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Highlights

- Amylin, pituitary adenylate cyclase-activating polypeptide (PACAP) and their receptors contribute to migraine pathogenesis and are additional novel targets yet to be clinically exploited.
- The amylin receptor subunit, the calcitonin (CT) receptor, and the PACAP receptor (PAC_1) splice variants are expressed in migraine-relevant sites in the central and peripheral nervous system.
- The CT and PAC_1 splice variants display unique structural, pharmacological and behavioral properties. However, there are limited studies examining how drugs (approved and in development) targeting these receptors act comparatively at their variants.
- Tissue- and disease-specific expression of the receptor variants has been observed and expression may be influenced by sex hormones.
- Targeting specific CT or PACAP receptor splice variants could provide additional therapeutic benefit to migraine patients.

Outstanding Questions

- Which splice variants are present in migraine-relevant structures?
- Do the variants activated and targeted in preclinical models translate to migraine patients?
- Do splice variants contribute to migraine pathophysiology equally, or do specific variants have a greater involvement?
- Would targeting specific variants improve therapeutic safety, as both PACAP and CGRP have protective roles in the cardiovascular system?
- Do the variants display different signalling or behavioral profiles, such as biased signalling or upregulation in disease states, which can be exploited to improve clinical outcomes?

Calcitonin/PAC_1-receptor splice variants: A blindspot in migraine research

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Abstract:
The neuropeptides calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating peptide (PACAP), and their receptors are linked to migraine neurobiology. Recent antimigraine therapeutics targeting these neuropeptides signaling are effective, however, some patients respond sub-optimally, indicating an incomplete understanding of migraine pathophysiology. The CGRP- and PACAP-responsive receptors can be differentially spliced. It is known that receptor splice variants can have different pathophysiology in other receptor-mediated pain pathways. Despite considerable knowledge of the structural and pharmacological differences of the CGRP- and PACAP-responsive receptor splice variants and their expression in migraine-relevant tissues, their role in migraine is rarely considered. Here we shine a spotlight on the calcitonin and PAC_1 receptor splice variants and examine what implications they may have for drug activity and design.

Calcitonin and PAC_1 receptor splice variants, emerging antimigraine targets

In the last 30 years, two key neuropeptides, calcitonin gene-related peptide (**CGRP**) and pituitary adenylate cyclase-activating polypeptide (**PACAP;** see glossary), have been identified as playing a role in craniofacial pain modulation and migraine pathophysiology [1, 2] (Box 1). The past five years have seen the rapid development and approval of several antimigraine drugs targeting CGRP or its receptor, providing relief for many individuals [3]. However, up to 40-50% of migraine patients do not benefit from CGRP-targeted therapies. Furthermore, despite promising data in pre-clinical models, an antibody targeting the PAC_1 receptor failed to show efficacy in human trials [4-6]. This highlights that our current understanding of migraine pathophysiology is incomplete, and that further research into the molecular mechanisms could address this significant unmet clinical need.

Studies have shown that a subunit of a CGRP-responsive receptor, the calcitonin receptor (**CTR**), and the **PAC_1** receptor (see glossary) are promising targets for the treatment of migraine. However, it is important to consider that both of these receptors can be differentially spliced, with variants observed or speculated to be expressed in migraine-relevant tissues (Figure 1) [7-9]. Despite this, the presence and potential role of these splice variants in pain and migraine are rarely considered. Given that the splice variants differ between species and have differences in pharmacology, regulation, and signaling, we believe the lack of consideration is an oversight in the current field of migraine research, both in the context of pharmacological tools and therapeutic design [10-12]. Additionally, many splice variants have differences in structure, including the absence and addition of amino acids in the extracellular and juxtamembrane domains, which could affect the efficacy of novel therapeutics targeting these regions [13, 14]. Recently, research of other G protein-coupled receptors (**GPCR**, see glossary), such as the μ-opioid receptor, has proven to be a successful strategy to reveal and refine analgesic targets with fewer side effects [15-17].

Given the rapid development of CGRP-targeted therapies and the recent failure of the anti-PAC_1 receptor antibody, AMG301, here we aim to shine a spotlight on the importance of researching CGRP- and PACAP-responsive receptor splice variants. We hope that highlighting the presence of these splice variants and their possible role in migraine will encourage the scientific community

and pharmaceutical companies to consider these receptor splice variants when researching the underlying mechanisms of migraine pathophysiology and in the drug development pipeline.

Calcitonin receptor splice variants in migraine

Beyond the canonical CGRP receptor

Studies consistently support the role of CGRP in migraine headache pathophysiology [18]. However, the CGRP signaling pathway is complex, and current therapies only target one receptor, whereas multiple receptors can be activated by CGRP (Figure 2A). The key CGRP-responsive receptors in humans are: the "canonical" CGRP receptor, a heterodimer of calcitonin receptor-like receptor (**CLR**) and receptor activity-modifying protein (**RAMP**) 1, and the amylin 1 (**AMY_1**) receptor (see glossary), comprised of the CTR and RAMP1, which form high-affinity receptors for CGRP [19]. Interestingly, the AMY_1 receptor is a dual receptor that is also potently activated by amylin, a neuroendocrine hormone closely related to CGRP, which has recently been linked to migraine pathophysiology [19-21]. The adrenomedullin receptors (AM_1 and AM_2) and AMY receptors (AMY_2 and AMY_3), comprised of RAMP2 and RAMP3 with CLR and CTR, respectively, can also be activated by CGRP but to a much lesser extent (Figure 2A), and their physiological relevance is currently unclear.

Several recent discoveries suggest that the AMY receptors, such as the AMY_1 receptor, play a role in migraine pathophysiology. The AMY_1 receptor subunits are reported to be expressed in many migraine-relevant sites, including trigeminal fibers, trigeminal ganglia (TG) and spinal trigeminal nucleus (STN) neurons and vasculature [7, 22, 23]. Infusion of an AMY receptor agonist, pramlintide, can induce migraine-like attacks, and pharmacological data indicate the presence of functional amylin receptors in rodent trigeminal ganglia cultures [20, 24]. Interestingly, AMY receptors may not be acting solely as CGRP-responsive receptors, as there is emerging evidence suggests that amylin itself could also play a role in migraine. For example, amylin was elevated in the plasma of chronic migraine patients, indicating that it may be released during migraine attacks, similar to CGRP [21, 25]. Therefore, it is possible that AMY receptors may underlie both CGRP-dependent and -independent mechanisms in migraine.

The exact mechanism by which AMY receptors contribute to trigeminovascular activation and sensitization is yet to be elucidated, however, recent studies are beginning to provide clues. For

example, pramlintide infusion in migraine patients induced limited facial flushing and temporal artery dilation [20]. In addition, an increase in mean arterial pressure was observed [20]. Overall, this indicates pramlintide infusion had minimal vasodilatory effects, and the resultant migraine-like attacks were not exclusively reliant on dilation of the cranial vasculature [20]. Another clue to determining the molecular contributions of AMY receptors in migraine is the frequent co-expression of CTR with CGRP in TG C-fiber neurons [7]. This suggests that CGRP could activate these AMY receptors in an autocrine fashion to mediate trigeminovascular activation and/or sensitization [7]. Furthermore, CGRP has previously been shown to upregulate its expression in an autoregulatory and autocrine way [25, 26]. The upregulation of CGRP is involved in migraine chronification and cannot be eliminated by CGRP receptor-specific antagonists [25-27]. AMY receptors could mediate this process. Overall, the evidence suggests that multiple potential mechanisms drive AMY receptor-mediated activity in the trigeminovascular system, providing novel targets for migraine therapeutics.

Unfortunately, at present, there are no AMY receptor-specific antagonists under development for the treatment of migraine. Many of the current therapeutics, such as erenumab and the gepants, target the canonical CGRP receptor, potently blocking receptor activation. Remarkably, these drugs also have some ability to act at the AMY_1 receptor, although they are 30- to 270-fold less potent at blocking CGRP at the AMY_1 receptor than at the CGRP receptor [28-30]. Circulating concentrations of erenumab and gepants are unlikely to effectively block the AMY_1 receptors present in migraine-relevant sites; although, it is worth noting that the concentration at the site of action is unknown [31]. This may explain why some patients have a limited response to these therapeutics, particularly if there is any individual variation in the AMY or CGRP receptors contribution to trigeminovascular activation and sensitization [20]. The development of therapeutics which specifically target the AMY_1 receptor to block the nociceptive actions of CGRP, and potentially amylin, could be an exciting area of opportunity to address the gaps in existing migraine treatments, particularly for the patients who experience limited relief from their current regiment.

Considering CTR splice variants in migraine therapeutic design

Unlike the CLR gene (CALCRL), there are several human (hCTR) and rodent (rCTR) CTR isoforms which arise from alternative splicing of the CALCR gene (Figure 2B, C). These CTR

splice variants exhibit a wide array of structural variations, including longer (o-hCT) or shorter extracellular domains ($hCT_{(\Delta 1\text{-}47)}$), inserts in the intracellular ($hCT_{(b)}$) or extracellular ($rCT_{(b)}$) loops, and premature terminations in the transmembrane helices (hCT5, hCT6) [32-36]. The existence of a diverse complement of CTR isoforms has long been established in the literature; however, the majority of studies focus on the human and rodent $CT_{(a)}$ variant, which contains no insertions or deletions and is considered the "reference" sequence (Figure 2B, C). Consequently, the physiological relevance of each isoform is yet to be elucidated. It is also unclear whether targeting one or more variants is optimal or inhibiting the activity of a particular variant could have unintended side effects.

CTR splice variants display not only unique structural differences but also complex pharmacological and behavioral profiles, alone and as part of AMY receptors [7, 34, 37, 38] (Figure 2D, Table 1). These isoforms could make distinct contributions to nociceptive signaling that could mechanistically underlie different sensitivities to amylin, CGRP, and antimigraine treatments. For example, when the $hCT_{(\Delta 1\text{-}47)}$ variant is part of an AMY_1 receptor, it has a significantly increased activation of cAMP signaling in response to CGRP and amylin, compared to when an AMY_1 receptor is formed with $hCT_{(a)}$ [32]. $AMY_{1(\Delta 1\text{-}47)}$ receptors expressed in migraine-relevant tissues could mediate amylin or CGRP sensitivity through elevated hyper-excitability of neurons, resulting from increased cAMP signaling [32, 39, 40]. In addition, antagonists have reduced efficacy at the $AMY_{1(\Delta 1\text{-}47)}$ receptor, likely due to the absence of the first 47 amino acids containing residues and a glycosylation site important for binding [32, 41]. Therefore, $AMY_{1(\Delta 1\text{-}47)}$ receptors may also underlie the poor effect of antagonists for some migraine patients.

Another variant speculated to play an important role in the trigeminovascular system is the $hCT_{(b)}$ variant [7]. This variant maintains agonist affinity but has reduced cAMP and calcium signaling, likely due to the additional 16 amino acids in ICL1 sterically interfering with G-protein binding [14, 29, 37]. Interestingly, the 16 amino acid insert does not appear to impact the potent induction of ERK1/2 phosphorylation [33]. Phosphorylated ERK is reported to be a key signaling molecule in CGRP-induced nociception, with ERK1/2 specific inhibitors suppressing neuronal excitation in rat spinal neurons [42, 43]. Activation of $hCT_{(b)}$-based AMY receptors may promote and bias signaling towards this pro-nociceptive molecule. Interestingly, the $hCT_{(b)}$ receptor isoform has

lower rates of internalization relative to $hCT_{(a)}$ [33, 44], which have been reported to be important in CGRP receptor-mediated nociceptive signaling [42]. Nevertheless, the dimerization of $hCT_{(a)}$ with RAMP1 significantly decreases receptor internalization with no reduction in activation of signaling pathways [30, 45, 46]. Therefore, it is unclear what effect the different regulatory profiles of the CTR isoforms might have on AMY receptor function *in vivo*.

While multiple CTR isoforms have been observed in the sites important to migraine, such as the trigeminovascular system of rodents (including the TG), as well as the brainstem, hypothalamus and cortex, the expression profiles of the CTR splice variants largely remain unknown [7, 34, 37]. Determining the relative distribution and abundance of the CTR throughout the body could help shed light on which isoform(s) are the best candidates to target and which may lead to unwanted side effects. For example, $hCT_{(\Delta 1\text{-}47)}$ mRNA was identified in multiple brain regions and in the kidney, where it is involved in calcium secretion [39]. This suggests that blocking this variant could have off-target effects on kidney function and calcium homeostasis. Future studies should focus on delineating where each of the specific variants is expressed and may be potentially contributing to pain transmission or sensitization. In addition, most studies examining isoform expression focus on rodents that do not express human CTR variants, making translational inferences difficult [34, 38]. Research using humanized CTR rodent models could bridge this translational gap, allowing in-depth examination into the distribution of human CTR isoforms and analysis of the impact of activation or inhibition of these isoforms on nociception in health and disease [47].

PACAP: an emerging target in migraine

Despite the great advances in our understanding of migraine headache pathophysiology, a high percentage of patients do not benefit from current antimigraine treatment options; therefore, novel pharmacological targets are needed. Due to the ability of PACAP to induce migraine-like attacks and its location in structures previously associated with migraine pathophysiology (Figure 1), attention was drawn to this neuropeptide and its potential role as a promising target for migraine treatment [48-51].

AMG301, a cautionary tale

In order to develop effective drugs that target the PACAP-signaling pathway, it is important first to understand its pharmacology. PACAP belongs to a wider family of peptides that also comprises the Vasoactive Intestinal Peptide (VIP) [52]. These peptides act via three receptors, the PAC_1, $VPAC_1$ and $VPAC_2$ receptors [52, 53]. While PACAP and VIP bind to both $VPAC_{1/2}$ receptors with similar affinity, PACAP has exhibited a 100-fold higher activity than VIP at the PAC_1 receptor (Figure 3A). As PACAP, but not VIP, induced migraine-like attacks in the initial infusion studies, it was widely accepted in the field that the receptor involved in migraine was the PAC_1 receptor [54], and an antibody against this receptor (AMG301) was developed for the preventative treatment of migraine, with no positive results [51, 55].

Even though the failure of AMG301 in Phase II trials was unfortunate [55], it was not entirely unexpected. Alternative splicing of the PAC_1 receptor gene results in several receptor variants with different ligand-binding properties (Table 1, Figure 3B, C) [56, 57]. Currently, more than 12 variants have been described, characterized by shorter extracellular domains (PAC_{1s}, PAC_{1vs}), inserts in an intracellular loop important for G-protein interaction (PAC_{1hip}, PAC_{1hop1}, PAC_{1hop2}, $PAC_{1hiphop1}$, $PAC_{1hiphop2}$) and/or discrete sequences located in transmembrane domains (PAC_{1TM4}); yet, most studies focus on PAC_{1null}, a receptor variant with no insertions or deletions [56]. Therefore, while the amino acid sequence recognized by AMG301 was never disclosed, one could speculate that if this antibody was developed based on the structure of the PAC_{1null} receptor variant, expression of a variant with a shorter extracellular domain in migraine-relevant structures would result in a lack of binding and, subsequently, of efficacy. In line with this, studies have reported the presence of mRNA of the PAC_{1s} receptor variant in the trigeminal ganglion of rodents [58]. It would be interesting to assess whether the infusion of PACAP or VIP triggers vasodilation of the middle meningeal artery, correlating with the onset of a migraine-like attack, in patients who did not respond to AMG301. This could further suggest that the antibody does not bind the receptor due to the presence of receptor variants with deletions in the extracellular domain (i.e., $PAC_{1s/vs}$).

A question that arises from the lack of efficacy of AMG301 is whether pre-clinical studies could have predicted this. Interestingly, intravenous administration of a rodent-specific PAC_1 receptor antibody (Ab181) inhibited the nociceptive responses to dural stimulation [6], a model that has proven highly predictive for pharmacological screening of potential antimigraine compounds.

Nonetheless, similar to AMG301, the amino acid sequence recognized by Ab181 was not disclosed, and the splice variant involved was never evaluated. Based on mRNA studies, PAC_{1s} and $PAC_{1hiphop}$ receptor variants have been described in the rodent trigeminovascular system; however, it is not yet clear whether these variants are expressed in the trigeminovascular system of humans and, more specifically, of migraine patients. This adds a new layer to the complexity of targeting receptor splice variants, since it is not yet known whether there are species-specific expression differences in migraine-relevant structures, which may have also contributed to this translational challenge.

Refining our understanding of the role of PAC_1 receptor variants in migraine

Alternative splicing of the PAC_1 receptor results in different profiles of ligand-binding properties (Table 1, Figure 2D). Understanding this can improve our knowledge of the role of the PACAP-responsive receptors in migraine pathophysiology. In line with this, recent studies have shown that VIP is a more potent agonist at the PAC_{1s} receptor than at the PAC_{1null} receptor (Figure 3D) [59], suggesting that, in fact, PACAP is not as selective for the PAC_1 receptor as previously thought. Remarkably, a recent study showed that infusion of VIP also provokes migraine-like attacks [4], which could be mediated *via* activation of the $VPAC_{1/2}$ receptors or a splice variant of the PAC_1 receptor with affinity for VIP. Since all three receptors have been reported in trigeminal ganglia [60], this broadens the therapeutic target options but requires properly designed studies to evaluate the receptor(s) behind the actions of these peptides. For this, it is important to consider the properties and limitations of current pharmacological tools. For example, all the antagonists of the $VPAC_{1/2}$ and PAC_1 receptors (i.e. PG 97-269, $PACAP_{6-38}$ and M65) have displayed ligand-dependent antagonism, being more effective at inhibiting VIP-mediated responses than PACAP-mediated [59]. More importantly, for the PAC_1 receptor, M65 and $PACAP_{6-38}$, have long been considered its antagonists [61, 62]; however, in rodent trigeminal ganglia primary cultures both have been shown to behave as agonists [63]. Therefore, for studies in pre-clinical models of migraine, where the trigeminovascular system is fundamental, there is an urgent need for novel pharmacological tools that allow us to characterize the different PACAP-responsive receptors.

Besides the differences in ligand-binding properties, the PAC_1 receptor splice variants couple to different signaling pathways (Figure 3D). While binding to Gs protein is considered the

predominant pathway, the PAC_1 receptor can also couple to Gq proteins [59]. This is particularly relevant in migraine where cAMP accumulation can lead to vasodilation, whereas hydrolyzation of phosphatidylinositol phosphate would result in vasoconstriction. Although the role of vasculature in migraine headache is still a highly debated topic, provocation studies have consistently shown vasodilatory responses after PACAP and, more recently, after continuous VIP infusion [4, 54, 64], suggesting that in migraine patients activation of a Gs-coupled receptor variant is likely. Future studies should not only assess the splice variants expressed in the different components of the trigeminovascular system, but also determine the predominant signaling pathways since it is not unlikely that more than one receptor splice variant is expressed in the same structure.

Are neuropeptide-targeting antibodies the solution?

Due to the promising role of PACAP in migraine pathophysiology and the failure of AMG301 in clinical trials, an antibody against PACAP was developed (Lu AG09222) with positive preliminary results [65]. As seen with the antibodies against CGRP, neuropeptide-targeting antibodies offer a novel therapeutic approach when receptor pharmacology is complex. However, this should not discourage the development of novel antagonists for scientific and therapeutic reasons. As seen with the μ-opioid receptor [16], understanding the pharmacology and expression profile of the PAC_1 receptor splice variants and their role in migraine pathophysiology could lead to the successful refinement and development of novel antimigraine drugs with fewer adverse effects.

Further considerations and unanswered questions regarding the CTR and PAC_1 splice variants in migraine

There is currently limited information on which isoforms are expressed in migraine-relevant sites, with even less known about their presence in the vasculature (Figure 3). However, it would not be unexpected for multiple variants to be present or for the expression of particular variants to change during disease [8]. Indeed, tissue-, pain- and disease-specific expression of the PAC_1 isoforms has previously been observed [8, 66, 67]. If a drug has varying ability to interact with different splice variants, then sub-optimal or excess efficacy could occur. This might also contribute to the diverse side-effect profiles between similar groups of drugs (e.g., CGRP receptor antagonists have different constipation rates [68]). Targeting a predominantly neuronal splice variant might be beneficial for treating migraine, especially in patients with preexisting

cardiovascular risk factors, where blocking vascular neuropeptide receptors, such as the CGRP and PACAP receptors, is not recommended.

It is important to consider the full complement of receptor splice variant expression in tissues, not just their individual expression. Combinatorial expression of receptor isoforms appears to modify signaling profiles, with different combinations of receptor variants demonstrating synergistic enhancement in the amount and rate of signaling or promoting bias, which could result in a different pharmacological action compared to that expected if characterized at the "canonical" receptor alone [8]. Under this paradigm, for example, co-expression of $hCT_{(a)}$ and $hCT_{(b)}$ receptors could bias signaling towards ERK1/2, a pro-nociceptive molecule.

Data indicating sex-dependent responses to CGRP, amylin and PACAP are beginning to emerge in migraine and other conditions [20, 69-72]. There may be different populations of receptor variants expressed in males and females that could mediate this susceptibility in both healthy and diseased states. In line with this, differences in the expression of CTR and PAC_1 receptor variants between the sexes have been observed [7, 73]. Certainly, female sex hormones play a role in migraine pathophysiology, with hormonal fluctuations influencing CGRP release and migraine attack occurrence during different reproductive milestones, such as menstruation, pregnancy, and menopause [74, 75]. However, this may also be partially mediated by changes in receptor expression, as upregulation of CT and PAC_1 receptors in response to sex hormones has recently been observed [76, 77]. Given the profound differences in migraine prevalence in males and females, it is crucial to determine what population of receptors, including variants, are present and whether they mechanistically contribute to migraine pathophysiology.

Another consideration is how environmental factors, such as circadian rhythm, sleep, other medications, and age, may affect the expression or signaling of the receptor variants and whether this alters the efficacy of migraine drugs. For example, pain sensitivity appears to be closely linked to circadian rhythm and sleep debt, with a greater sensitivity observed at night [78]. Interestingly, patients who primarily experience migraine in the evening had greater brain activity during a migraine attack than those who experience migraine in the morning [79]. It is unknown whether this is due to neurons regulated by circadian rhythm altering the integration of sensory information in the brain or changes in receptor expression and signaling.

Finally, several animal models of migraine have been developed throughout the last decades, which allowed us to increase our understanding of migraine pathophysiology and successfully identify drug targets, such as CGRP, and evidence of the translation potential of these models [80]. Although mice and rats are the most used pre-clinical models, the rodent CT and PAC_1 receptor isoforms are poorly characterized. This may lead to incorrect inferences between species and non-significant antimigraine properties. Humanized rodent models of migraine harboring CGRP- or PACAP-responsive receptor splice variants might reduce these translational issues. Previously, humanized CTR mice have been generated; however, splice variants were not examined [47].

Concluding Remarks and Future Perspectives

The past years have marked an exciting time for the research of GPCRs and migraine with the approval of several CGRP system-targeted treatments and the positive preliminary results of LU AG09222, an antibody directed against PACAP. Equally, there have been disappointments, such as the AMG301 antibody against the PAC_1 receptor being ineffective in migraine prevention. Despite these advances, there is still a limited understanding of the molecular contributions of these receptors in migraine pathophysiology or why some patients receive little to no benefit from the currently approved therapeutics. In this review, we have looked beyond the "canonical" CGRP and PACAP-responsive receptor variants and considered the role(s) the other splice variants may have in migraine (Figure 4).

Determining which variants are expressed in migraine-relevant sites and how they might contribute to trigeminal nociceptive transmission or sensitization is a major gap in our knowledge (see Outstanding Questions). Investigating the expression of these receptors is complicated as tools, such as antibodies and ligands, tend not to be sufficiently selective between the splice variants or are poorly validated and characterized. Techniques including mass spectrometry and single-cell RNA-Seq have proven effective in illuminating the distribution of GPCR splice variants, identifying disease-, tissue- and cell-specific receptor expression [8, 81, 82]. They could be employed to determine the relative abundance of splice variants in migraine-relevant sites. Similarly, genetic approaches such as tissue-specific knockdown or upregulation of splice variants in animal models could help untangle the contribution of each isoform.

Understanding the role of each receptor variant is important not only for migraine but also for other conditions where these peptides and receptors are clinically relevant, such as cardiovascular and metabolic diseases. Determining which variant(s) can be best exploited for therapeutic gain could enhance efficacy, reduce off-target effects and lead to more personalized medicine. In conclusion, it is essential to consider receptor variants not only when developing therapies targeting the amylin and PACAP receptors but all GPCRs with isoforms.

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Declaration of Interests

All authors declare no conflict of interest.

Highlights

- Amylin, pituitary adenylate cyclase-activating polypeptide (PACAP) and their receptors contribute to migraine pathogenesis and are additional novel targets yet to be clinically exploited.
- The amylin receptor subunit, the calcitonin (CT) receptor, and the PACAP receptor (PAC_1) splice variants are expressed in migraine-relevant sites in the central and peripheral nervous system.
- The CT and PAC_1 splice variants display unique structural, pharmacological and behavioral properties. However, there are limited studies examining how drugs (approved and in development) targeting these receptors act comparatively at their variants.
- Tissue- and disease-specific expression of the receptor variants has been observed and expression may be influenced by sex hormones.
- Targeting specific CT or PACAP receptor splice variants could provide additional therapeutic benefit to migraine patients.

Outstanding Questions

- Which splice variants are present in migraine-relevant structures?
- Do the variants activated and targeted in pre-clinical models translate to migraine patients?
- Do splice variants contribute to migraine pathophysiology equally, or do specific variants have a greater involvement?
- Would targeting specific variants improve therapeutic safety, as both PACAP and CGRP have protective roles in the cardiovascular system?
- Do the variants display different signaling or behavioral profiles, such as biased signaling or upregulation in disease states, which can be exploited to improve clinical outcomes?

Glossary

AMY_1: formed by CTR and RAMP1 and is a dual receptor for CGRP and amylin.

Amylin: 37 amino acid hormone that is co-secreted from the pancreas in response to food intake to promote satiety and hypoglycemia.

CGRP: 37 amino acid neuropeptide that is highly expressed in sensory nerves. Two variants of CGRP exist, α and β, which are derived from distinct genes.

CLR: the calcitonin-like receptor is a class B GPCR, closely related to the CTR. Has no known splice variants.

Cranial meninges: refers to the three layers of membranes that envelope and protect the brain. From superficial to deep, the meninges are the dura mater, arachnoid mater, and pia mater.

CTR: the calcitonin receptor, which has multiple splice variants. It can interact with the three RAMPs to generate three amylin receptors. Has multiple splice variants.

GPCR: G protein-coupled receptor, a family of ~ 800 members characterized by seven transmembrane domains, an extracellular N-terminus, three extracellular and intracellular loops, and an intracellular C terminal region. Class B GPCRs have a comparatively large N-terminus which participates in peptide hormone binding. The juxtamembrane domain (extracellular loops and transmembrane helices on the extracellular face) contains the residues which interact with the peptide to activate the GPCR. The intracellular loops, C-terminus and transmembrane helices

PAC_1: pituitary adenylate cyclase-activating polypeptide 1 receptor. Has multiple splice variants and can interact with intracellular signaling molecules to exert their (patho)physiological effects.

PACAP: a neuropeptide that is highly expressed in sensory nerves. Two variants exist, a 38 amino acid (PACAP-38) and 27 amino acid (PACAP-27) variant encoded by the same gene.

Receptor splice variants: Alternative splicing of the exons encoding a receptor results in different exon combinations at the mRNA level and, consequently, multiple isoforms of the receptor when translated to protein.

RAMP: receptor activity-modifying protein, a single transmembrane protein with a large extracellular N-terminus and small intracellular C-terminus. Three known RAMPs (RAMP1, RAMP2 and RAMP3) interact with GPCRs and alter their pharmacology and behavior.

Sensitization: refers to an increased responsiveness of sensory neurons to either normal or sub-threshold afferent inputs

Trigeminal afferents: refers to trigeminal neurons that carry sensory information from the face, mouth, nasal sinuses and meninges.

Trigeminovascular system: consists of pseudounipolar neurons peripherally innervating the cranial meninges and their associated blood vessels, whose somas are in the trigeminal ganglion and centrally projecting axons to the trigeminocervical complex that transmits nociceptive signals to the thalamus and higher-order cortical areas.

Box 1: Current understanding of migraine

Migraine is a neurological disorder that affects approximately 15% of the population, with a twofold or threefold higher prevalence in females than males [83]. According to the Global Burden of Disease initiative, migraine is highly disabling and represents the second cause of global disability and first among women under 50 years of age [83, 84]. Although the exact mechanisms underlying the onset of migraine remain unclear, it has been established that the development of a migraine *headache* is mediated by the activation and **sensitization** of the **trigeminovascular system** [18, 85], a functional pathway of sensory neurons innervating the **cranial meninges** (see glossary).

Tables

Receptor	Variation	Effect	Comment	Reference
hCTR ($hCT_{(a)}$ reference)				
$hCT_{(b)}$	16 amino acid insert in ICL1	- peptide binding ↓ signaling (cAMP/Ca^{2+}) - signaling (ERK1/2) ↓ internalization	+ RAMPs: amylin and CGRP binding and potency similar to $hCT_{(a)}$ + RAMPs	[33, 86]
$hCT_{(\Delta 1\text{-}47)}$	Deletion of first 47 amino acids from the N-terminus	↓ Peptide binding ↓/↑ signaling (cAMP) ↓ expression	+ RAMP1: amylin and CGRP potency similar/increased compared to $hCT_{(a)}$+RAMP1	[32]
o-hCT	Extended signal sequence (18 amino acids)	↑ peptide binding ↓ signaling (cAMP)	Reduced potency and maximal cAMP response. Interaction with RAMPs unknown	[36]
hCT5	Premature stop codon in TM4	↓ peptide binding ↓ signaling (cAMP)	Interaction with RAMPs unknown	[35]
hCT6	Premature stop codon in TM + 16 amino acid insert in ICL1	↓ peptide binding ↓ signaling (cAMP)	Interaction with RAMPs unknown	[35]
rCTR ($rCT_{(a)}$ reference)				
$rCT_{(b)}$	37 amino acid insert in ECL1	↓ peptide binding ↓ signaling (cAMP)	Interaction with RAMPs unknown	[34, 87]
$hPAC_1$ ($hPAC_{1n}$ reference)				
$hPAC_{1\ sv1}$	28 amino acid insert in ICL3	↑ peptide binding (VIP) -/↑ signaling (cAMP, VIP)	In rodents PAC_{1hip}	[11, 56]

hPAC$_{1\ sv2}$	28 amino acid insert in ICL3	- peptide binding ↑ signaling (Ca^{2+})	In rodents PAC$_{1hop}$	[14, 56]
hPAC$_{1\ sv3}$	56 amino acid insert in ICL3	- peptide binding ↓ signaling (cAMP/ Ca^{2+})	In rodents PAC$_{1hiphop}$	[56, 88]
hPAC$_{1\ \delta5,6}$	Deletion of 21 amino acid from N-terminus	- ↑ peptide binding (VIP) - signaling	In rodents PAC$_{1short}$	[11, 56, 59]
hPAC$_{1\ \delta5,6\ hip}$	Deletion of 21 amino acid from N-terminus + insertion of 28 amino acid in ICL3	↓ signaling (cAMP/ Ca^{2+})		[11, 56]
hPAC$_{1\ \delta5,6\ hop}$	Deletion of 21 amino acid from N-terminus + insertion of 28 amino acid in ICL3	↑ signaling (cAMP/ Ca^{2+})		[11, 56]
hPAC$_{1\ \delta4,5,\ 6}$	Deletion of 21 amino acid from N-terminus	↓ signaling (cAMP/ Ca^{2+})	In rodents PAC$_{1veryshort}$	[11, 56]
hPAC$_{1\ \delta5}$	Deletion of 7 amino acid from N-terminus	- ↑ peptide binding (VIP) ↑/↓ signaling (cAMP/ Ca^{2+})		[11, 56]
hPAC$_{1\ \delta5\ hip}$	Deletion of 7 amino acid from N-terminus + insertion of 28 amino acid in ICL3	↓ signaling (cAMP/ Ca^{2+})		[11, 56]
hPAC$_{1\ \delta5\ hop}$	Deletion of 7 amino acid from N-terminus + insertion of 28 amino acid in ICL3	↓ signaling (Ca^{2+})		[11, 56]

$rPAC_1$ ($rPAC_{1n}$ reference)				
	$rPAC_{1\ hop2}$	27 amino acid insert in ICL3	↑ peptide binding - signaling	Similar affinity for PACAP and VIP	[88]
	$rPAC_{1\ 3a}$	24 amino acid insertion in N-terminal	↑ peptide binding ↓ signaling (cAMP/IP)		[89]
	$rPAC_{1\ TM4}$	Substitution and deletion of two amino acid in the TM4 + substitutions in N-terminal and TM2	↓ VIP binding No cAMP/IP signaling	Activation of L-type Ca^{2+} channels	[90]

Table 1. **Summary of CT and PAC_1 receptor splice variant differences**. Structural, pharmacological and behavioral comparison of each variant to the reference receptor variant for each species ($hCT_{(a)}$, $rCT_{(a)}$, $hPAC_{1n}$ or $rPAC_{1n}$). ↓ decrease, ↑ increase, - no change, when compared to the reference variant.

Figure Legends

Figure 1: Expression of CGRP, amylin, PACAP and their splice variant receptors in the peripheral and central trigeminovascular system, as well as central nervous system locations. Expression profiles based on overall data for mRNA and/or protein with relative levels of expression not indicated. h/rCTR (?) indicates the reported expression of human or rat CTR, but the specific splice variant is unknown. PAC_1 (?) indicates the reported expression of PAC_1, but the specific splice variant is unknown. PAC_1 receptors may contain the "hip" and "hop" inserts and expression of both inserts in a region may indicate expression of a $PAC_{1hiphop1}$ receptor. LC, locus coeruleus; PAG, periaqueductal gray; PBN, parabrachial nucleus; TCC, trigeminocervical complex; TG, trigeminal ganglia. Data summarized from [9, 56, 91, 92].

Figure 2: Calcitonin receptor splice variants. (A) The human calcitonin (CT) receptor family subunits, receptors and pharmacology. Solid arrows indicate relatively potent activity compared with dashed arrows, which indicate weaker activity. AM, adrenomedullin; AM2, adrenomedullin 2 or intermedin; CLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein. Adapted from [92]. (B) Schematic indicating which exons (blue boxes with numbers) code for which portions of the CT receptor variants. hCT, human CTR; o-hCT, ovarian-human CTR; r/mCT, rat/mouse CTR; ECD, extracellular domain; H, transmembrane helix; I, intracellular loop; E, extracellular loop; ICD, intracellular domain. (C) Location of variation for each splice variant and the structural difference compared to $CT_{(a)}$ receptor. (D) Known pharmacology of the CTR splice variants. hCT peptide, human calcitonin; sCT peptide, salmon calcitonin; rCT peptide, rat calcitonin.

Figure 3: PAC_1 receptor splice variants. (A) The human PACAP receptor family and pharmacology. Solid arrows indicate relatively potent activity compared with dashed arrows, which indicate weaker activity. PACAP, Pituitary adenylate cyclase-activating polypeptide; VIP, Vasoactive intestinal peptide. (B) Schematic indicating which exons (blue boxes with numbers) code for which portions of the PAC_1 receptor variants. h, human CTR; r/m, rat/mouse; ECD, extracellular domain; H, transmembrane helix; I, intracellular loop; E, extracellular loop; ICD, intracellular domain. (C) Location of variation for each splice variant and the structural difference compared to PAC_{1n} receptor. (D) Known pharmacology of the PAC_1 splice variants. In the case

of receptor variants with species-specific nomenclature, the rodent nomenclature has been included between brackets.

Figure 4: Overview of antimigraine drugs targeting the activity of the CGRP, AMY_1 and PAC_1 receptors, which have multiple splice variants and exhibit differences in therapeutically relevant properties. Solid lines indicate relatively potent activity compared with dashed lines, which indicate weaker activity.

Figure

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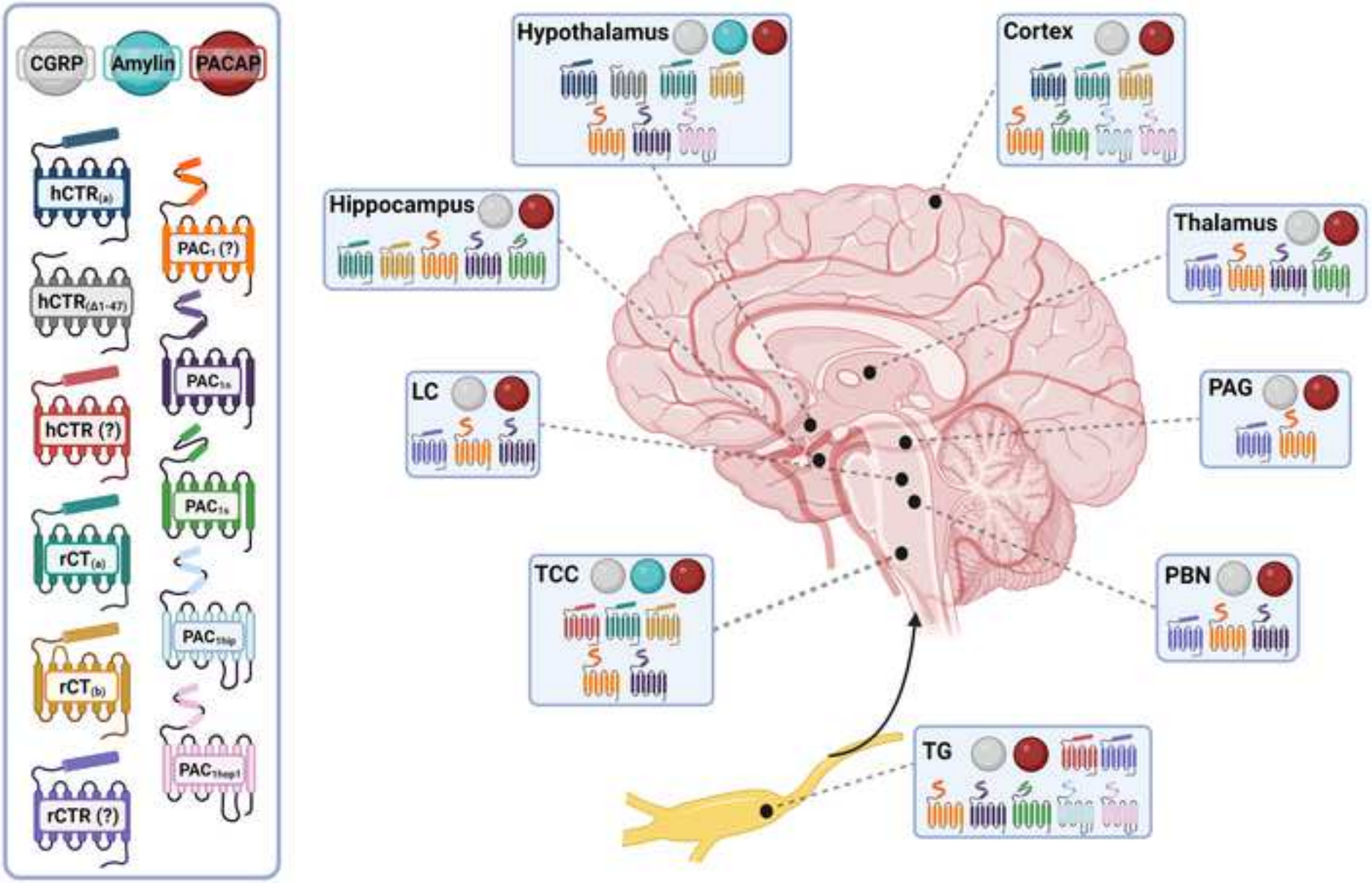

Figure

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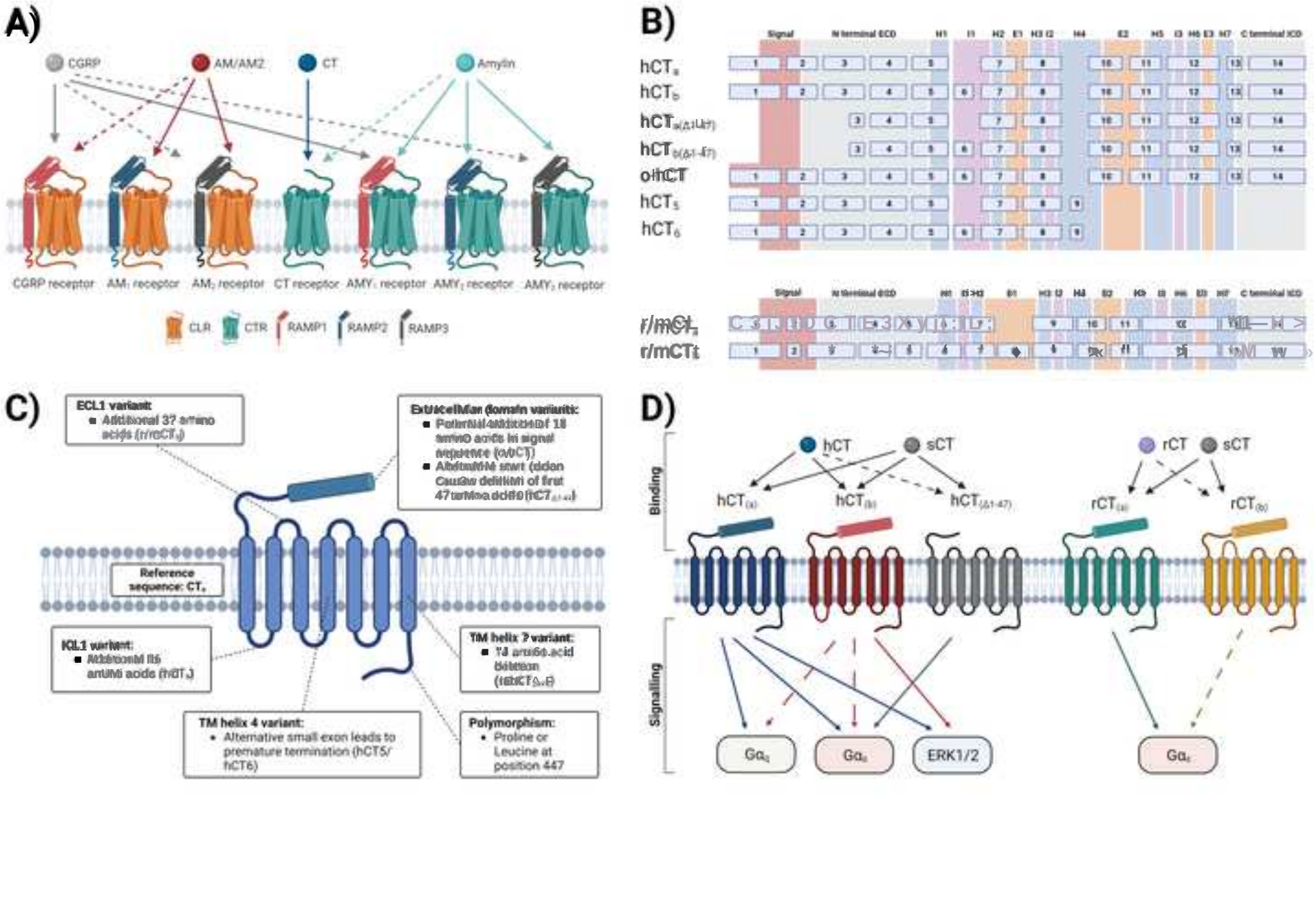

Figure

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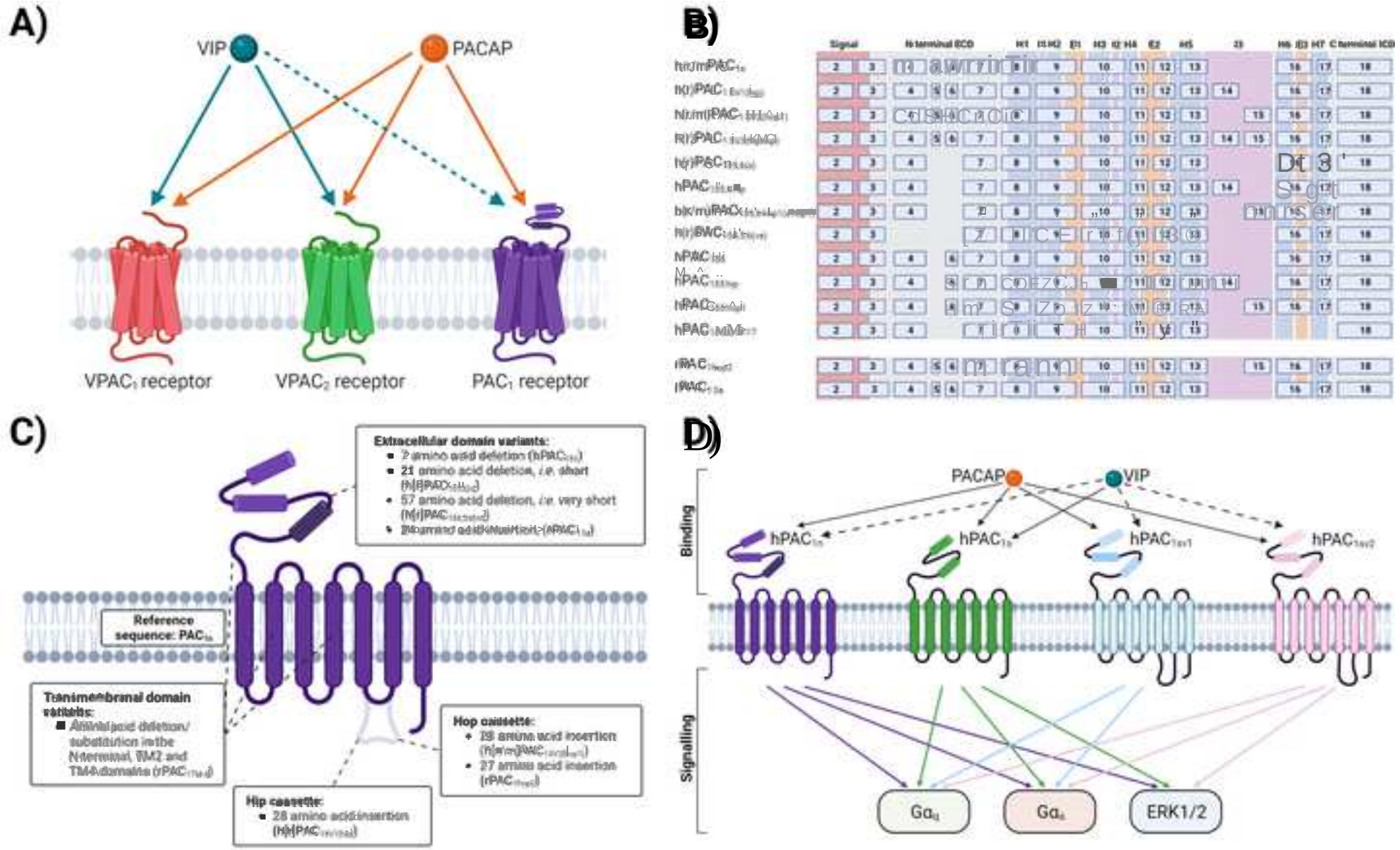

Figure