ receptor and the $AMY_{1(b)}$ receptor was noted in previous studies (Udawela *et al*., 2008, Tilakaratne *et al*., 2000, Hay and Walker, 2017, Hay *et al*., 2006, Leuthauser *et al*., 2000). More recent work has confirmed that the $AMY_{1(a)}$ receptor can respond as well to CGRP as it does to amylin (Walker *et al*., 2017, Walker *et al*., 2015, Hay and Walker, 2017) (Figure 5). Even more importantly, there is evidence that *in vivo*, CGRP may exert effects by activating AMY_1 receptors. This has potentially important implications for understanding CGRP biology and for using antagonists; it may be necessary to use agents that block both CLR/RAMP1 and CTR/RAMP1 to fully antagonise the effects of CGRP *in vivo* (Walker *et al*., 2015). Where high concentrations of non-peptide antagonists are used, this may already be the case because these show only limited selectivity between CGRP and AMY_1 receptors (Hay and Walker, 2017).

The classification of CLR/RAMP1 as the CGRP receptor does not rule out the possibility of other endogenous CGRP receptors, such as AMY_1. The ongoing interest in the CGRP system as a drug target in migraine makes it especially important to remember the early reports of

functional heterogeneity which in many cases still do not have a molecular correlate (Hay *et al*., 2008). Perhaps unfortunately, the name AMY_1, does not easily lend itself to an obvious role in CGRP biology. The dual activation of this receptor by both CGRP and amylin creates problems for nomenclature. There is insufficient information regarding the location and function of this receptor *in vivo* either as a CGRP or amylin receptor at the present time. Readers are urged to consider this receptor both in amylin and CGRP biology to assist with refining receptor nomenclature.

2.2 Endogenous agonists

2.2.1 AM2/IMD

AM2/IMD remains poorly understood. It has a wide range of effects on the cardiovascular system, adipose tissue and macrophages and the kidney. It increases prolactin release and in the CNS it reduces food intake and causes activation of the sympathetic nervous system (Zhang *et al*., 2017, Hong *et al*., 2012). It is sometimes reported to be more potent *in vivo* than AM and the distribution of its mRNA is distinct from that of AM, being preferentially expressed in the thyroid and kidney, compared to the placenta and adipocytes where AM mRNA is most highly expressed (Figure 6). When reviewing the current data at human CLR-based receptors, AM2/IMD appears to be most potent at the AM_2 receptor (Figures 3 and Figure 7), being equipotent to AM at this receptor. Equal potency for AM and AM2/IMD at the AM_2 receptor has also been reported for rat and mouse receptors (Halim and Hay, 2012). This profile is different to the AM_1 receptor where AM has higher potency than AM2/IMD (Figures 3 and 7), however these data are only available for cloned human receptors. AM2/IMD can also activate CTR and AMY receptors but there is much less data. When comparing data between species, the activity of AM2/IMD may be greater at rat $AMY_{3(a)}$ receptors, compared to the human receptor but this needs more investigation (Bailey *et al*., 2012, Hay *et al*., 2005). However, the pattern appears similar to the situation with AM, which may also have more activity at rat, compared to human $AMY_{3(a)}$ receptors (Bailey *et al*., 2012). It has been suggested that a distinct receptor for AM2/IMD may exist (Hashimoto *et al*., 2007; Taylor *et al*., 2006). Given its affinity at several CLR and CTR-based receptor complexes, we consider this to be unlikely and that one or more existing complexes are likely to mediate the effects of this peptide, although we acknowledge that some results in the literature are difficult to explain (Taylor *et al*., 2006). The lack of useful antagonists makes this a continuing problem. Furthermore, signalling bias has been little explored at these receptors and it is unclear what distinctive features may come

from AM/IMD activating each of the individual receptors. Thus the pharmacology and physiology of AM2/IMD remain somewhat elusive.

The actions of AM2/IMD are further complicated due to its metabolism, where it can potentially exist in a number of N-truncated forms, all of which retain the key disulphide bond which is considered essential for full activity. It remains far from clear what the most physiologically important form of the peptide is and what are the implications of the potential metabolism for its activity (Hong *et al*., 2012, Zhang *et al*., 2017). Another AM, AM5 has also been reported in some mammals but its actions are not well understood (Takei *et al*., 2008).

2.2.2 βCGRP and CRSP

βCGRP is encoded by a different gene to αCGRP and has a different pattern of expression, being particularly prominent in the enteric nervous system. This has led to the view that βCGRP has restricted expression but this is not necessarily the case, and is found throughout the CNS (Amara *et al*., 1985). It is found in only a small number of species, chiefly rodents and primates. The differences between the forms are species-dependent (Figure 1). In rat βCGRP, there are two differences at positions 17 and 35, compared to rat αCGRP. In humans, there are three differences, at positions 3, 22 and 25. There are suggestions of subtle differences in receptor activity of human and rat α and β CGRP, although this has not been explored in any detail (Bailey and Hay, 2006, Bailey *et al*., 2012). In other species (but not humans), a second CGRP-like peptide named CRSP is expressed. There is an interesting paradox with this peptide. Its sequence clearly marks it as a CGRP variant (Figure 1); however, it is reported to activate CTR-based receptors and to have very little activity on CLR-based receptors, including the CGRP receptor (Katafuchi *et al*., 2009, Katafuchi *et al*., 2004, Katafuchi and Minamino, 2004, Katafuchi *et al*., 2003). The reason for this is not known and this peptide would benefit from further study. However, matching sequence to pharmacology for these peptides and complex receptors is not an easy task. CGRP and amylin are the most closely related of the CT family of peptides in mammals, yet CGRP activates CTR and CLR-based receptors with RAMP1, whereas amylin is much more selective for CTR/RAMP complexes. The nature of the C terminal amino acid seems like a good place to look to explain this, with Phe37 in CGRP and Tyr37 in amylin, yet AM shares a C-terminal Tyr with amylin but has a strong preference for CLR/RAMP complexes.

3. Developments with agonists

Recent attention has focussed on the development of metabolically stable peptide agonists because the members of this peptide family can be metabolised by a range of peptidases and have several cleavage sites (Kim *et al*., 2013, Schonauer *et al*., 2016). For CGRP, a fatty acid attached to a serine at position 1 of human αCGRP gives an analogue with markedly prolonged *in vivo* biological activity (Nilsson *et al*., 2016). AM has been modified by palmitoylation, lactam cyclisation and N-methylation to produce an analogue with prolonged half-life (Schonauer *et al*., 2016). For pramlinitide, a non-aggregating analogue of human amylin, glycosylation has been used as an approach to enhance stability (Yule *et al*., 2016, Kowalczyk *et al*., 2014, Tomabechi *et al*., 2013). The key to these peptide mimetic development programmes is the identification of sites on the peptide that allow derivatisation without compromising either receptor binding or activation. In principle, this will be facilitated by the availability of structures showing the peptides bound to their cognate, full-length receptors, although the difficulty of predicting where an elongated substituent such as a fatty acid might bind should not be underestimated. In principle, similar problems might be anticipated in the preparation of other derivatives such as fluorescent peptides (Cottrell *et al*., 2005) where their use at relatively high concentrations may be needed to counter reduced affinity. The activity of analogues is usually only tested against cAMP production, leaving open the formal possibility that they have altered signalling bias.

Salmon CT has historically been used to treat Paget's disease and osteoporosis in people (Gennari and Agnusdei, 1994). However, due to side effects, relative efficacy compared to other treatments and lack of cost effectiveness, its use has declined. Particularly concerning was the suggestion that salmon CT may increase the risk of metastases. However, in a recent meta-analysis the relationship was described as weak and there is no clear biological mechanism (Wells *et al.*, 2016). Given the clinical usage, it is unsurprising that salmon CT has been explored in other disorders. Numerous studies have suggested that salmon CT could treat metabolic disorders by lowering body weight, elevating energy expenditure, limiting food intake and improving glucose handling in rats (Lutz *et al*., 2000, Eiden *et al*., 2002, Wielinga *et al*., 2007, Feigh *et al*., 2012, Feigh *et al*., 2014). Recently a number of CT mimetics, known by "KBP" codes e.g. KBP-042, KBP-088 and KBP-089 have been described (Patent WO 2015/071229). These molecules are reported to maintain the high efficacy of salmon CT, whilst improving tolerability in rats (Gydesen *et al*., 2017a). A similar strategy appears to have been

employed for the development of Davalintide which displays enhanced effects to reduce body weight and food intake compared to amylin in rats (Mack *et al*., 2010). Development of Davalintide has apparently been discontinued. Interestingly, the KBP compounds maintain the long-acting ability of salmon CT to stimulate signalling in cell culture models (Gydesen *et al*., 2016, Andreassen *et al*., 2014b). The receptor pharmacology of the KBP peptides has not been extensively studied and so far the peptides have only been tested at CTR, AMY_3 and CGRP receptors (Andreassen *et al*., 2014a, Gydesen *et al*., 2016, Gydesen *et al*., 2017a). They are reported to activate both CT and AMY_3 receptors but not CGRP receptors, similar to salmon CT and are known as "DACRAs" – dual amylin and CT receptor agonists; salmon CT is a natural DACRA. Their activity at other AMY receptors has not been tested but close sequence similarity to salmon CT of any peptide would make it likely that they show potent agonism at all CTR/RAMP complexes. The receptor pharmacology analysis of these peptides has relied on purchased stably transfected cell lines, which are not especially well characterised. It is not clear how much activity of the ligands occurs via free CTR in this transfected cell system (Andreassen *et al*., 2014a, Gydesen *et al*., 2017b, Gydesen *et al*., 2017a, Gydesen *et al*., 2016). As noted above, to determine affinity at a CTR/RAMP complex, displacement of ^{125}I-amylin (or ^{125}I-CGRP for the AMY_1 receptor) is the most reliable measure of true AMY receptor affinity. Further pharmacological characterisation is required to validate the DACRA nomenclature and confirm the relative activity of these peptides at different receptor complexes.

The future for novel peptides may be to follow the lead for the GLP-1 receptor, where a ligand has been designed based on a crystal structure of the receptor (Jazayeri *et al*., 2017). A number of structures are available showing the ECDs of CLR/RAMP complexes or the CTR in complex with bound ligands (Table 1, Figure 8). Many of these have been reviewed (Hay and Pioszak, 2016). In addition, a cryo-electron microscopy structure of the complete CTR bound to Gs and CT has been published (Liang *et al*., 2017), but the ECD and bound ligand in this are poorly resolved and are not included in the deposited co-ordinates. Therefore, there is still some way to go before there is a complete picture to enable structure-based peptide agonist design for these receptors

A series of small molecule agonists for the CTR have been identified and their binding site is probably at the junction of the ECD and the transmembrane domain of the receptor (Dong *et*

al., 2009). They probably work allosterically but their pharmacology remains largely unexplored.

4. Developments with antagonists

A major advance in the pharmacology of CGRP receptors came with the "gepant" class of antagonists, typified by olcegepant (BIBN4096BS) and telcagepant (MK0974), which were developed as part of the global effort to develop drugs that inhibit CGRP action in migraine. These compounds have a high selectivity for CGRP as opposed to AM receptors because they bind to the interface between CLR and RAMP1. Telcagepant showed therapeutic efficacy in migraine and although the development of this particular molecule was halted, non-peptide CGRP receptor antagonists continue to be tested in clinical trials. The pharmacology of olcegepant and telcagepant has been extensively reviewed previously but a number of developments should be noted. Both antagonists showed marked species selectivity in favour of primate receptors, restricting their use as experimental tools. Work to develop further gepant-type compounds continues (Civiello *et al*., 2016, Tora *et al*., 2013, Crowley *et al*., 2015). The wider pharmacological characterisation of these compounds has not been extensively pursued, but there are some significant exceptions. A study of olcegepant, telcagepant, MK-3207 and rimagepant (BMS-927711) on rat mesenteric arteries have shown that they all behave as simple competitive antagonists with pA_2 values ranging from 8.8 (MK-3207) to 6.45 (telcagepant). They have similar affinities on mesenteric arteries and in binding assays to rat brain apart from rimagepant, which shows a 50-fold lower affinity to brain. The reasons for this discrepancy are unclear (Sheykhzade *et al*., 2017). The selectivity of olcegepant and telcagepant for human CGRP and $AMY_{1(a)}$ receptors has been compared at receptors transfected into Cos 7 cells. Surprisingly, for olcegepant acting on the $AMY_{1(a)}$ receptor, this depends on the pathway being measured; it is 5-fold more potent at blocking CGRP when CREB phosphorylation is measured compared to cAMP (Walker *et al*., 2017). Thus if cAMP is measured, olcegepant has over 100-fold selectivity for CGRP over $AMY_{1(a)}$ receptors; for CREB this drops to around a 25-fold selectivity. This is not seen to the same extent with telcagepant, nor is the differential antagonism observed at the CGRP receptor. The implications of this will be considered further below.

The development of the gepant antagonists has tended to draw attention away from other small molecule antagonists such as SB-273779 (Aiyar *et al*., 2001) and other compounds. These compounds show little selectivity between CGRP and AM receptors; the binding site for the

Merck compounds variously known as compound 4 or compound 16 appears to include part of the TMD and extracellular loop (ECL3), well away from the ECD interface between CLR and RAMP1 used by the gepants (Salvatore *et al*., 2006). It is possible that SB-273779 binds in a similar place. However, mutagenesis suggests that there are RAMP effects on ECL3 and so it may be possible to develop selective antagonists which bind to this region (Watkins *et al*., 2016).

There has been work to develop shortened peptide antagonists. A substituted version of the final 11 amino acids of CGRP has been reported to bind with a sub-micromolar affinity (Rist *et al*., 1998) and a crystal structure of this bound to the ECD of RAMP1 and CLR has been solved (Booe *et al*., 2015) (Table 1, Figure 8). Chimeras between $CGRP_{8-37}$, AM_{22-52} and $AM2/IMD_{16-47}$ produced analogues with novel specificities but whose activities remain difficult to understand (Robinson *et al*., 2009). Homology models of amylin receptors are facilitating the development of novel CTR and amylin receptor antagonists, based on the related CT family receptor ECD structures (Lee *et al*., 2016).

A recent development has been the use of antibodies to block the actions of CGRP, as an alternative to the use of classic antagonists for the therapy of migraine. The majority of these act against CGRP itself (Mason *et al*., 2017; Tso and Goadsby, 2017), but some success has been achieved with antibodies directed to the CGRP receptor, both in experimental models (Miller *et al*., 2016) and human studies (Shi *et al*., 2016; Tso and Goadsby, 2017).

5. The challenges of pharmacology in non-transfected cell systems

Whilst studies with transfected cells are essential for defining the pharmacology of individual receptor subtypes, they have some limitations. In particular, if pharmacology is influenced by cell-specific factors such as G proteins (see below) or accessory proteins, then this will only be properly revealed by experiments in the physiologically relevant cell. Even if the receptors are identical, differences in responses can be produced by their level of expression and factors such as differential expression of peptidases. There are particular considerations where CTR is expressed with RAMPs, as it is highly likely that both AMY receptors and free CTR will be present at the cell surface. This section provides some examples of pharmacology emerging from cells that endogenously express receptors and highlights some of the challenges of using "model" cell lines that endogenously express receptor components.

5.1. Primary cells

In cultured rat trigeminal neurons, CTR, CLR and RAMP1 are present, giving a particularly complex situation. The data suggest that different cells have either CLR or CTR, sometimes with RAMP1 so there are difficulties in comparing functional data of pooled responses to individual cells with one or more functional receptors. CGRP-mediated cAMP production is blocked by the CT and amylin receptor antagonist AC187 with a pA_2 appropriate to the $AMY_{1(a)}$ receptor. Antagonism of CGRP responses by olcegepant, however were consistent with the presence of the CGRP receptor, CLR/RAMP1, supporting the notion that two populations of CGRP-responsive receptors are present in these cells (Walker *et al*., 2015).

Somewhat similar complexities have been observed with rat embryonic dissociated spinal cord cells. In this case, CGRP, AM and AM2/IMD responses have been investigated in two separate studies. These cells express high affinity binding sites for both AM and CGRP and both peptides also produce cAMP, consistent with the presence of CGRP and AM receptors. A selection of antagonists were used to try and define the receptors that mediated cAMP responses to each agonist. The response of CGRP was effectively blocked by olcegepant. However, the data for AM and AM2/IMD are less straightforward to interpret (Takhshid *et al*., 2006). AM2/IMD showed biphasic high and low affinity displacement of bound ^{125}I-AM but monophasic high affinity displacement of ^{125}I-CGRP. Despite high affinity for the CGRP binding site, antagonism of AM2/IMD by olcegepant was weak, which is not consistent with AM2/IMD acting through a canonical CGRP receptor (Owji *et al*., 2008). It is likely that there are mixed populations of receptors, potentially within the same or different cells within these cultures, creating mixed pharmacology. It is possible that an amylin receptor could partially explain this. Indeed, using the same spinal cell system, amylin responses have also been studied. This highlights another mismatch between this endogenous system and transfected cells; the potency of $amylin_{8\text{-}37}$. In this study, it achieved a pA_2 of 7.94, which is far greater than the highest value achieved in transfected cell systems (rat) of 6.16 (Bailey *et al*., 2012). The reason for this is not known.

These few observations serve as examples that reflect the difficulty of working with systems that endogenously express one or more populations of receptors. A common problem is that it is difficult to test all of the different combinations of agonists and antagonists that are currently necessary to tease apart the pharmacology of these receptors. Therefore it is common that limited concentrations and ranges of pharmacological tools are used. This is of course a

consequence of using cells that are only available in small amounts, and the problem with generating very pure cultures. We have used the studies discussed in the preceeding paragraph because they are more helpful than many which use only a single concentration of agonist or antagonist in an "all or nothing" approach and thus cannot quantify parameters or define pharmacology in any meaningful way. For example, if CGRP were to be used in any study of rodent tissues or cells at 100 nM or greater concentration, it could potentially act through CGRP, AM_2, AMY_1 or AMY_3 receptors. Blockade of this response with 1 μM or greater $CGRP_{8-37}$ would not rule in or out any of these receptors because this concentration of antagonist can block all of these receptors. Hence, the concentrations and combinations of agents used are very important and further work is needed on *ex-vivo* cells, to establish the pharmacology that they display. Similar issues are often faced in cell lines.

5.2. Cell lines

Despite the challenges associated with endogenously expressed receptors, the SK-N-MC cell line (derived from a human neuroblastoma) has proven invaluable for understanding CGRP receptor pharmacology (Poyner *et al*., 1992). SK-N-MC cells have been extensively characterised and display pharmacology consistent with a functional CGRP receptor in transfected cells (Bailey and Hay, 2006). These cells have been used as a starting point for the pharmacological characterisation for several CGRP receptor antagonists (Moore and Salvatore, 2012). However, this model is not perfect. They reportedly express RAMP2 in addition to CGRP receptor components (CLR and RAMP1) and lose their CGRP receptor phenotype over passages (Choksi *et al*., 2002). Similarly, the human breast cancer cell line, T47D displays pharmacology consistent with a CTR and may represent an appropriate model for studying the pharmacology of this receptor (Muff *et al*., 1992, Zimmermann *et al*., 1997). Thus, SK-N-MC and T47D cells appear to be appropriate models to study the pharmacology of CGRP and CT receptors respectively. However, it should be noted that the compliment of downstream intracellular signalling proteins may be very different between these cell lines and a physiological tissue. They may therefore not be suitable for deciphering intricate biological activities.

Using a similar rationale other human cell lines including Col 29 (colonic epithelial) and MCF-7 (breast cancer) have been examined for their responsiveness to CGRP and related peptides (Hay *et al*., 2002, Zimmermann *et al*., 1997). However the pharmacology reported for these cell lines is not straight forward. Despite this, MCF-7 cells have been used in several studies

as an amylin receptor model (Sisnande *et al*., 2015, Shi *et al*., 2016). These cells are reported to express mRNA encoding two distinct splice variants of CTR, RAMP1 and RAMP3 (Chen *et al*., 1997, Ellegaard *et al*., 2010). MCF-7 cells therefore have the potential to contain functional CTR, AMY_1 and AMY_3 receptors. In these cells, CT stimulated cAMP production potently and ^{125}I-CT binding was not displaced by amylin or CGRP, suggesting that the CTR may be present. However, the potent cAMP response to amylin, coupled with the weak displacement of ^{125}I-amylin binding by CGRP relative to amylin is consistent with the AMY_3 receptor in transfected cell models (Zimmermann *et al*., 1997; Hay *et al.*, 2005). Yet, in functional assays, CGRP and amylin have similar potencies for the stimulation of cAMP production (Zimmermann *et al*., 1997; Ellegaard *et al.*, 2010). This suggests that these cells may contain functional AMY_1 and/or CGRP receptors. Curiously in direct contradiction to this, ^{125}I-CGRP was reported not to bind to MCF-7 cells under the conditions used suggesting that neither AMY_1 nor CGRP receptors were present (Zimmermann *et al*., 1997). It is not clear whether CLR is expressed by MCF-7 cells. MCF-7 cells highlight the difficulties involved in the study of this family of heterodimeric receptors where cells may express multiple interchangeable receptor components. Overall, MCF-7 cells likely contain a mixture of receptors and therefore are not recommended as a model system for this peptide family.

6. Receptor signalling

6.1. Biased signalling

Whilst it has been recognised for many years that CLR and CTR-based receptors signal through a variety of pathways, most work focussed on cAMP. Recently, work has started both to document the extent of signal bias and also to understand underlying mechanisms.

In transfected HEK293 cells, a significant Gi-component was observed to the response to AM at CGRP receptors and to CGRP at AM_1 and AM_2 receptors; this Gi-component was not seen with CGRP or AM acting at their cognate receptors. The Gi component was not seen in HEK293S cells, perhaps reflecting low expression of this G protein. The results are broadly consistent with data obtained in *Saccharomyces cerevisiae* engineered to express versions of Gs and Gi, where AM is more potent than CGRP acting through the CGRP receptor and CGRP is more potent than AM at the AM_1 receptor when measuring coupling to the Gi construct (Weston *et al*., 2016). Taken at face value, these results suggest that ligand bias can significantly change receptor selectivity. Caution is needed; the results have only been shown in a single, transfected, cell line; it remains to be established whether the effects are seen in

native cells. None-the-less, the data indicate the potential importance of biased signalling. This conclusion is reinforced by the pathway-selective antagonism previously discussed for olcegepant (Walker *et al*., 2017). In this study strong cell-dependent differences were seen in signalling with respect to ERK and p38. In rat trigeminal ganglion neuron cultures (which probably express both AMY_1 and CGRP receptors), rat αCGRP stimulated cAMP, CREB and p38 phosphorylation but not ERK. In Cos 7 cells transfected with human CGRP and $AMY_{1(a)}$ receptors, human αCGRP stimulated cAMP, CREB and ERK phosphorylation, but not p38.

There are processes such as stimulation of angiogenesis where cAMP-mediated mechanisms may be expected to be of minor importance compared to stimulation of pathways such as Akt (Nikitenko *et al*., 2006; Walker *et al*., 2010) and so biased agonists might be particularly useful, either to avoid or promote this effect. However, even here a contribution from cAMP is sometimes observed (Miyashita *et al*., 2003; Jin *et al*., 2008). There is a clear need to study signalling in physiologically relevant tissues and cells, to take into account all aspects of the inherent variability of receptor signalling.

An important contribution to understanding the mechanism behind biased signalling has come from comparing the effects of human and salmon CT on G protein activation. Human CT has a higher efficacy of the two ligands. The two agonists stabilise forms of CTR which differ in their ability to interact with Gs (Furness *et al*., 2016). The molecular explanation for this observation remains to be established.

6.2 Receptor internalisation and recycling

In both transfected HEK cells and rat mesenteric smooth muscle cells, following challenge with CGRP, the ligand/CLR/RAMP1 complex is targeted to the early endosome. Cleavage of CGRP by endothelin-converting enzyme-1 within this organelle leads to the release of beta arrestins and recycling of the CLR/RAMP1 complex to the cell surface (McNeish *et al*., 2012). There may be significant cell and tissue variability in this response. Thus it has been reported that in human microvascular endothelial cells, AM but not CGRP could cause internalisation of both AM and CGRP receptors (Nikitenko *et al*., 2006). Curiously, there is a report that over-expression of beta arrestin 1 or 2 both inhibit AM_1 receptor internalisation in HEK cells (Kuwasako *et al*., 2017), although activation of GRKs 5 and 6 cause the expected internalisation (Kuwasako *et al*., 2016).

Internalisation of the CTR is well characterised. The internalisation rate differs between the $hCT_{(a)}$ and $hCT_{(b)}$, perhaps linked to the different signalling profiles of these splice variants (Moore *et al.*, 1995). Interestingly it has been noted that the internalised CTR may continue to stimulate adenylate cyclase when stimulated by salmon but not human CT (Andreassen *et al*, 2014b). The C-terminus of the CTR plays an important role in determining the fate of the internalised receptor; the rabbit CTR can bind to the actin-binding protein filamin and this promotes recycling (Seck *et al.*, 2003); it is not known if this applies to other species as there are differences in the sequences of the C-terminus. In contrast, the fate and mechanisms of CTR trafficking in the presence of RAMPs is not known.

It seems likely that internalisation of CLR- and CTR-based receptors depends on a combination of the cell line, the agonist, the splice variant (for CTR) and the RAMP, with both the C-terminus (Bomberger *et al*., 2005a, Bomberger *et al*., 2005b) and the TMD (Kuwasako *et al*., 2012) of the RAMP containing important determinants. The significance, if any, of signalling directed by internalised CTR or CLR complexes is unexplored.

7. Unresolved questions, challenges and recommendations

Since the identification that RAMPs are required for formation of AM, CGRP and amylin receptors great strides have been made in understanding their biology (McLatchie *et al*., 1998; Christopolous *et al*., 1999). However, the heterodimeric nature of these receptors results in unique challenges in understanding the pharmacological and physiological roles and several complications or questions have arisen in the field.

1. *Which amylin and AM receptors are biologically relevant?* Although the combinations of CTR and RAMPs are described as amylin receptors, there is little protein data on the co-expression of these subunits in tissues and it is not clear whether one or all of these 'amylin receptors' form functional complexes *in vivo*. To address this question highly specific probes (antibodies, labelled ligand and/or antagonists) for CTR alone and individual CTR/RAMP complexes are required. A very similar situation exists for AM, when it is extremely difficult to distinguish pharmacologically between AM_1 and AM_2 receptors.

2. *Amylin receptor studies may be complicated by co-expression with free CTR.* CTR can reach the cell surface in the absence of a RAMP to form a receptor for CT or in the presence of a

RAMP to form an amylin receptor (Christopolous *et al*., 1999). The potential contribution of free CTR to the pharmacological profiles of amylin receptors in transfected cell models was discussed earlier in this review. Whether free CTR reaches the cell surface in the presence of RAMPs *in vivo* is not clear. It is possible that amylin receptors are commonly co-expressed with variable amounts of free CTR, complicating interpretation.

3. *Is the AMY_1 receptor responsible for physiological actions of CGRP?* Although the actions of CGRP are often assumed to be via the CGRP receptor in many cases a mixture of receptors may be involved or the receptor has simply not been identified. Given the high potency CGRP displays at the AMY_1 receptor and the widespread distribution of components for the AMY_1 receptor in the nervous system and peripheral tissues it would be surprising if CGRP did not act endogenously at the AMY_1 receptor (McLatchie *et al*., 1998; Oliver *et al*., 2001; Tolcos *et al*., 2003). This requires clarification.

4. *AM2/IMD has two different names and has activity at several receptors.* AM2 or IMD was initially described by two different research groups (Roh *et al*., 2004, Takei *et al*., 2004). No consensus has been reached regarding a single name for this peptide and it is now generally referred to by both names as AM2/IMD (Hong *et al*., 2012). It is important to note that intermedin is an alternative name for melanocyte-stimulating hormone and was also used to describe plant compounds (Li *et al*., 2008). The dual name for AM2/IMD may cause confusion, especially for those unfamiliar with the field. Given that IMD does not exclusively describe the AM relative, we recommend the use of AM2 or AM2/IMD but never just IMD. It is also important that when referring to the CLR/RAMP3 receptor complex, a subscript 2 character is used i.e. AM_2 receptor to clearly identify descriptions of the AM2 peptide and AM_2 receptor. It is likely that existing complexes of CLR and/or CTR with RAMPs can explain AM2/IMD actions without needing to invoke alternative receptors. Better antagonists are needed and an awareness of differences in pharmacology between species should be acknowledged.

5. *βCGRP has widespread expression.* βCGRP is often described as being predominantly expressed in the enteric nervous system. However, it is more correct to state that βCGRP is the predominant form of CGRP expressed in the enteric nervous system (Schutz *et al*., 2004). αCGRP and βCGRP are reportedly expressed throughout the nervous system with variable and overlapping distributions (Amara *et al*., 1985, Schutz *et al*., 2004). For example, αCGRP and βCGRP are both expressed in the dorsal root ganglia and dorsal horn of the spinal cord, whereas

αCGRP appears to be the predominant form expressed in the ventral horn of the spinal cord and at the neuromuscular junction (Schutz *et al*., 2004). Hence βCGRP should not be ignored as a widespread ligand for CGRP receptors.

8. Conclusions

The pharmacological classification of receptors for the CT/CGRP family as first proposed by NC-IUPHAR in 2002 remains a useful framework. There are however a number of conceptual challenges, many of which are highlighted in the previous section. Perhaps the most significant of these is that receptors of the AMY_1 type may be activated physiologically by CGRP. There is also a lack of agents that can discriminate between AM_1 and AM_2 receptors, or any of the AMY receptors. This represents a major barrier to our understanding of the *in vivo* role of these subtypes. Whilst it is likely that coupling to Gs and cAMP is the main transduction pathway for receptors of this family, a much better exploration of ligand bias is needed. The development of new pharmacological agents will be facilitated by our increased molecular understanding of the receptors within this family, drawing on insights from both structural and computational biology. As these become available, our understanding of the physiology of these peptides and their potential therapeutic uses will increase.

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 (Southan *et al*., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al*., 2015).

Conflicts of Interest

The authors declare no conflict of interest.

Statement of authorship

All authors analysed data and contributed to the writing.

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Table 1 Summary of structures of the of CTR and CLR/RAMP complexes with bound ligands.

RAMP	GPCR	ligand	RSCB PDB ID/ Reference	Comment
$RAMP1_{26-117}$	CLR_{22-133}	Telcagepant	3N7R, (ter Haar *et al*., 2010)	
$RAMP1_{26-117}$	CLR_{22-133}	Olcegepant	3N7S, (ter Haar *et al*., 2010)	
MBP-$RAMP1_{24-111}$-$(GSA)_3$-CLR_{29-144}-$(H)_6$		$CGRP_{27-37}$ [D^{31},P^{34},F^{35}]	4RWG, (Booe *et al*., 2015)	CGRP has beta I-turn, with terminal F facing W84 of RAMP1 (Fig 7)
MBP-$RAMP2[L106R]_{55-140}$-$(GSA)_3$-CLR_{29-144}-$(H)_6$		AM_{25-52}	4RWF, (Booe *et al*., 2015)	AM has beta I-turn, with terminal Y facing E101 of RAMP2 (Fig 7)
-	H_6-CTR_{25-144}	[$BrPhe^{22}$]$sCT_{8–32}$	5II0, (Johansson *et al*., 2016)	CT has beta II-turn, with terminal P facing W79/Y131 of CTR (Fig 7).
-	CTR with Gsαβγ and stabilising nanobody 35	sCT	5UZ7, (Liang *et al*., 2017)	Cryo-em structure. The ECD and ligand are not resolved.

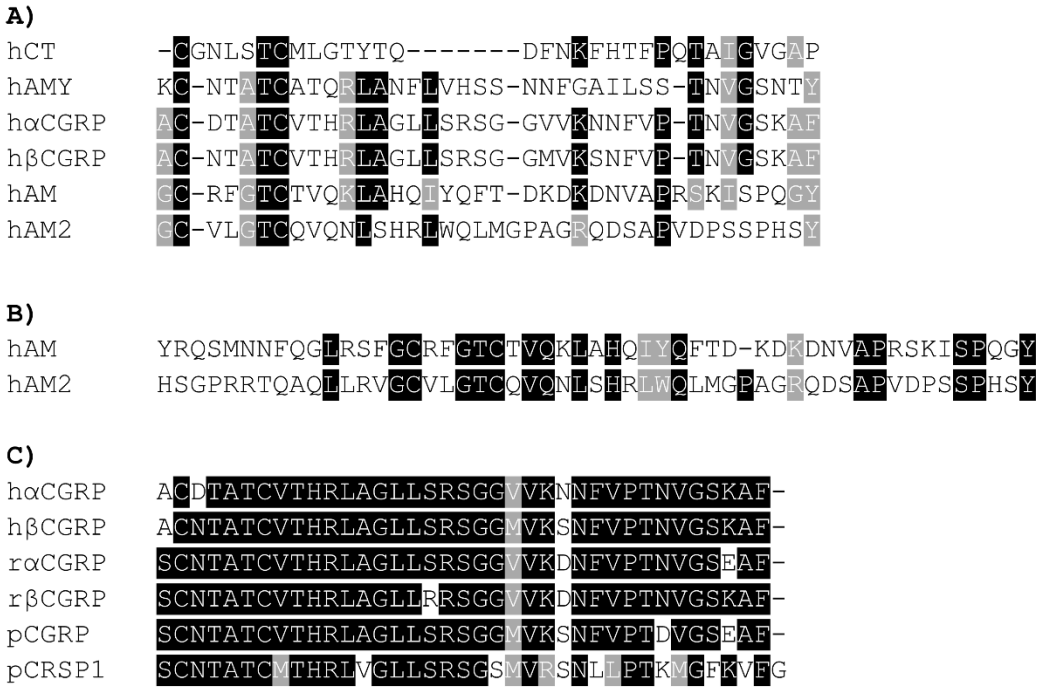

Figure 1. Amino acid sequence alignments of the CT peptide family. In all peptides, a disulphide bond is formed between the two N-terminal cysteines and they each have a C-terminal amide. For pCRSP1 this would occur on Phe37, presuming that the glycine is removed during processing like the other peptides. A) The human CT peptide family, omitting the N-terminal extensions of AM and AM2. B) Alignment of full-length human AM and AM2. C) Sequence alignment of human α and βCGRP, rat α and βCGRP and pig αCGRP and CRSP1. h-human, r-rat, p-pig. Alignment performed in COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web) and analysed using BoxShade (http://ch.embnet.org/software/BOX_form.html). Black indicates exact match, grey indicates 70-100% similarity and white indicates <70% similarity.

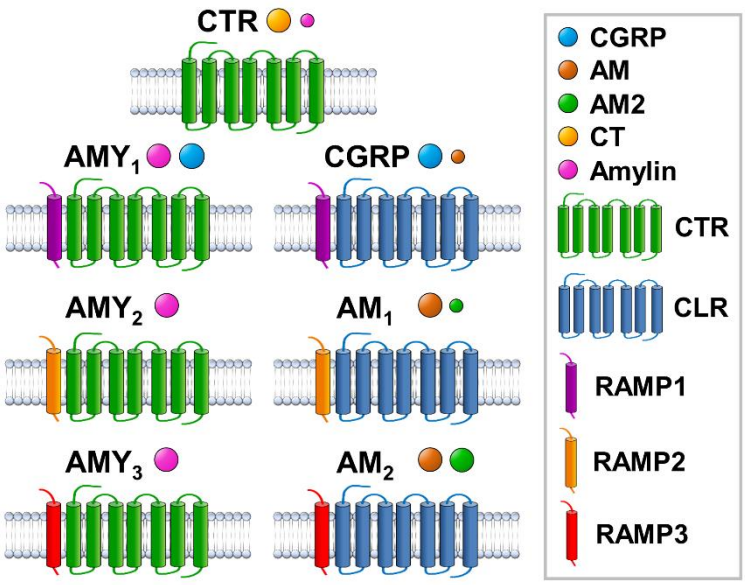

Figure 2. The subunit composition and current classification of human calcitonin-family receptors. The legend is shown in the box. Ligands are indicated by spheres with relative sizes reflecting relative potency at each receptor, with the smaller sphere indicating lower potency of a given ligand.

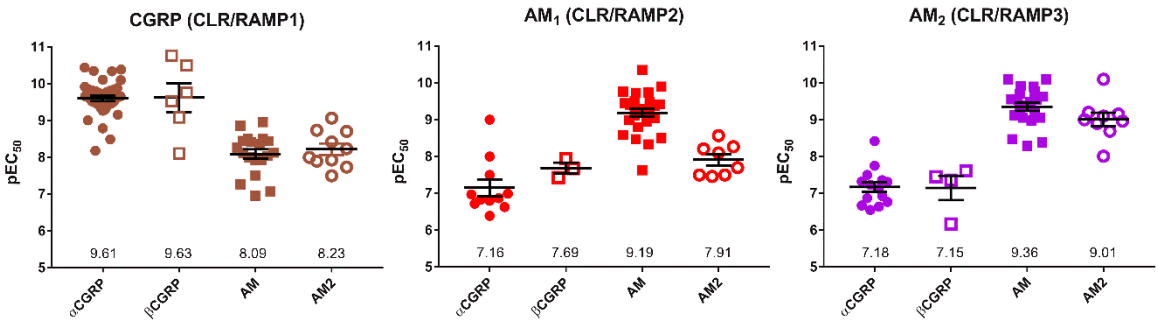

Figure 3. The pharmacology of selected ligands across CGRP, AM_1 and AM_2 receptors. All receptors are human and data are pEC_{50} values for cAMP production in cells transfected to express receptors. Each point is an individual value from independent publications, except where different cell lines were used within a single study and two values are therefore used from that study. The individual values and references can be found in Supplementary Data. The mean pEC_{50} is shown; error bars represent S.E.M. The CGRP receptor (CLR/RAMP1) is brown, AM_1 receptor (CLR/RAMP2) is red, AM_2 receptor (CLR/RAMP3) is purple. For ligands, αCGRP is a filled circle, βCGRP in an open square, AM is a filled square, AM2/IMD is an open circle.

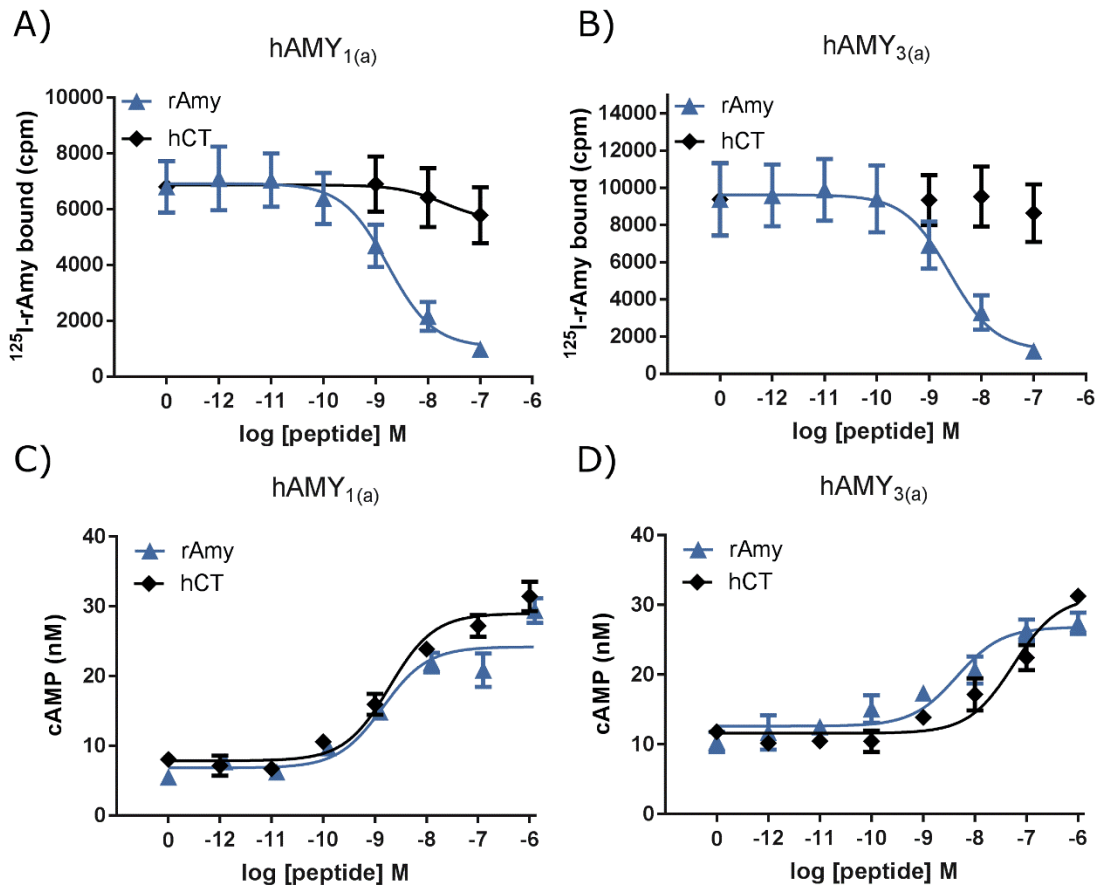

Figure 4. The binding of and cAMP production by rat amylin (rAmy) and human CT (hCT) in $AMY_{1(a)}$ and $AMY_{3(a)}$ transfected Cos7 cells. A) Displacement of I^{125}-amylin by amylin and CT at the $AMY_{1(a)}$ receptor. B) Displacement of I^{125}-amylin by amylin and CT at the $AMY_{3(a)}$ receptor. C) cAMP responses to amylin and CT at the $AMY_{1(a)}$ receptor. D) cAMP responses to amylin and CT at the $AMY_{3(a)}$ receptor. Data replotted from Hay *et al*., 2005.

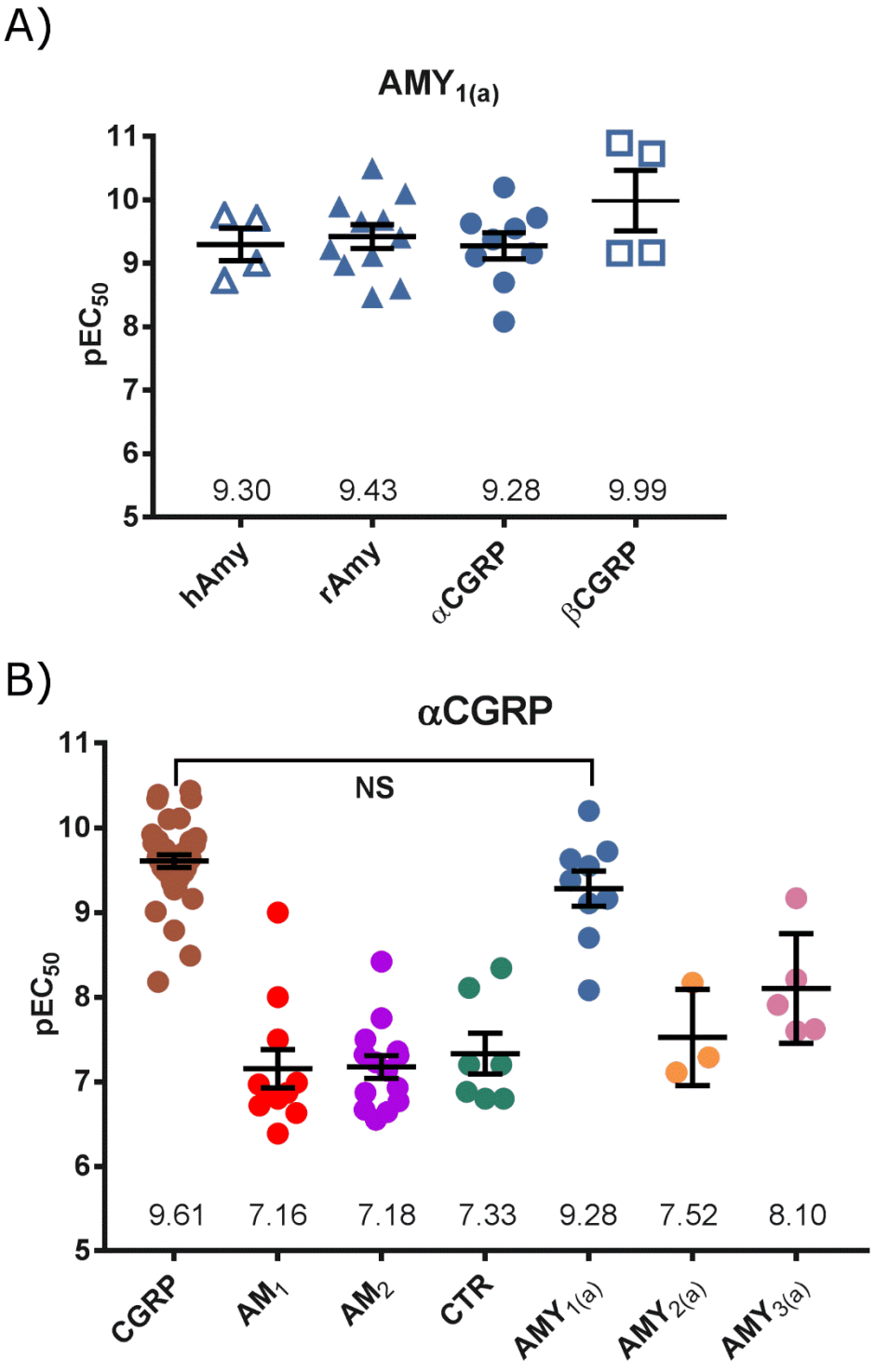

Figure 5. The pharmacology of A) the $AMY_{1(a)}$ receptor and B) αCGRP across various receptors. All receptors are human and data are pEC_{50} values for cAMP production in cells transfected to express receptors. Each point is an individual value from independent

publications, except where different cell lines were used within a single study and two values are therefore used from that study. The individual values and references can be found in Supplementary Data. The mean pEC_{50} is shown; error bars represent S.E.M. The CGRP receptor (CLR/RAMP1) is brown, AM_1 receptor (CLR/RAMP2) is red, AM_2 receptor (CLR/RAMP3) is purple, CTR is green, the AMY_1 receptor (CTR/RAMP1) is blue, AMY_2 receptor (CTR/RAMP2) is orange, and AMY_3 receptor is pink. For ligands, αCGRP is a filled circle, rat amylin (rAmy) is a filled triangle, human amylin (hAmy) is an open triangle and βCGRP is an open square. Data were analysed by one-way ANOVA followed by Tukey's test; in B) only the key comparison is shown.

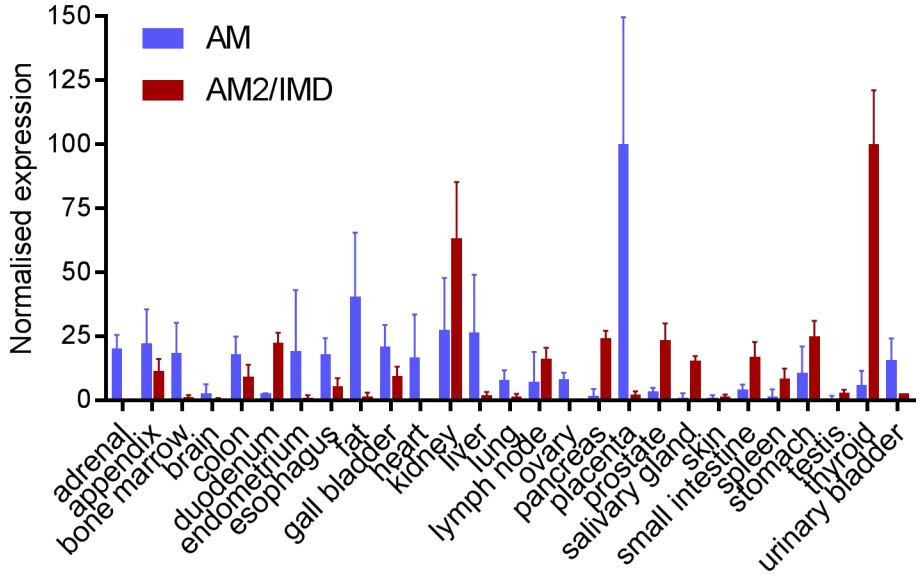

Figure 6. Relative distribution of the mRNA for AM and AM2/IMD. The data was taken from the HPA RNA-seq normal tissues database, available via the NCBI Gene website https://www.ncbi.nlm.nih.gov/gene). Values were normalised to 100% for the highest expressing tissue for both peptides. Similar expression profiles can be seen at http://www.proteinatlas.org/ENSG00000148926-ADM/tissue and http://www.proteinatlas.org/ENSG00000128165-ADM2/tissue.

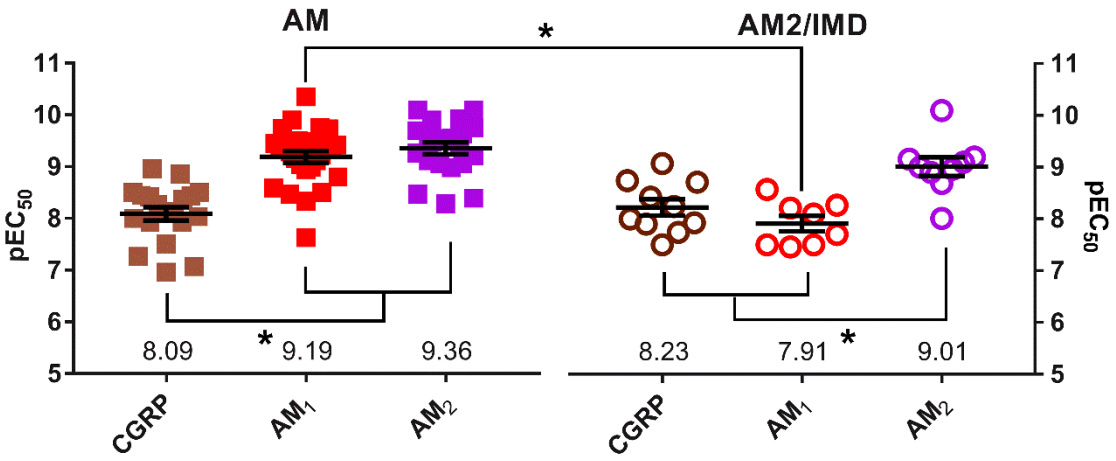

Figure 7. The pharmacology AM and AM2/IMD at CGRP, AM_1 and AM_2 receptors. All receptors are human and data are pEC_{50} values for cAMP production in cells transfected to express receptors. Each point is an individual value from independent publications, except where different cell lines were used within a single study and two values are therefore used from that study. The individual values and references can be found in Supplementary Data. The mean pEC_{50} is shown; error bars represent S.E.M. The CGRP receptor (CLR/RAMP1) is brown, AM_1 receptor (CLR/RAMP2) is red, AM_2 receptor (CLR/RAMP3) is purple. AM is a filled square, AM2/IMD is an open circle. Data were analysed by one-way ANOVA followed by Tukey's test; *$p<0.05$.

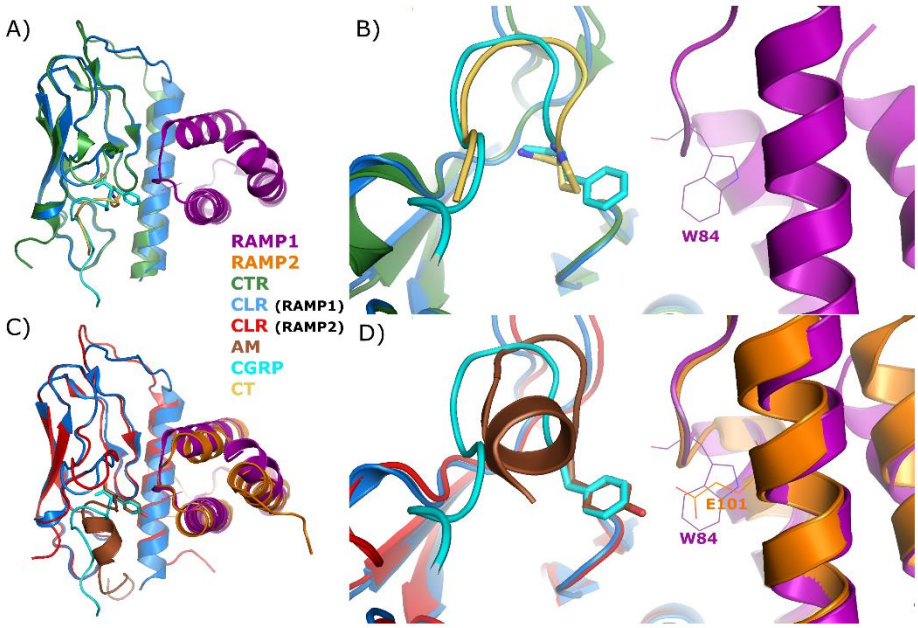

Figure 8. Structural alignment of CTR and CLR based receptor ECDs with bound ligands. A) Far and B) near views of the CTR and CLR/RAMP1 ECDs bound to [BrPhe^{22}]sCT$_{8-32}$ or [D^{31},P^{34},F^{35}]hαCGRP$_{27-37}$, respectively. C) Far and D) near views of the CLR/RAMP1 and CLR/RAMP2 ECDs bound to [D^{31},P^{34},F^{35}]hαCGRP$_{27-37}$ or hAM$_{22-52}$, respectively. All receptor ECDs are human. The C-terminal residue of each peptide is shown in stick format and the RAMP residue important for peptide interactions (RAMP1 W84/RAMP2 E101) shown in line format. Images created in pymol and aligned based on similarities between CTR and CLR or CLR and CLR. Images rotated 90° in the Z-plane between near and far views