REVIEW ARTICLE

Pituitary adenylate cyclase-activating polypeptide receptors in the trigeminovascular system: implications for migraine

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Received 18 June 2017; **Revised** 24 August 2017; **Accepted** 11 September 2017

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The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has been implicated in a wide range of functions including vasodilatation, neuroprotection, nociception and neurogenic inflammation. PACAP activates three distinct receptors, the PAC₁ receptor, which responds to PACAP, and the VPAC₁ and VPAC₂ receptors, which respond to both PACAP and vasoactive intestinal polypeptide. The trigeminovascular system plays a key role in migraine and contains the trigeminal nerve, which is the major conduit of craniofacial pain. PACAP is expressed throughout the trigeminovascular system and in higher brain regions involved in processing pain. Evidence from human clinical studies suggests that PACAP may act outside the blood–brain barrier in the pathogenesis of migraine. However, the precise mechanisms involved remain unclear. PACAP potentially induces migraine attacks by activating different receptors in different cell types and tissues. This complexity prompted this review of PACAP receptor pharmacology, expression and function in the trigeminovascular system. Current evidence suggests that the PAC₁ receptor is the likely pathophysiological target of PACAP in migraine. However, multiple PACAP receptors are expressed in key parts of the trigeminovascular system and further work is required to determine their contribution to PACAP physiology and the pathology of migraine.

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Abbreviations

BBB, blood–brain barrier; GRF, growth hormone releasing factor; ICL, intracellular loop; PACAP, pituitary adenylate cyclase-activating polypeptide; PHM, peptide histidine methionine; STN, spinal trigeminal nucleus; TG, trigeminal ganglia; VIP, vasoactive intestinal peptide

Introduction

Migraine is a complex, painful and disabling neurovascular disorder that poses a significant social and economic burden to society. Migraine attacks are typically characterized by a unilateral pulsating headache which normally last 4–72 h and can be aggravated by physical activity. Migraine attacks are associated with a range of other neurological symptoms including nausea, photophobia and phonophobia (Headache Classification Committee of the International Headache Society, 2013). In approximately 30% of migraine sufferers, the headache phase of an attack is preceded by a complex neurological phenomenon called aura. In the majority of migraine sufferers, aura is characterized by visual disturbances (Headache Classification Committee of the International Headache Society, 2013). The prevalence of migraine continues to rise, and it is currently estimated that migraine affects over 1 billion people worldwide and is rated as the sixth highest cause of disability worldwide by the World Health Organization (Whiteford *et al.*, 2015). Given the high prevalence and disabling nature of this disorder, it is unsurprising that migraine is listed as a priority target for new treatments (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; Steiner *et al.*, 2015).

The mechanisms underlying migraine pathophysiology are unclear. However, the disorder appears to involve both peripheral, outside the blood–brain barrier (BBB) and central, inside the BBB components (May and Goadsby, 1999; Buture *et al.*, 2016). The trigeminovascular system, which includes the trigeminal nerve, the cerebral vasculature and nuclei within the brainstem/spinal cord, appears to play a role in this neurovascular disorder (Akerman *et al.*, 2017). The trigeminal nerve is important for processing craniofacial pain signals and innervates the cerebral vasculature (May and Goadsby, 1999). Sensory neurons of the trigeminal nerve reside in the trigeminal ganglia (TG) and are the only sensory afferent to project peripherally to innervate the cerebral vasculature and project centrally descending into the brainstem *via* the spinal trigeminal tract and terminating within the spinal trigeminal nucleus (STN) and the dorsal horn in the spinal cord.

Several GPCRs have been identified as potential targets for migraine treatment (Olesen and Ashina, 2011). Major efforts have focused on blocking activation of the calcitonin gene-related peptide (**CGRP**) receptor, with both small molecules and antibodies having entered phase III clinical trials (Bigal *et al.*, 2015; Karsan and Goadsby, 2015). Given the heterogeneity of migraine, it is not surprising that some patients do not appear to benefit from blocking CGRP receptor activity (Tfelt-Hansen, 2011; Hou *et al.*, 2017). It is unlikely that a single therapy will be sufficient to treat all patients. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is present at multiple sites within the trigeminovascular system and shares overlapping biological activities with CGRP. There is now strong evidence that PACAP plays an important role in migraine pathogenesis, leading to its emergence as a new target for migraine treatment (Edvinsson, 2013; Kaiser and Russo, 2013). This review will focus on the expression and function of PACAP and its receptors in the trigeminovascular system and the role they may play in migraine.

Pituitary adenylate cyclase-activating polypeptide

PACAP was initially identified as a 38 amino acid polypeptide in ovine hypothalamus and named for its ability to stimulate adenylate cyclase activity in cultured rat anterior pituitary cells (Miyata *et al.*, 1989). Subsequent research identified a 27 amino acid, C-terminally truncated variant of PACAP (Miyata *et al.*, 1990). These two forms of PACAP are highly conserved across mammals, displaying identical sequences between humans and rodents (Sherwood *et al.*, 2000). They are encoded by the *ADCYAP1* gene in humans, which produces a precursor protein. PreproPACAP is processed into 38 amino acid (**PACAP-38**) and 27 amino acid (**PACAP-27**) forms (Vaudry *et al.*, 2009; Harmar *et al.*, 2012). PACAP-38 is reported to be the more prevalent form, accounting for most PACAP immunoreactivity in the nervous system (Arimura *et al.*, 1991). However, this conclusion has been drawn from a relatively small number of studies and may not reflect the biologically active form of PACAP at its sites of action. PACAP is closely related to **vasoactive intestinal peptide (VIP)** and **peptide histidine methionine (PHM)**. The preproVIP peptide gives rise to both the 28 amino acid peptide VIP and the 27 amino acid PHM in humans or **peptide histidine isoleucine (PHI)** in rodents (Miyata *et al.*, 1989; Vaudry *et al.*, 2009). A C-terminally extended form of PHM/I, called **peptide histidine valine (PHV)**, has also been identified (Vaudry *et al.*, 2009).

PACAP-38 and PACAP-27 are widely distributed throughout the body, including the central and peripheral nervous system (Vaudry *et al.*, 2009). It is therefore unsurprising that PACAP has been implicated in a wide range of biological processes, including the regulation of circadian rhythms, reproduction and development, cognitive behaviour, neuroprotection, neuromodulation and pain transmission (Hashimoto *et al.*, 1996; Laburthe *et al.*, 2007; Rat *et al.*, 2011; Markovics *et al.*, 2012; Nakajima *et al.*, 2013). Interestingly, the ability of PACAP-38 to cross the BBB is negligible; approximately 0.05% of an injected dose was reported to cross the BBB (Banks *et al.*, 1996). This suggests that the peripheral and central actions of PACAP are likely to be compartmentalized. Evidence suggests that PACAP acts in the periphery during a migraine attack. Several research groups have reported that plasma PACAP-38 concentrations were elevated during the ictal phase of migraine (Tuka *et al.*, 2013; Zagami *et al.*, 2014). Furthermore, the intravenous infusion of PACAP-38 into migraine sufferers induced migraine-like attacks (Schytz *et al.*, 2009). Interestingly in patients that do not suffer from migraine, PACAP-38 infusion induced a mild transient headache (Schytz *et al.*, 2009). This is similar to the mild headaches reported following infusion of migraine sufferers with VIP. VIP infusion did not induce migraine-like attacks in people with migraine (Rahmann *et al.*, 2008). It is not known whether PACAP-27 or PHM is capable of inducing migraine-like attacks.

PACAP receptors

Early pharmacological studies suggested that there was significant **PACAP receptor** heterogeneity and PACAP

receptors were initially divided into two receptor subtypes: the 'PACAP type I receptor', which displayed approximately 1000-fold greater affinity for PACAP over VIP, and the 'PACAP type II receptor', which displayed approximately equal affinity for PACAP and VIP (Figure 1; Harmar *et al.*, 2012; Vaudry *et al.*, 2009). The 'PACAP type I receptor', encoded by the *ADCYAP1R1* gene, is now known as the **PAC₁** receptor (Pisegna and Wank, 1993; Alexander *et al.*, 2015). Several pharmacological tools are used to study this receptor, including the reportedly selective agonist **maxadilan** (Moro and Lerner, 1997) and the antagonists **M65** and **Max.d.4** (Uchida *et al.*, 1998; Tatsuno *et al.*, 2001). It should be noted that **PACAP-(6-38)** is commonly described and used as a specific PAC₁ receptor antagonist; however, PACAP-(6-38) has been reported to be an equally potent antagonist of **VPAC₂ receptors**, with IC₅₀ values of 30 and 40 nM respectively (Gourlet *et al.*, 1995).

Figure 1

The PACAP/VIP receptor family. Schematic representations of the PAC₁ (green), VPAC₁ (red) and VPAC₂ (blue) receptors. The relative potency of PACAP and VIP is shown below the appropriate receptor.

Understanding the activity of the PAC₁ receptor is complicated by the array of PAC₁ receptor splice variants which have been identified (Table 1; Blechman and Levkowitz, 2013; Furness *et al.*, 2012). The major reported PAC₁ splice variants fall into two categories: N-terminal deletion and intracellular loop 3 (ICL3) insertion variants (Table 1). Combinations of N-terminal deletion and ICL3 alternatively spliced forms have also been reported (Braas and May, 1999; Lutz *et al.*, 2006). Although PAC₁ splice variants are typically reported to have 100 to 1000-fold higher affinity for PACAP than VIP, in one study, PACAP and VIP are reported to display similar binding affinity and intracellular signalling properties at human PAC₁ short (Dautzenberg *et al.*, 1999; Blechman and Levkowitz, 2013). Many GPCRs, including PAC₁, are reported to form homodimers (Yu *et al.*, 2012). The stoichiometry of different PAC₁ receptor splice variants is unclear. The pharmacological behaviours of pharmacological tools reported to be selective for the PAC₁ receptor including, maxadilan, M65 and Max.d.4 have not been extensively characterized at these diverse PAC₁ splice variants and splice-dependant differences in function cannot be ruled out.

The molecular identity of the 'PACAP type II receptor' proved to be two closely related GPCRs, the **VPAC₁** and **VPAC₂** receptors, which share approximately 50% sequence homology with PAC₁ receptors (Ishihara *et al.*, 1992; Lutz *et al.*, 1993; Alexander *et al.*, 2015). VPAC₁ and VPAC₂ receptors display similar pharmacology and bind PACAP-38, PACAP-27 and VIP with similar affinity (Harmar *et al.*, 2012). To pharmacologically differentiate these two receptors, a number of pharmacological tools have been developed. For example, the antagonist **PG 99-465** is at least

Table 1

Common PAC₁ receptor splice variants

Splice variant name(s)	Amino Acids (species)	Comment	References
N-terminal variants			
PAC1-3a	492 (rat)	24 amino acid insert between exons 3 and 4.	Daniel <i>et al.</i> , 2001
PAC1null or PAC1n	468 (human) 467 (rat)	Full length PAC1	Spengler <i>et al.</i> , 1993; Pisegna and Wank, 1996; Dautzenberg <i>et al.</i> , 1999
PAC1 short	447 (human)	21 amino acid deletion of exons 5 and 6	Dautzenberg <i>et al.</i> , 1999
PAC1 very short	411 (human)	57 amino acid deletion of exon 4, 5 and 6	Dautzenberg <i>et al.</i> , 1999
Intracellular loop 3 variants			
PAC1-hip or PAC1 sv-1	496 (human) 495 (rat)	28 amino acid insert of exon 14	Spengler <i>et al.</i> , 1993; Pisegna and Wank, 1996; Lutz <i>et al.</i> , 2006
PAC1-hop1 or PAC1 sv-2	496 (human) 495 (rat)	28 amino acid insert of exon 15	Spengler <i>et al.</i> , 1993; Pisegna and Wank, 1996; Lutz <i>et al.</i> , 2006
PAC1-hop2	494 (rat)	27 amino acid insert of truncated exon 15	Spengler <i>et al.</i> , 1993
PAC1-hiphop1 or PAC1 sv-3	524 (human) 523 (rat)	56 amino acid insert of exons 14 and 15	Spengler <i>et al.</i> , 1993; Pisegna and Wank, 1996; Lutz <i>et al.</i> , 2006
PAC1-hiphop2	522 (rat)	55 amino acid insert of exon 14 and truncated exon 15	Spengler <i>et al.</i> , 1993; Journot <i>et al.</i> , 1995

10-fold more potent at the VPAC₂ receptor (Moreno *et al.*, 2000). Similarly, the agonist [Lys¹⁵, Arg¹⁶, Leu²⁷] VIP(1-7)/GRF(8-27)-NH₂ and antagonist PG 97-269 are at least 100-fold selective for the VPAC₁ receptor (Gourlet *et al.*, 1997a,b).

The VPAC₁ and VPAC₂ receptors do not display the same diversity as the PAC₁ receptor due to splice variants. Deletions in transmembrane helix 5, ICL3 and the C-terminus have been reported (Grininger *et al.*, 2004; Bokaei *et al.*, 2006; Miller *et al.*, 2006). However, both the VPAC₁ and VPAC₂ receptor have been shown to interact with single-transmembrane spanning receptor activity-modifying proteins (RAMPs) (Christopoulos *et al.*, 2003; Muller *et al.*, 2007; Wootten *et al.*, 2013). Three RAMPs have been identified, **RAMP1**, **RAMP2** and **RAMP3**, which have been shown to interact with a subset of GPCRs (McLatchie *et al.*, 1998; Hay *et al.*, 2016; Hay and Pioszak, 2016). The formation of a heterodimer between a RAMP and a GPCR can result in altered ligand binding, receptor trafficking and/or intracellular signalling (Hay *et al.*, 2016). In the case of VPAC₁ and VPAC₂, co-expression with RAMP1, 2 or 3 may result in altered G protein-coupling and subsequent changes to downstream signalling events (Christopoulos *et al.*, 2003; Wootten *et al.*, 2013). Interestingly, the co-expression of RAMP2 with the VPAC₂ receptor is also reported to increase the binding affinity of PHM (Muller *et al.*, 2007). This preliminary finding suggests that the VPAC₂/RAMP2 heterodimer may represent the molecular identify for a biologically important PHM receptor, but a more thorough investigation is needed. There is currently no evidence that the PAC₁ receptor forms heterodimers with RAMPs. However, given the lack of published data, interactions between the PAC₁ receptor and RAMPs cannot be ruled out. It is worth noting that RAMP-receptor interactions can be difficult to detect. This was the case for the VPAC₂ receptor, which was initially reported not to interact with RAMPs (Christopoulos *et al.*, 2003).

PACAP receptor signalling

Interestingly, despite reported differences in physiological functions, PAC₁, VPAC₁ and VPAC₂ receptors display similar signalling profiles and activate a diverse signalling network (Vaudry *et al.*, 2009). They are reportedly coupled to G_{as}, which results in cAMP accumulation and G_{aq}, which results in phospholipase C activation and IP₃ production (Sreedharan *et al.*, 1994; Delporte *et al.*, 1995; Van Rampelbergh *et al.*, 1997; Dickson *et al.*, 2006). The PACAP receptors have also been shown to induce the phosphorylation of signalling proteins including, p38 mitogen-activated protein kinase (p38), ERK and cAMP responsive element-binding protein (CREB) (Kopp *et al.*, 1997; Shi *et al.*, 2006; Monaghan *et al.*, 2008; Walker *et al.*, 2014). The activation of several distinct signalling pathways, coupled with multiple endogenous ligands, provides the ideal environment for biased signalling to occur. Biased signalling is the phenomenon where different ligands can activate specific signalling pathways at the same receptor (Kenakin and Christopoulos, 2013). The PAC₁ receptor provides one of the earliest examples of biased signalling. At the rat PAC₁ receptor, PACAP-38 and PACAP-27 were reported to stimulate cAMP

accumulation with similar potency, but when IP₃ production was measured, only PACAP-38 produced a measureable response (Spengler *et al.*, 1993). This suggests that in this model, PACAP-27 is biased towards the activation of cAMP signalling over IP₃ signalling. This phenomenon was observed regardless of the presence of ICL3 splice variants. Similar results were also reported in the rat PC-12 cell line (Deutsch and Sun, 1992). Further, in primary rat TG glia, PACAP-38, but not PACAP-27, reportedly activated ERK phosphorylation (Walker *et al.*, 2014). However at the human PAC₁ receptor, PACAP-38 and PACAP-27 displayed similar potencies for total IP turnover (Pisegna *et al.*, 1996). Further research is required to elucidate how extensive signalling bias may be for these receptors.

Receptor internalization is a key regulator of GPCR signalling (Pierce and Lefkowitz, 2001). Traditionally, internalization of an activated GPCR was thought to simply shut down signalling. However, it is now clear that while this process shuts down some signalling pathways, it can activate or potentiate other pathways (Reiter *et al.*, 2012). A key player in this process is β -arrestin, which mediates both internalization and can activate specific signalling pathways (Reiter *et al.*, 2012). Studies in PAC₁ receptor transfected cells have shown that PACAP-38 can induce β -arrestin recruitment and receptor internalization resulting in ERK signalling (Broca *et al.*, 2009; May *et al.*, 2014). Although the VPAC₁ and VPAC₂ receptors undergo desensitization and internalization, a direct interaction with β -arrestin is less well described (Shetzline *et al.*, 2002; Langer *et al.*, 2005; Nachtergaele *et al.*, 2006; Murthy *et al.*, 2008). Interestingly, ADP-ribosylation factor (ARF), which can induce clathrin-mediated receptor internalization, has been reported to interact with VPAC₁, VPAC₂ and PAC₁ receptors resulting in phospholipase D activation (McCulloch *et al.*, 2000; McCulloch *et al.*, 2001; Donaldson and Jackson, 2011). This suggests that receptor internalization is an important component of PACAP-induced signalling.

The precise intracellular signalling mechanisms activated by PACAP during a migraine attack are not known. However, human clinical studies utilizing **cilostazol**, a phosphodiesterase inhibitor that elevates cAMP, have suggested that cAMP may play an important role. Initial studies showed that cilostazol may induce headache or migraine-like attack in migraine patients (Birk *et al.*, 2006). This has recently been confirmed in a follow-up study which showed that cilostazol robustly induces a migraine-like attack in all migraine patients examined (Khan *et al.*, 2017). Interestingly, PACAP was originally characterized and named based on its ability to activate cAMP production in pituitary cells and has been shown to stimulate cAMP accumulation in TG neurons and glia (Miyata *et al.*, 1989; Nakajima *et al.*, 2013; Walker *et al.*, 2014). Studies examining signalling molecules downstream of cAMP in migraine have not been performed; however, animal models suggest that the phosphorylation of signalling proteins, including p38, ERK and CREB, is involved in nociception and could therefore play a role in migraine pain (Ji *et al.*, 2009; Edelmayer *et al.*, 2014). Further studies are required to elucidate the signalling pathways activated by PACAP in migraine; however, given that animal models typically examine a single feature of this disorder for migraine, this may prove difficult.

PACAP and PACAP receptors in the trigeminovascular system

The precise role played by PACAP in the pathophysiology of migraine is unclear. However, given the potential importance of the trigeminovascular system in migraine, PACAPs pathophysiological effects likely occur within this system. The specific site(s) of PACAP action in the trigeminovascular system and the receptor(s) or receptor variant(s) that PACAP activates to induce migraine have not been identified.

The cranial vasculature and vasodilatation

Neurons expressing PACAP and VIP have been identified innervating the cranial vasculature (Uddman *et al.*, 1993; Edvinsson *et al.*, 2001). PACAP expressing neurons originate from several sources that are known to play a role in cranial blood flow, including the trigeminal and sphenopalatine ganglia (Edvinsson *et al.*, 2001). PACAP and VIP released from these neurons are proposed to act locally on PACAP receptors

in vascular smooth muscle cells. This is hypothesized to result in vasodilatation of cranial arteries. In the case of migraine and other headaches with a vascular component, vasodilatation caused by neuropeptides released by the trigeminal nerve has been proposed as a potential cause of mechanical pain. However, more recent evidence suggests that migraine pain may not result from vasodilatation (Amin *et al.*, 2013; Schytz *et al.*, 2017).

The expression of PACAP receptors in the cranial vasculature has been investigated using qPCR, *in situ* hybridization and immunohistochemistry (Table 2). These studies indicate that VPAC₁, VPAC₂ and PAC₁ receptors are widely expressed throughout the vasculature. For example, mRNA for VPAC₁, VPAC₂ and PAC₁ receptors has been reported in human and rat middle meningeal artery (MMA) (Boni *et al.*, 2009; Baun *et al.*, 2011; Chan *et al.*, 2011; Syed *et al.*, 2012). The expression of VPAC₁, VPAC₂ and PAC₁ receptor mRNA has also been reported in rat and human middle cerebral arteries (MCA) and basilar arteries (Knutsson and Edvinsson, 2002; Baun *et al.*, 2011). This has been followed up with

Table 2

Summary of selected studies where PACAP receptors have been detected in the trigeminovascular system

Tissue	PAC ₁	VPAC ₁	VPAC ₂	Species	Reference	Notes
Cranial vasculature						
Middle cerebral artery	mRNA	mRNA	mRNA	Human	Knutsson and Edvinsson, 2002	–
	mRNA	mRNA	mRNA	Rat	Syed <i>et al.</i> , 2012	–
	–	mRNA	–	Rat	Fahrenkrug <i>et al.</i> , 2000	1
	mRNA	mRNA	mRNA	Rat	Baun <i>et al.</i> , 2011	–
	Protein	Protein	Protein	Rat	Erdling <i>et al.</i> , 2013	1
Middle meningeal artery	mRNA	mRNA	mRNA	Rat	Chan <i>et al.</i> , 2011	–
	mRNA	mRNA	mRNA	Rat	Baun <i>et al.</i> , 2011	–
	mRNA	–	mRNA	Rat	Syed <i>et al.</i> , 2012	–
	mRNA	mRNA	mRNA	Rat	Boni <i>et al.</i> , 2009	–
Basilar artery	mRNA	mRNA	mRNA	Human	Knutsson and Edvinsson, 2002	–
	mRNA	mRNA	mRNA	Rat	Baun <i>et al.</i> , 2011	–
Trigeminal ganglia						
Intact	mRNA	mRNA	mRNA	Human	Knutsson and Edvinsson, 2002	–
	mRNA	–	–	Rabbit	Fukiage <i>et al.</i> , 2007	–
	mRNA	mRNA	mRNA	Rhesus	Nakajima <i>et al.</i> , 2013	–
	mRNA	ND	mRNA	Rat	Chaudhary and Baumann, 2002	–
Neurons	mRNA	ND	mRNA	Rat	Walker <i>et al.</i> , 2015	2, 3
	Protein	–	Protein	Rat	Chaudhary and Baumann, 2002	2, 3
	Protein	–	–	Rhesus	Nakajima <i>et al.</i> , 2013	3
	Protein	–	–	Rat	Markovics <i>et al.</i> , 2012	–
Schwann cells (Glia)	Protein	–	–	Rhesus	Nakajima <i>et al.</i> , 2013	3
Brainstem						
Spinal trigeminal nucleus	Protein	Protein	Protein	Protein	Joo <i>et al.</i> , 2004	–
	Protein	–	–	Rat	Markovics <i>et al.</i> , 2012	–
	mRNA	–	–	Rat	Hashimoto <i>et al.</i> , 1996	–

RNA, detected by PCR or *in situ* hybridization. Protein, detected by histology. Entries marked with a '–' were not examined in that study. ND denotes 'not detected'. 1, co-localized with actin in VSMC. 2, VPAC₂ receptor expression (mRNA) was low relative to PAC₁ receptors or not on the cell surface (Protein). 3, primary culture.

immunohistochemistry, which suggests that VPAC₁, VPAC₂ and PAC₁ receptors are co-expressed with actin in the vascular smooth muscle of the MCA (Erdling *et al.*, 2013). However, the specificity of the anti-receptor antibodies used in this study and others is uncertain. Immunohistochemical studies examining GPCRs have traditionally suffered from the use of antibodies which recognize off-target epitopes, and as such, the specificity cannot be conclusively established. For example, a recent study of four commercial anti-MAS receptor antibodies showed that they were unsuitable for use in histology (Burghi *et al.*, 2017). For this reason measuring mRNA is often relied upon when determining receptor expression. These types of observations have led to the development of proposed guidelines for the validation of antibodies, which should be carefully considered in future studies (Uhlen *et al.*, 2016).

Pharmacological analysis has been employed to confirm the presence of functional PACAP receptors. However, this suggested that expression may not be a good indicator of the PACAP receptors involved in vasodilatation. In one study in isolated rat MMA, PACAP-38 was approximately 100-fold more potent at inducing vasodilatation than VIP and could be effectively blocked by PACAP-(6-38), suggesting that a PAC₁ receptor is involved (Syed *et al.*, 2012). However, in a similar study, no response to PACAP or VIP was observed in isolated rat MMA (Baun *et al.*, 2011). Furthermore, in a closed cranial rat MMA model, VIP and PACAP-38 displayed equivalent vasodilatation, which could be blocked by PG 97-269, a VPAC₁ antagonist. PG 99-465, a VPAC₂ antagonist, and PACAP-(6-38) failed to antagonize PACAP responses, suggesting that the VPAC₁ receptor was responsible for the observed vasodilatation (Boni *et al.*, 2009). The translation of these findings into isolated human MMA has not elucidated the situation. VIP induced only weak vasodilatation, which was slightly more potent than PACAP-38. Curiously, neither PACAP-(6-38) nor PG 97-269 could block the weak PACAP-38 or VIP activity (Chan *et al.*, 2011). For comparison in the same study, PACAP-38, PACAP-27 and VIP induced strong vasodilatation in human coronary arteries and the VPAC₁ receptor agonist, (Lys¹⁵,Arg¹⁶,Leu²⁷)-VIP(1-7)-GRF(8-27), induced partial dilation. Interestingly, PG 97-269 and PACAP-(6-38) both partially blocked VIP suggesting that both VPAC₁ and VPAC₂ receptors are functional (Chan *et al.*, 2011). The reasons for this inconsistency in MMA responses are unclear but may reflect differences between the regions of the MMA sampled, experimental differences related to tissue preparation or the different methods of vascular precontraction employed.

Pharmacological analysis indicated that the rat MCA and basilar arteries were relaxed by VIP, PACAP-27 and PACAP-38 (Baun *et al.*, 2011; Erdling *et al.*, 2013), whereas maxadilan did not dilate either vessel (Baun *et al.*, 2011). Interestingly, VIP and PACAP-induced vasodilatation was partially blocked by PG 97-269, whereas PG 99-465 had no effect (Baun *et al.*, 2011). Conversely, in another study, VIP was blocked by PG 99-465 suggesting that a VPAC₂ receptor was at least partially responsible for the observed vasodilatation (Erdling *et al.*, 2013). Partial antagonist blockade may suggest that multiple receptor subtypes are present or that the antagonist displays weak partial agonism. Overall the data suggest that a pharmacologically diverse range of PACAP and VIP receptors are

responsible for PACAP-induced vasodilatation in the cranial vasculature. It should be noted that although there is a wealth of pharmacological data describing PACAP activity in the cranial vasculature, it is not clear that endogenous PACAP can induce these effects physiologically.

The trigeminal ganglia and brainstem

PACAP-expressing nerve fibres have been shown to innervate the cranial vasculature (Uddman *et al.*, 1993). However, the source of these fibres appears to be distinct. Nerve fibres that co-express PACAP and VIP appear to originate from parasympathetic ganglia (Nielsen *et al.*, 1998a; Uddman *et al.*, 1999; Edvinsson *et al.*, 2001; Csati *et al.*, 2012), whereas nerve fibres expressing PACAP alone appear to originate from the TG. Using *in situ* hybridization and immunohistochemical methods, PACAP expression alone was localized to small-medium-sized sensory neurons in rat TG (Moller *et al.*, 1993; Mulder *et al.*, 1994; Nielsen *et al.*, 1998a; Eftekhari *et al.*, 2015). Similar results have been observed in human TG where PACAP was detected in ~20% of neuron cell bodies (Tajti *et al.*, 1999). Consistent with these findings, PACAP-expressing nerve fibres, presumably originating in the TG, have been observed in the STN and the dorsal horn (laminae I and II) at the C1/C2 level in the spinal cord. VIP-expressing nerve fibres were not observed in the STN or the dorsal horn (Larsen *et al.*, 1997; Nielsen *et al.*, 1998b; Uddman *et al.*, 2002).

The expression of PACAP receptors in the TG and brainstem has been examined by qPCR, *in situ* hybridization and immunohistochemistry (Table 2). mRNA extracted from intact human or rhesus monkey TG revealed the presence of transcripts for PAC₁, VPAC₁ and VPAC₂ receptors (Knutsson and Edvinsson, 2002; Nakajima *et al.*, 2013). This is perhaps unsurprising given that the TG is a highly vascularized structure that is permeable to circulating factors (Eftekhari *et al.*, 2015). The pattern of PACAP receptor mRNA expression observed is likely to be a combination of neuronal, glial and vascular cell types. PACAP receptor expression in TG neurons has been further elucidated by examining mRNA expression and using immunohistochemical and pharmacological approaches in neuron-enriched TG culture models. In rat TG neuron cultures mRNA encoding PAC₁ and VPAC₂, but not VPAC₁ receptors, was reported (Chaudhary and Baumann, 2002; Walker *et al.*, 2015). This included mRNA encoding PAC_{1n}, PAC_{1s} and ICL 3 insertion splice variants (Chaudhary and Baumann, 2002). Immunohistochemical approaches confirmed the presence of PAC₁ receptors on the surface of small-medium-sized rat TG neurons and on neurons and glial (schwann) cells in rhesus monkey TG (Chaudhary and Baumann, 2002; Markovics *et al.*, 2012; Nakajima *et al.*, 2013). Interestingly, diffuse intracellular staining of VPAC₂ receptors was reported in rat TG neurons, suggesting that VPAC₂ receptors may not be functional in these preparations (Chaudhary and Baumann, 2002). Consistent with the presence of PAC₁ receptors in TG neurons, pharmacological analysis indicated that PACAP was 100- to 1000-fold more potent than VIP at inducing cAMP accumulation and neurite outgrowth (Nakajima *et al.*, 2013; Walker *et al.*, 2014). However, PACAP, maxadilan, a VPAC₂ receptor agonist BAY 55-9837 and several antagonists have been reported to stimulate Ca²⁺ responses in TG

neurons, suggesting that functional PAC₁ and VPAC₂ receptors may both be present in some TG neuron preparations (Saghy *et al.*, 2015). It is not clear how these receptors may be regulated and differ under different experimental conditions or paradigms.

Although the data are more limited, the presence of PACAP-expressing nerve fibres projecting to the STN and the dorsal horn appears to correlate with PACAP receptor expression. Specific PACAP binding sites are present in rat brainstem and spinal cord membranes (Lam *et al.*, 1990; Cauvin *et al.*, 1991; Suda *et al.*, 1991). Evidence suggests that PAC₁ is the major PACAP receptor subtype present; PACAP binding was not effectively blocked by VIP in brainstem membranes and PACAP binding is reduced to background levels in PAC₁ knockout mouse spinal cord (Suda *et al.*, 1991; Jongsma *et al.*, 2001). The presence of PAC₁ receptors has been demonstrated in STN neurons using immunohistochemistry and *in situ* hybridization to detect PAC₁ receptor protein and mRNA respectively (Hashimoto *et al.*, 1996; Joo *et al.*, 2004; Markovics *et al.*, 2012). However, there has also been a report of VPAC₁ and VPAC₂ receptors in the STN (Joo *et al.*, 2004). The majority of studies indicate that a PAC₁ receptor is present in the TG, brainstem and spinal cord. However, the PAC₁ splice variant(s) present is unclear, and the consequences of physiological processes, such as inflammation on PAC₁, VPAC₁ and VPAC₂ expression in these tissues require further research.

PACAP receptors and pain transmission

Behavioural studies have examined the effect of PACAP administration on pain transmission. The pathophysiological role for PACAP in trigeminal sensitization and pain transmission is supported primarily by rodent studies, where administration of PACAP to rodents sensitized neurons to pain. Conversely, PACAP antagonists reduced pain sensitivity (Akerman and Goadsby, 2015). Furthermore, intrathecal PACAP-(6-38) reduced mechanical and thermal hyperalgesia suggesting that PAC₁ or VPAC₂ receptors are important for PACAP-induced pain (Davis-Taber *et al.*, 2008). The role of PAC₁ receptors has been further highlighted through the use of knockout models. Mice deficient in PAC₁ receptors display diminished chronic responses to chemical, thermal and mechanical stimuli and had no neuropathic pain in response to carrageenan or nerve transection (Jongsma *et al.*, 2001; Mabuchi *et al.*, 2004). It should be noted that some rodent studies have suggested that PACAP may display acute peripheral antinociceptive properties (Nemeth *et al.*, 2006; Helyes *et al.*, 2007; Sandor *et al.*, 2009). However, in healthy volunteers, PACAP-38 and VIP induced acute nociceptive cutaneous responses (Schytz *et al.*, 2010). Interestingly, in a model of photophobia, peripheral administration PACAP-38 induced light aversion in wild-type mice but had no effect in PAC₁^{-/-} mice (Markovics *et al.*, 2012). Although the mechanisms underlying migraine photophobia are not well understood, there is some evidence suggesting that the trigeminal nerve projections, which innervate the eye, could be involved (Kowacs *et al.*, 2001; Juhl *et al.*, 2007; Okamoto *et al.*, 2009). PACAP has also been implicated in the higher order processing of pain and related behaviours in brain

regions including the thalamus and amygdala (Martin *et al.*, 2003; Vaudry *et al.*, 2009; Missig *et al.*, 2014; 2017).

PACAP receptor genetics

The genetic basis of migraine has been well documented through the use of familial and twin studies, which suggest that this disorder may be inheritable (Sutherland and Griffiths, 2017). A recent meta-analysis of 22 genome-wide association studies which included nearly 60 000 migraine sufferers identified 38 distinct genetic loci that were significantly linked to migraine risk, highlighting the polygenic nature of the disorder (Gormley *et al.*, 2016). However, despite the body of evidence pointing to a key role for PACAP-38 and PACAP receptors in migraine, linkages to variants in these genes were not observed. This analysis does not rule out the possibility that a genetic variant of PACAP or a receptor may provide a protective benefit for migraine or that several variants may collectively contribute to the overall risk of suffering from migraine.

Several disease-associated genetic variants have been identified for the PAC₁, VPAC₁ and VPAC₂ genes (*ADCYAP1R1*, *VIPR1* and *VIPR2*, respectively). For example, a VPAC₁ single nucleotide polymorphism (SNP) (rs437876) is associated with achalasia and gallstone development, and VPAC₂ SNPs are associated with schizophrenia (rs3812311) and myopia (rs2071625 and others) (Krawczyk *et al.*, 2010; Yiu *et al.*, 2013; Jin *et al.*, 2016). Interestingly, reported genetic variants for the PAC₁ receptor include an intronic SNP (rs12668955), which was reported to be a risk factor for cluster headache (Bacchelli *et al.*, 2016) and an SNP (rs2267735) in an oestrogen response element that regulates PAC₁ receptor expression, which was reported to increase the risk of developing post-traumatic stress disorder or depression in women (Ressler *et al.*, 2011; Lowe *et al.*, 2015). Given that PACAP-38 is elevated in the blood during cluster headache (Tuka *et al.*, 2016) and the important role stress plays as a trigger of migraine, a closer assessment of these two genetic loci may prove enlightening.

Conclusions and implications

Despite the high prevalence and large burden placed on society by migraine, very little is known about mechanisms involved in migraine pathogenesis. In human studies, a number of endogenous factors have been shown to induce a migraine-like attack (Olesen and Ashina, 2011). The neuropeptide PACAP is one of these factors and likely acts in the trigeminovascular system during a migraine attack. However, the complexity of the PACAP receptor family, coupled with the lack of well-characterized molecular tools and the lack of animal migraine models, has made determining which PACAP receptor(s) are involved in migraine pathophysiology challenging.

In this review, we have described the evidence indicating that multiple PACAP receptors are expressed in key parts of the trigeminovascular system. The current opinion is that the PAC₁ receptor is the likely pathophysiological target of PACAP in migraine. This is based on the important but

indirect observation that VIP can induce a vascular headache and does not induce migraine attacks, suggesting that the activation of VPAC₁ and VPAC₂ receptors is not sufficient to cause a migraine (Edvinsson and Uddman, 2005; Hansen *et al.*, 2006). However, there is no direct evidence tying PAC₁ receptors to migraine pathophysiology. Further work is required to determine the contribution of all three PACAP receptors and their respective heterodimers or splice variants to PACAP physiology and migraine. New molecular tools that display greater receptor specificity or discrimination are required to dissect out this complex biology. Small molecules or antibodies that specifically block the PAC₁ receptor will be critical to understand the role of PACAP in migraine and determine if the PACAP receptor system can be used to treat other forms of craniofacial pain.

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).

Acknowledgements

The authors thank Professor Debbie Hay for her helpful discussions. C.S.W. is the recipient of a Sir Charles Hercus Health Research Fellowship from the Health Research Council of New Zealand. This work was supported by a Marsden Fast-start grant from the Royal Society of New Zealand to C.S.W.

Conflict of interest

The authors declare no conflicts of interest.

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