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REVIEW

CGRP in the trigeminovascular system: a role for CGRP, adrenomedullin and amylin receptors?

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The neuropeptide calcitonin gene-related peptide (CGRP) is reported to play an important role in migraine. It is expressed throughout the trigeminovascular system. Antagonists targeting the CGRP receptor have been developed and have shown efficacy in clinical trials for migraine. However, no CGRP antagonist is yet approved for treating this condition. The molecular composition of the CGRP receptor is unusual because it comprises two subunits; one is a GPCR, the calcitonin receptor-like receptor (CLR). This associates with receptor activity-modifying protein (RAMP) 1 to yield a functional receptor for CGRP. However, RAMP1 also associates with the calcitonin receptor, creating a receptor for the related peptide amylin but this also has high affinity for CGRP. Other combinations of CLR or the calcitonin receptor with RAMPs can also generate receptors that are responsive to CGRP. CGRP potentially modulates an array of signal transduction pathways downstream of activation of these receptors, in a cell type-dependent manner. The physiological significance of these signalling processes remains unclear but may be a potential avenue for refining drug design. This complexity has prompted us to review the signalling and expression of CGRP and related receptors in the trigeminovascular system. This reveals that more than one CGRP responsive receptor may be expressed in key parts of this system and that further work is required to determine their contribution to CGRP physiology and pathophysiology.

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Abbreviations
AM, adrenomedullin; AMY, amylin receptor; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CREB, cAMP response element binding protein; CTR, calcitonin receptor; DRG, dorsal root ganglia; RAMP, receptor activity-modifying protein; STN, spinal trigeminal nuclei; TG, trigeminal ganglia; VSMC, vascular smooth muscle cell

Introduction

GPCRs control numerous aspects of physiology. They are strategically positioned on the cell surface to control cellular signalling; this makes them excellent drug targets. GPCRs are divided into different families, according to amino acid sequence similarity (Fredriksson *et al.*, 2003). The focus of this review is the subfamily of receptors that respond to the neuropeptide calcitonin gene-related peptide (CGRP), which are members of the B or secretin family of GPCRs, and their role in the trigeminovascular system and migraine.

Migraine is a worldwide concern and is estimated to affect 11–15% of people (Stovner *et al.*, 2007). This painful and debilitating neurovascular disorder has some existing treatments but they vary greatly in effectiveness and have side effects that prevent their use in many sufferers (Goadsby, 2002; Silberstein, 2004). The trigeminal nerve is the major pain conduit in the head. This innervates the vasculature and is activated during a migraine attack (Messlinger *et al.*, 2011). It is essential to understand how this nerve functions in the generation of migraine pain. New medications for migraine treatment and management are vital for improving the quality

of life for sufferers. Studies describing the elevation of CGRP content in the blood or saliva of migraine sufferers and the triggering of migraine by CGRP infusion have provided compelling evidence that CGRP is important in this disease (Goadsby *et al.*, 1990; Cady *et al.*, 2009; Hansen *et al.*, 2010). However, there is some controversy; when CGRP content was examined during a migraine attack (between ~100 and 300 min), jugular CGRP content was not increased (Tvedskov *et al.*, 2005).

The CGRP receptor is a promising candidate for relieving migraine pain and is targeted by the now discontinued CGRP receptor antagonists; olcegepant and telcagepant (Tfelt-Hansen, 2012). Olcegepant (BIBN4096BS) was first reported over a decade ago (Doods *et al.*, 2000). This drug showed efficacy in phase II clinical trials but did not progress further because it was unsuitable for oral administration (Olesen *et al.*, 2004). The related, orally bioavailable, telcagepant (MK-0974) progressed to multiple phase III clinical trials for the acute treatment of migraine, where it displayed comparable efficacy to zolmitriptan (Ho *et al.*, 2008). However, elevated transaminase levels in a few patients with longer-term treatment apparently halted the progression of this compound. Other CGRP receptor antagonists, such as MK-3207, BI-44370 and BMS-927711, have reached clinical trials but less is known about these molecules (Diener *et al.*, 2011; Hewitt *et al.*, 2011; Luo *et al.*, 2012). There is unlikely to be a single reason why there are currently no CGRP receptor antagonists available for migraine treatment or management. One contributing factor that has been widely discussed in the literature could be low central penetration of these drugs leading to lower than expected clinical efficacy (Olesen and Ashina, 2011; Tfelt-Hansen, 2011). Also, translation of therapies for migraine is challenging, given that few models are available and the complexity of this disorder. Therefore, there has been a strong emphasis on preclinical testing in vascular models, which effectively demonstrate antagonism of CGRP receptors. However, there are many potential peripheral and central sites involved in CGRP action that the antagonists may need to reach to be effective (Silberstein, 2004; Eftekhari and Edvinsson, 2010). A further concept that we highlight in this review, is that the receptor type which is involved at each of these potential sites still needs to be defined. There is an assumption that the CGRP receptor mediates all of the actions of CGRP but as discussed in the next section, it is worthwhile considering the possible contribution of other CGRP-responsive receptors.

There are other forms of pain in which CGRP has been implicated, or where reducing its action could be useful. In particular, the small molecule CGRP receptor antagonists, which were intended to target the trigeminovascular system, could have other uses beyond migraine and be used to treat other forms of craniofacial pain which involve the trigeminal nerve; including some forms of headache, temporomandibular disorders, trigeminal neuralgia and dental pain (Awawdeh *et al.*, 2002; Ambalavanar and Dessem, 2009; Rapoport, 2010; Cady *et al.*, 2011; Messlinger *et al.*, 2011; Sacerdote and Levrini, 2012). Moreover, the potential benefits of medications which target the CGRP system stretch far beyond this panel of disorders. In cancer patients, pain resulting from the metastatic invasion of bone tissue is one of the most debilitating symptoms (Hay *et al.*, 2011). Intriguingly, tumour-associated pain is linked with elevated CGRP content and can be blocked by the antagonist, $CGRP_{8-37}$ (Wacnik *et al.*, 2005). Furthermore, in a model of invasive bone cancer, irradiation treatment was associated with a reduction in both pain and spinal cord CGRP content (Park *et al.*, 2005). Given the success of the relatively selective CGRP antagonists in clinical trials for migraine, it would be particularly interesting to see whether they could also be used to treat tumour-associated pain, trigeminal neuralgia or other painful conditions. However, as several CGRP-responsive receptors have been reported, the ideal receptor or receptors to target remains unclear and needs resolving. In this article, we review current knowledge of CGRP receptors and their activity in the trigeminovascular system.

What is a 'CGRP receptor'?

Receptors that bind, or are activated by CGRP, are found throughout the body (Brain and Grant, 2004). However, their pharmacological characteristics are diverse and this led to the former subdivision of CGRP-responsive receptors into $CGRP_1$ and $CGRP_2$ subtypes. Other receptors, such as RDC1 and L1 (also known as ADMR), were also proposed, but they are no longer considered viable receptors for CGRP or related peptides (Hay *et al.*, 2011). It is important that care is taken to distinguish reports citing these 'CGRP receptors' (Moreno *et al.*, 1999; Tajti *et al.*, 1999), from later studies examining what is now accepted as the CGRP receptor (Eftekhari *et al.*, 2010; Tajti *et al.*, 2011).

The molecular composition of the receptor formerly known as the $CGRP_1$ receptor is now accepted to be the calcitonin receptor-like receptor (CLR) with receptor activity-modifying protein 1 (RAMP1); this has been renamed simply as the CGRP receptor (McLatchie *et al.*, 1998; Alexander *et al.*, 2009). This receptor is characterized by its high affinity for CGRP, the peptide antagonist $CGRP_{8-37}$, olcegepant and telcagepant (Alexander *et al.*, 2009; Moore and Salvatore, 2012). For these latter two antagonists, species selectivity is apparent and should be taken into account when interpreting data (Mallee *et al.*, 2002; Salvatore *et al.*, 2008). The CGRP receptor can also be activated by adrenomedullin (AM) and more weakly by other related peptides that together comprise the calcitonin peptide family (Bailey and Hay, 2006). These are calcitonin, amylin and AM2. These related peptides share receptor subunits, leading to inevitable complexity and overlapping pharmacology. CLR pairs with either RAMP 2 or 3 to generate AM receptors (Poyner *et al.*, 2002). The RAMPs can also interact with the calcitonin receptor (CTR) to form high affinity receptors for amylin, called AMY receptors (Poyner *et al.*, 2002). Several of these receptors can be activated by CGRP (Figure 1).

This array of receptors that can be activated by CGRP has enabled observations of '$CGRP_2$ receptor' pharmacology to be explained, but a '$CGRP_2$ receptor' *per se* is not recognized by the International Union of Pharmacology committee on receptor nomenclature (IUPHAR-NC). However, this does not mean that other CGRP-responsive receptors should be ignored. Quite the opposite is true; we should carefully consider their potential contributions to CGRP physiology and pathophysiology. Once we have more information, the clas-

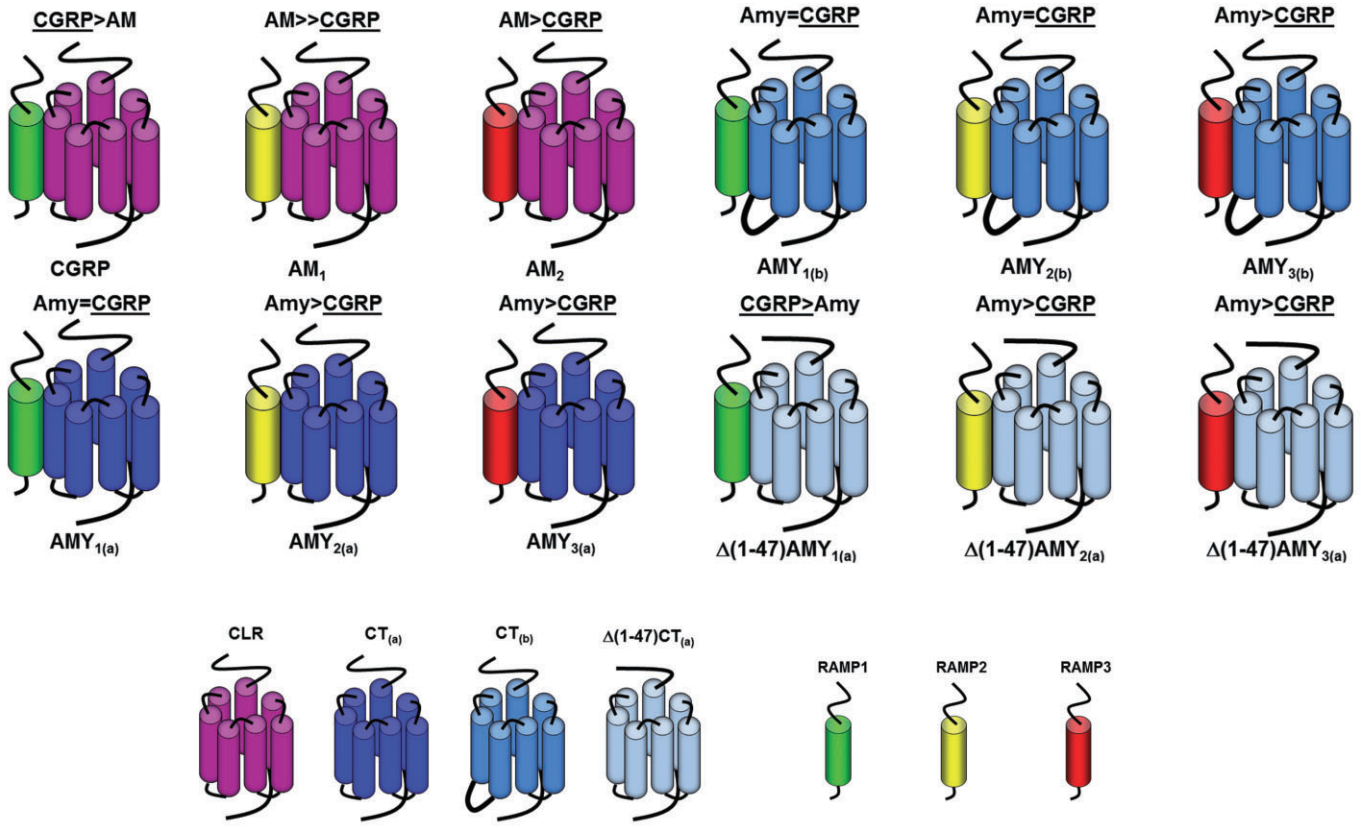

Figure 1

The calcitonin receptor family. The relative potency of CGRP compared to AM or amylin (Amy) is shown above a schematic representation of the appropriate receptor complex (Alexander *et al.,* 2009; Qi *et al.,* 2012; Udawela *et al.,* 2008). The different receptor components are described in the legend, beneath the receptors.

sification of CGRP receptors may need to be reconsidered. The AMY_1 receptor is particularly notable because several studies have reported that amylin and CGRP are equipotent at this receptor in transfected cells (Hay *et al.,* 2005; Udawela *et al.,* 2008). The physiological and pathophysiological actions of CGRP could be mediated although multiple receptor subtypes, including the CGRP receptor, the AM_2 receptor and the AMY_1 receptor. Figure 1 demonstrates this inherent complexity and illustrates the problem of assigning terms like $CGRP_2$ or even $CGRP_3$ to receptors that are also potently activated by related peptides with distinct physiologies.

Although cell-based studies infer that there are potentially many receptors that could be activated by CGRP, precisely how much of this translates to *in vivo* CGRP physiology or pathophysiology requires considerably more work to delineate (Udawela *et al.,* 2008; Alexander *et al.,* 2009; Qi *et al.,* 2012). In individual tissues, the pharmacological phenotype is complicated by likely co-expression of multiple receptor components which can give rise to several different receptor subtypes (McLatchie *et al.,* 1998). Only recently have good antibodies become available for localizing individual receptor components (Eftekhari *et al.,* 2010; Wookey *et al.,* 2012). However, not all of these are commercially available and there has still not been adequate characterization of commercial RAMP2 and RAMP3 antibodies to verify their specificity. Thus, there is understandably still a heavy reliance on measuring receptor mRNA in tissues for the purposes of assigning a receptor to an effect. This is only of limited value because several mRNAs for different RAMPs are often detected, which may or may not be processed into functional receptor complexes. Furthermore, these methods may collect mRNA from multiple cell types and thus may not be reflective of the situation in the precise cell type of interest.

The advent of small molecule CGRP receptor antagonists such as olcegepant and telcagepant has been helpful in providing researchers with pharmacological tools with a known binding mechanism (ter Haar *et al.,* 2010). These antagonists effectively block CGRP-mediated vasodilation, although their relative effectiveness appears to vary according to vessel type (Edvinsson *et al.,* 2007). Given the well-described vascular aspect of migraine pathology (Silberstein, 2004) and the role of CGRP receptors in the relaxation of cerebral arteries (Jansen-Olesen *et al.,* 2003; Edvinsson *et al.,* 2010), it seems likely that blockade of this receptor would offer some relief.

Although usually considered as selective molecules, olcegepant and telcagepant are only approximately 200-fold less effective at the human $AMY_{1(a)}$ receptor than the human CGRP receptor (Table 1) (Moore and Salvatore, 2012). In clinical trials, olcegepant and telcagepant can apparently achieve blood concentrations of 200 nM and 4–6 μM, respectively

Table 1

Summary of selected small molecule CGRP receptor antagonist potencies at human CGRP and AMY_1 receptors in cell culture models

Antagonist	CGRP receptor	AMY_1 receptor	Model	Reference
Olcegepant (BIBN4096BS)	14.4 pM (K_i)		SK-N-MC/binding	Doods *et al.*, 2000
	11.0 (pK_B)		SK-N-MC/cAMP	Doods *et al.*, 2000
	10.5 (pK_B)		SK-N-MC/cAMP	Hay *et al.*, 2002
	9.73 (pK_B)	7.49 (pK_B)	Transfected Cos-7/cAMP	Hay *et al.*, 2006
	10.6 (pA_2)		Transfected Cos-7/cAMP	Miller *et al.*, 2010
Telcagepant (MK-0974)	2.2 nM (IC_{50})		Transfected HEK-293/cAMP	Salvatore *et al.*, 2008
	8.9 (pA_2)		Transfected HEK-293/cAMP	Salvatore *et al.*, 2008
	0.77 nM (K_i)		Transfected HEK-293/binding	Salvatore *et al.*, 2008
	0.78 nM (K_i)		SK-N-MC/binding	Salvatore *et al.*, 2008
		190 nM (K_i)	Transfected HEK-293/binding	Moore and Salvatore, 2012
	9.74 (pA_2)		Transfected Cos-7/cAMP	Miller *et al.*, 2010
MK-3207	0.024 nM (K_i)		SK-N-MC/binding	Salvatore *et al.*, 2010
	0.12 nM (IC_{50})		Transfected HEK-293/cAMP	Salvatore *et al.*, 2010
	10.3 (pA_2)		Transfected HEK-293/cAMP	Salvatore *et al.*, 2010
	0.022 nM (K_i)	0.74 nM (K_i)	Transfected HEK-293/binding	Salvatore *et al.*, 2010
BI-44370	–	–	**	Diener *et al.*, 2011
SB-273779	7.10 (pA_2)	*	Transfected Cos-7/cAMP	Miller *et al.*,2010
	310 nM (K_i)		SK-N-MC/binding	Aiyar *et al.*, 2001
	250 nM (K_i)		Transfected HEK-293/binding	Aiyar *et al.*, 2001
	390 nM (IC_{50})		Transfected HEK-293/cAMP	Aiyar *et al.*, 2001
BMS-927711	27.0 pM (K_i)	–	SK-N-MC/binding	Luo *et al.*, 2012
	0.14 nM (IC_{50})		SK-N-MC/cAMP	Luo *et al.*, 2012
BMS-694153	12.8 pM (K_i)		SK-N-MC/binding	Degnan *et al.*, 2008
	16.1 pM (K_B)		SK-N-MC/cAMP	Degnan *et al.*, 2008
		>5 μM (K_i)	Transfected CHO/cAMP	Degnan *et al.*, 2008

Entries marked with a '–' were not reported or have not been examined. * SB-273779 does not bind (>3 μM Ki) to CTR expressing T47D cells. **Shows efficacy for the treatment of migraine in a phase II study; no pharmacology reported. cAMP, antagonism of cAMP responses; Binding, antagonism of radioligand binding. Data represent reported mean.

(Tfelt-Hansen and Olesen, 2011). Taking into consideration the existing pharmacological data on these compounds (Table 1), these concentrations are potentially sufficient to, at least partially, antagonize peripheral AMY_1 receptors as well as antagonizing the CGRP receptor. Therefore, can the AMY_1 receptor be completely ruled out in terms of contributing to the actions of CGRP in migraine? Although other factors, such as the antagonist free fraction and target accessibility should be considered, it will be interesting to see how effectively a more CGRP receptor specific antagonist, such as BMS-694153, alleviates migraine (Degnan *et al.*, 2008). How alternative CGRP blocking strategies, using CGRP binding entities, such as antibodies which act peripherally or RNA-spiegelmers behave when translated into the clinic will also provide valuable information and help define a peripheral versus central site of action (Edvinsson *et al.*, 2007; Olesen and Ashina, 2011).

In this review, we consider the available data on CGRP receptor expression and signalling in the trigeminovascular system. In light of the possible actions of CGRP at other receptors, we also cover the yet limited amount of literature on other receptors that are CGRP-responsive. Work of this nature may ultimately reveal whether any of these other CGRP-responsive receptors should seriously be considered as therapeutic targets.

CGRP and receptor localization in the trigeminovascular system: an overview

The trigeminovascular system represents a major control centre for the regulation of blood flow in the head and is a key conduit for the transmission of pain. It is comprised of the trigeminal nerve, the cranial vasculature it innervates and the spinal trigeminal nuclei (STN) in the brainstem (Figure 2). The cell bodies of pseudounipolar neurons that make up the sensory arm of the trigeminal nerve extend their projections from the trigeminal ganglia (TG) to both the periphery; where they innervate the cerebral vasculature; including pial, meningeal and cerebral arteries (Uddman *et al.*, 1985;

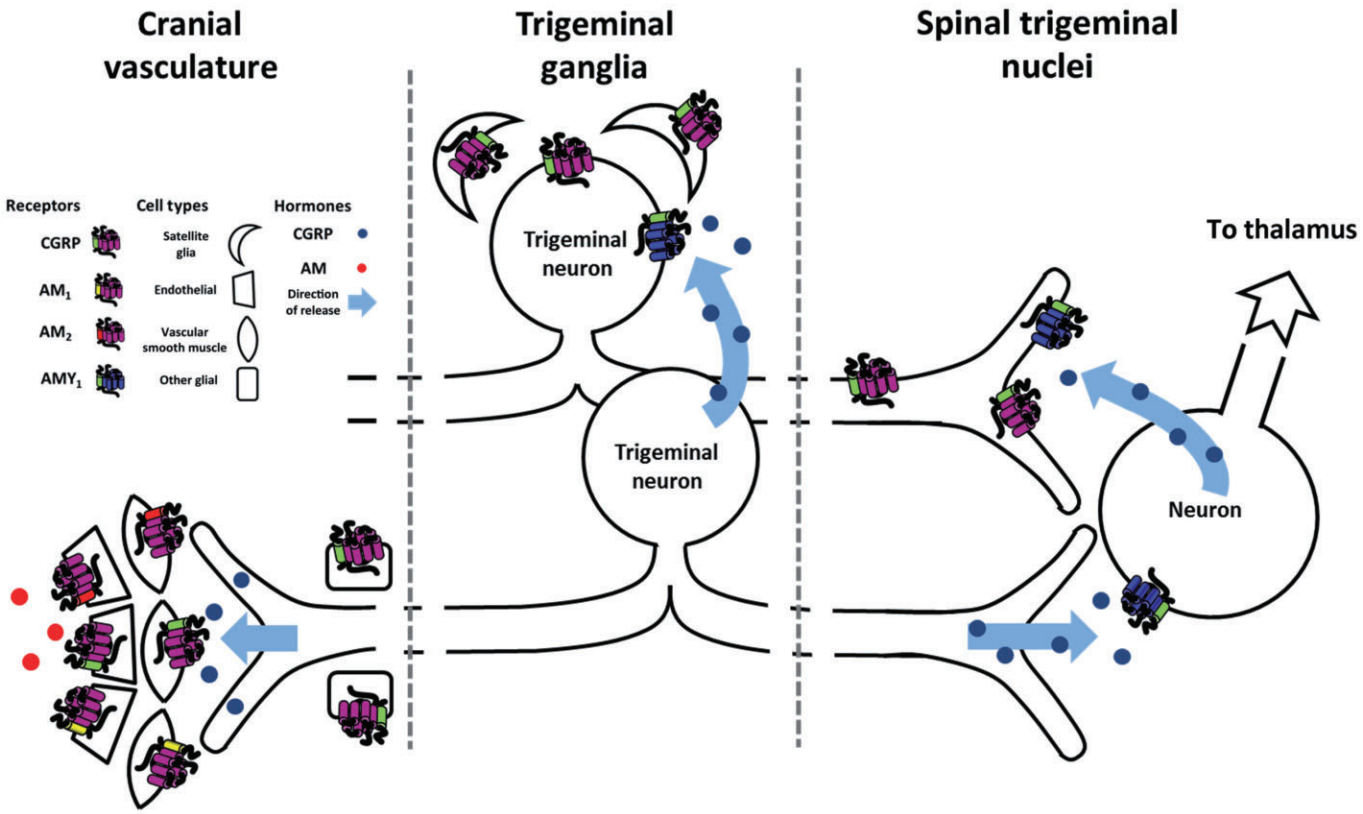

Figure 2

Overview of potential CGRP and CGRP-responsive receptor expression in the trigeminovascular system. A composite of two stylized TG neurons is shown innervating the cranial vasculature and the spinal trigeminal nucleus (Oliver *et al.*, 2002; Tolcos *et al.*, 2003; Lennerz *et al.*, 2008; Eftekhari *et al.*, 2010; Bower *et al.*, 2011; Eftekhari and Edvinsson, 2011; Walker and Hay, 2011).

Edvinsson and Uddman, 2005) and to the brainstem, where they synapse in the STN (Cuello *et al.*, 1978; Arbab *et al.*, 1988). Although the focus of this review is CGRP in the trigeminovascular system (Lennerz *et al.*, 2008), it should be noted that AM is reported to be expressed in the vasculature, the TG and the brainstem (Sone *et al.*, 1997; Moreno *et al.*, 1999; Serrano *et al.*, 2000) and that amylin has been identified in TG neurons and in nerve fibres innervating the pial vasculature (Edvinsson *et al.*, 2001). The significance of AM and amylin in the trigeminovascular system is unclear, but their presence indicates that their receptors may also be present, and highlights an increasing appreciation of their role in pain (Gebre-Medhin *et al.*, 1998; Ma *et al.*, 2006; Fernandez *et al.*, 2010).

CGRP and receptor localization in the trigeminovascular system: the cranial vasculature

CGRP-expressing neurons that originate in the TG have been well documented to innervate the cerebral vasculature (Uddman *et al.*, 1985; Edvinsson *et al.*, 1987a,b). CGRP released from these neurons is proposed to act locally in the vascular smooth muscle cell (VSMC) layer of cranial arteries, on mast cells or on glia associated with neuronal processes; where histological methods have identified the CGRP receptor components, CLR and RAMP1 (Table 2) (Oliver *et al.*, 2002; Lennerz *et al.*, 2008; Edvinsson *et al.*, 2010). In one study, the neuronal processes did not express CGRP receptors (Lennerz *et al.*, 2008). Related receptor components have also been identified in the cranial vasculature (Table 2). For example, mRNA encoding proteins capable of forming both CGRP and AM receptors (CLR, RAMP1, RAMP2 and RAMP3) have been identified on human meningeal, cerebral and cranial arteries (Sams and Jansen-Olesen, 1998) and in human cerebral and meningeal arteries that have had their endothelial layer removed (Jansen-Olesen *et al.*, 2003). Furthermore, immunoreactivity for CLR, RAMP1, RAMP2 and RAMP3 has been detected in the VSMC layer of cerebral and meningeal arteries (Oliver *et al.*, 2002).

CGRP and related peptides have been observed to induce different relative relaxation responses in independent vessel types, suggesting that different receptor subtypes or multiple receptors maybe involved. In functional studies, CGRP typically acts as a more potent vasodilator than AM in cranial vessels (Jansen-Olesen *et al.*, 2003; Petersen *et al.*, 2005; Edvinsson *et al.*, 2007). This may reflect dominant interactions of RAMP1 with CLR (Buhlmann *et al.*, 1999). On the

Table 2

Summary of studies where CGRP, AM and AMY receptor components have been detected in the trigeminovascular system

Tissue	CLR	RAMP1	RAMP2	RAMP3	CTR	Species	Reference	Notes
Cerebral vasculature								
Cerebral artery	RNA	RNA	RNA	RNA	–	Human	Sams and Jansen-Olesen, 1998.	
	RNA	RNA	RNA	RNA	–	Human	Jansen-Olesen *et al.*, 2003.	1
	Protein	Protein	–	–	–	Human	Edvinsson *et al.*, 2010.	2
	Protein	Protein	Protein	Protein	–	Human	Oliver *et al.*, 2002.	2
	Protein	Protein	–	–	–	Rat	Lennerz *et al.*, 2008.	
Meningeal arteries	Protein	Protein	Protein	Protein	–	Human	Oliver *et al.*, 2002.	2
	Protein	Protein	–	–	–	Human	Edvinsson *et al.*, 2010.	2
	RNA	RNA	RNA	RNA	–	Human	Sams and Jansen-Olesen, 1998.	
	RNA	RNA	RNA	RNA	–	Human	Jansen-Olesen *et al.*, 2003.	1
Pial arteries	Protein	Protein	Protein	Protein	–	Human	Oliver *et al.*, 2002.	2
Trigeminal ganglia								
Neuron cell bodies	Protein	Protein	–	–	–	Human, rat	Eftekhari *et al.*, 2010.	
	Protein	Protein	–	–	Protein	Rat	Walker and Hay, 2011.	
	Protein	Protein	–	–	–	Rat	Zhang *et al.*, 2007.	
					Protein	Rat	Tolcos *et al.*,2003	
		Protein	–	–	–		Tatji *et al.*, 2011	
Satellite glia	Protein	Protein	–	–	–	Human, rat	Eftekhari *et al.*, 2010	
	Protein	Protein	–	–	–	Rat	Zhang *et al.*, 2007.	
	Protein	Protein	–	–	–	Rat	Li *et al.*, 2008.	
Brainstem								
Spinal trigeminal nucleus	–	–	RNA	–	–	Rat	Stachniak and Krukoff, 2003.	
	Protein	Protein	–	–	–	Human	Eftekhari and Edvinsson, 2011.	
	Protein	Protein	–	–	–	Rat	Lennerz *et al.*, 2008.	
	–	Protein	–	–	Protein	Human	Bower *et al.*, 2011.	
	–	–	–	–	RNA	Mouse	Nakamoto *et al.*, 2000.	
					Protein	Rat	Tolcos *et al.*, 2003	
	–	–	–	–	Protein	Rat	Becskei *et al.*, 2004.	
Spinal trigeminal tract	Protein	Protein	–	–	–	Human	Eftekhari and Edvinsson, 2011.	

For vascular studies, human tissues have been cited in preference to those in animal models. RNA; detected by PCR or *in situ* hybridization. Protein; detected by histology. Entries marked with a '–' were not examined in that study. [1]Denuded of endothelium. [2]Co-localized with actin in VSMC.

other hand, the constitutive secretion of AM from vascular endothelial cells has been documented (Sugo *et al.*, 1994; Ishihara *et al.*, 1997) and suggests that AM receptors may be more important for longer-term effects, such as the maintenance of vascular tone (Nishio *et al.*, 1997) or integrity (Ichikawa-Shindo *et al*. 2008; Hagner *et al*., 2012).

Examining the relative pharmacological responses of CGRP and amylin or calcitonin in the vasculature may give insight into which CGRP-responsive receptor is involved in CGRP-induced vascular relaxation. The presence of an amylin response may indicate the presence of an AMY receptor, which either amylin or CGRP could act through. Amylin positive nerve fibres innervate the cat middle cerebral artery and amylin has been shown to induce relaxation in these arteries (estimated pIC_{50} 8.0) with similar potency to CGRP (pIC_{50} 8.3) but significantly lower maximal effect, whereas AM only caused weak relaxation. Interestingly, both Amy and CGRP responses were blocked equipotently by 1 μM $CGRP_{8–37}$, with pK_b's of 6.9 and 7.0, respectively, suggesting that the same receptor may be mediating these effects (Edvinsson *et al.*, 2001). In human cerebral arteries, amylin-induced relaxation (pIC_{50} 8.23) with 10-fold lower potency than CGRP (pIC_{50} 9.69), but higher potency than AM (pIC_{50} 7.21) (Jansen-Olesen *et al.*, 2003); suggesting that both CGRP and CTR or AMY receptors may be functional in these vessels.

CTR transcripts were observed in porcine coronary artery (Hasbak *et al.*, 2003) but functional AMY receptors did not appear to be present due to lack of amylin-induced relaxa-

tion. This is consistent with a lack of amylin-induced relaxation in human and rat coronary artery models (Sheykhzade and Nyborg, 1998; Hasbak *et al.,* 2001; 2003). Similarly, amylin has been reported to have only weak or no effect on relaxation of cerebral arterioles, basilar or middle cerebral arteries (Mori *et al.,* 1997; Jansen-Olesen *et al.,* 2001; Petersen *et al.,* 2005; Edvinsson *et al.,* 2007).

There is also heterogeneity in responsiveness to $CGRP_{8–37}$. Relatively weak antagonism of CGRP by $CGRP_{8–37}$ in meningeal, cerebral and coronary arteries (Wisskirchen *et al.,* 1999; Jansen-Olesen *et al.,* 2003) indicates that other receptors such as AMY receptors could mediate some effects of CGRP in blood vessels. Weak $CGRP_{8–37}$ antagonism could also be explained by poor tissue accessibility and a failure to reach equilibrium or partial agonist effects of $CGRP_{8–37}$ (Marshal and Wisskirchen, 2000). However, the human Col 29 cell line has also been reported to display a '$CGRP_2$' receptor-like phenotype with respect to $CGRP_{8–37}$ antagonism (Cox and Tough, 1994; Poyner *et al.,* 1998; Hay *et al.,* 2002). Interestingly, another study reported relatively high $CGRP_{8–37}$ affinity, alongside CLR, RAMP1 and RAMP2 expression (Choksi *et al.,* 2002). Therefore, even in the same cell type, CGRP receptor pharmacology can differ between studies. There is not a clear explanation as to why $CGRP_{8–37}$ sometimes has low affinity in tissues and cells where higher values would be predicted from the receptor expression pattern. On the other hand, in most cases, only a few receptor components are measured and it is always possible that multiple receptor phenotypes are present, complicating the interpretation of the experiment.

CGRP and receptor localization in the trigeminovascular system: the trigeminal ganglia

The TG houses the cell bodies of the sensory pseudounipolar neurons and also contains satellite glia and Schwann cells (Eftekhari *et al.,* 2010). The expression and localization of CGRP and the CGRP receptor has been extensively examined in both human and rat TG (Table 2). CGRP is expressed in small-medium neuron cell bodies, accounting for approximately 50% of neurons in the TG (Lennerz *et al.,* 2008; Eftekhari *et al.,* 2010). Stimulation of the TG by capsaicin, KCl or electrical impulse, results in the release of CGRP (Goadsby *et al.*, 1988; Capuano *et al.,* 2007). In fact, CGRP in the peripheral circulation, due to activation of the trigeminovascular system, is principally released from the TG (Hoffmann *et al.*, 2012). Primary cultured TG neurons have been shown to express functional CGRP receptors (Zhang *et al.,* 2007). In intact rat and human TG, an estimated 30–40% of neurons express the CGRP receptor components, CLR and RAMP1, with <1% of these neurons also co-expressing CGRP (Lennerz *et al.,* 2008; Eftekhari *et al.,* 2010). This suggests that CGRP could act directly on adjacent neurons or satellite glia. Several studies have observed both CLR and RAMP1 expression in satellite glia (Lennerz *et al.,* 2008; Li *et al.,* 2008; Eftekhari *et al.,* 2010); however, another study indicated that RAMP1 expression was low and up-regulated following organ culture (Tajti *et al.,* 2011). This is consistent with the finding that cultured TG glia were stained much less densely than TG neurons for CGRP receptor components (Zhang *et al.,* 2007). Furthermore, Eftekhari *et al*. (2010) noted that the expression was principally localized to the cytoplasm. The significance of these findings is unclear. However, functional effects of CGRP have been identified in cultured TG glia (Li *et al.,* 2008; De Corato *et al.,* 2011).

Studies reporting a link between the sensory nervous system and amylin suggest that other CGRP-responsive receptor subtypes may also be present in the TG (Gebre-Medhin *et al.,* 1998; Sibilia *et al.,* 2000). Furthermore, amylin is co-localized with CGRP in the cell bodies of TG (Edvinsson *et al.,* 2001) and dorsal root ganglia (DRG) neurons (Mulder *et al.,* 1997). Consistent with these findings, rat TG express CTR (Tolcos *et al.,* 2003) and cultured rat TG neurons, which respond to amylin, express AMY_1 receptor components, CTR and RAMP1 (Walker and Hay, 2011). Interestingly, some CLR-positive neurons in the TG were reported to be RAMP1 negative (Lennerz *et al.,* 2008). In the absence of a RAMP, CLR does not form a functional receptor (McLatchie *et al.,* 1998), suggesting that RAMP expression may be dynamic or that the TG could also contain either RAMP2 or RAMP3. The relatively weak cAMP response observed for AM in cultured TG neurons, however, suggests that RAMP2 or RAMP3 expression is unlikely (Walker and Hay, 2011).

CGRP and receptor localization in the trigeminovascular system: the brainstem

The brainstem is an important integration site for sensory signals from the periphery, including those from the TG, whose neural processes principally synapse in the STN (Cuello *et al.*, 1978; Arbab *et al.,* 1988). The brainstem is well documented to abundantly express neuropeptides, including CGRP (Van Rossum *et al.*, 1997) and AM (Serrano *et al.,* 2000). The STN contains CGRP binding sites (Van Rossum *et al.,* 1997). Neurons that project centrally from the TG co-express CGRP receptor components, CLR and RAMP1 (Lennerz *et al.,* 2008). This is consistent with the identification of co-expression of CLR and RAMP1 in the spinal trigeminal tract (Eftekhari and Edvinsson, 2011) and the detection of CGRP receptor positive projections in the STN (Lennerz *et al.,* 2008; Eftekhari and Edvinsson, 2011). Interestingly, CGRP expression in the spinal trigeminal tract (Smith *et al.,* 2002) was distinct from CGRP receptor expression (Eftekhari and Edvinsson, 2011). This stark division of CGRP and CGRP receptor expression is similar to that observed in TG cell bodies (Figure 2) (Lennerz *et al.,* 2008; Eftekhari *et al.,* 2010). In the STN, neuron cell bodies and astrocytes were observed to be devoid of CLR and RAMP1 expression (Lennerz *et al.,* 2008), suggesting that CGRP release in the STN may act directly, in a retrograde fashion on neurons originating from the TG. Alternatively, CGRP could act at a different receptor subtype.

The STN in rats expresses CTR as determined by immunohistochemistry (Tolcos *et al.,* 2003; Becskei *et al.,* 2004). In humans, neural projections and isolated cell bodies were identified which expressed CTR and RAMP1, although

co-localization has not been performed (Bower *et al.*, 2011). Salmon calcitonin binding sites have also been reported in the monkey STN, although amylin binding did not appear to be present in this study (Paxinos *et al.* 2004). Further, mRNA encoding RAMP2 has been identified in the rat STN (Stachniak and Krukoff, 2003), although there are little other data on RAMP2 or RAMP3 protein expression in the brainstem (Table 2). Nevertheless, the presence of CTR suggests that calcitonin and/or amylin receptors could be present in the STN. It should be noted that peptidergic neurons from the TG can also innervate other brainstem nuclei, including the nucleus of the solitary tract (South and Ritter, 1986). This suggests that stimulation of the trigeminovascular system may activate multiple regions of the brainstem and be much more complex than is currently appreciated.

CGRP mediated signal transduction in the trigeminovascular system: an overview

CGRP and related peptides are commonly accepted to couple $G\alpha_s$, which activates adenylate cyclase and subsequently elevates intracellular cAMP content (Walker *et al.*, 2010). In transfected cell line models, this robust activity has been utilized to comprehensively define the phenotype of CGRP, AM and amylin receptors (McLatchie *et al.*, 1998; Hay *et al.*, 2003; 2005; Bailey and Hay, 2006). However, GPCRs are often promiscuous and CGRP-mediated receptor activation and signalling likely involves coupling to other G-proteins or additional signalling adaptor proteins (Walker *et al.*, 2010). The signalling adaptor, β-arrestin, has been implicated in CGRP receptor signalling and regulation in cell lines (Walker *et al.*, 2010), but it is unclear if this adaptor is involved in the trigeminovascular system. Intracellular signalling is further complicated as distinct cell or tissue types may contain different compliments of intracellular signalling proteins or exhibit tissue-specific receptor post-translational modifications which can modulate downstream signalling (Tobin *et al.*, 2008). These processes have long been overlooked and may add new layers of complexity, which could be embraced to fine-tune new therapies in the future. In order to fully understand the role of CGRP in the trigeminovascular system, it is essential to define the signalling mechanisms triggered by CGRP in the individual component tissues.

CGRP mediated signal transduction in the trigeminovascular system: the cranial vasculature

The identification of CGRP as a potent vasodilator triggered interest in the mechanisms underlying CGRP-induced vascular relaxation. Although it is generally accepted that endothelium-independent vascular relaxation is mediated by the CGRP receptor in VSMCs, several findings discussed in this review indicate that an alternative CGRP-responsive receptor to the CGRP receptor may also be activated in VSMC layer.

Investigation of CGRP-mediated intracellular signalling in arteries and VSMCs shows that a $G\alpha_s$–mediated process is important in these vessels (Figure 3A). There are very few studies in cranial arteries detailing signalling downstream of cAMP; however, inferences can be made from other arteries, such as thymic or mesenteric (Champion *et al.*, 2003; Meens *et al.*, 2012). It should also be noted that use of different pre-contracting agents, including KCl, U46619 and noradrenaline, in arteries may complicate the interpretation (Yoshimoto *et al.*, 1998; Edvinsson *et al.*, 2007; 2010). CGRP-stimulated cAMP accumulation in arteries has been observed in numerous *in vivo* and *ex vivo* models, including rat pial arteries (Hong *et al.*, 1996), cat cerebral arteries (Edvinsson *et al.*, 1985), human coronary arteries (Gupta *et al.*, 2006), isolated rat aorta (Wisskirchen *et al.*, 1999) and cultured VSMCs (Kubota *et al.*, 1985; Ishizaka *et al.*, 1994; Casey *et al.*, 1997). Furthermore, CGRP-mediated relaxation was blocked by adenylate cyclase inhibitors, SQ-22536 and rp-cAMP in human thymic arteries (Champion *et al.*, 2003). The removal of the endothelium did not affect CGRP-induced cAMP accumulation (Edvinsson *et al.*, 1985; Champion *et al.*, 2003). A major consequence of CGRP-mediated cAMP accumulation in cranial and other arteries appears to be activation of PKA and a subsequent increase in inward K^+ current through ATP-sensitive K^+-channels (Kitazono *et al.*, 1993; Wellman *et al.*, 1998). However, in rat pial arteries, inhibition of ATP-sensitive K^+-channels resulted in blockade of both cAMP accumulation and relaxation (Hong *et al.*, 1996) suggesting that ATP-sensitive K^+-channels are not downstream of cAMP and that alternative mechanisms may be involved. The possibility that cAMP-independent pathways may be involved in VSMC mediated artery relaxation was highlighted in a recent study, where $G_{\beta\gamma}$ inhibitors blocked CGRP-mediated relaxation in mesenteric arteries, but increased cAMP accumulation in VSMCs (Meens *et al.*, 2012). Furthermore, in VSMCs, CGRP mediated acute activation of extracellular signal-regulated kinase 1/2 (ERK1/2), which was not blocked by PKA inhibitors (Iwasaki *et al.*, 1998). The activation of ATP-sensitive K^+-channels can increase ERK1/2 phosphorylation (Huang *et al.*, 2009). CGRP-induced relaxation in pial arteries was blocked by charybdotoxin, an inhibitor of Ca^{2+}-activated K^+ channels (Hong *et al.*, 1996). Overall, these studies provide evidence for the activation of both cAMP-dependent and independent pathways by CGRP in arteries. However, the relative importance of these pathways for each aspect of CGRP's function is unclear.

CGRP-mediated signalling in arteries is further complicated because AM and CGRP can also induce endothelium-dependent vasodilation (Figure 3B) (Brain and Grant, 2004). The relative importance of endothelium-induced vasodilation in the trigeminovascular system is unclear, but may account for some reports of CGRP or AM mediated cGMP or guanylate cyclase activation in arteries (Wisskirchen *et al.*, 1999; Champion *et al.*, 2003). CGRP or AM stimulate endothelium-induced vessel relaxation *via* cAMP mediated activation of nitric oxide synthase (NOS) and subsequent NO release. NO then acts on guanylate cyclase in associated VSMCs, resulting in cGMP accumulation and channel activation (Brain and Grant, 2004). Although CGRP clearly has effects in vessels, it is difficult to be as confident about the precise receptor responsible.

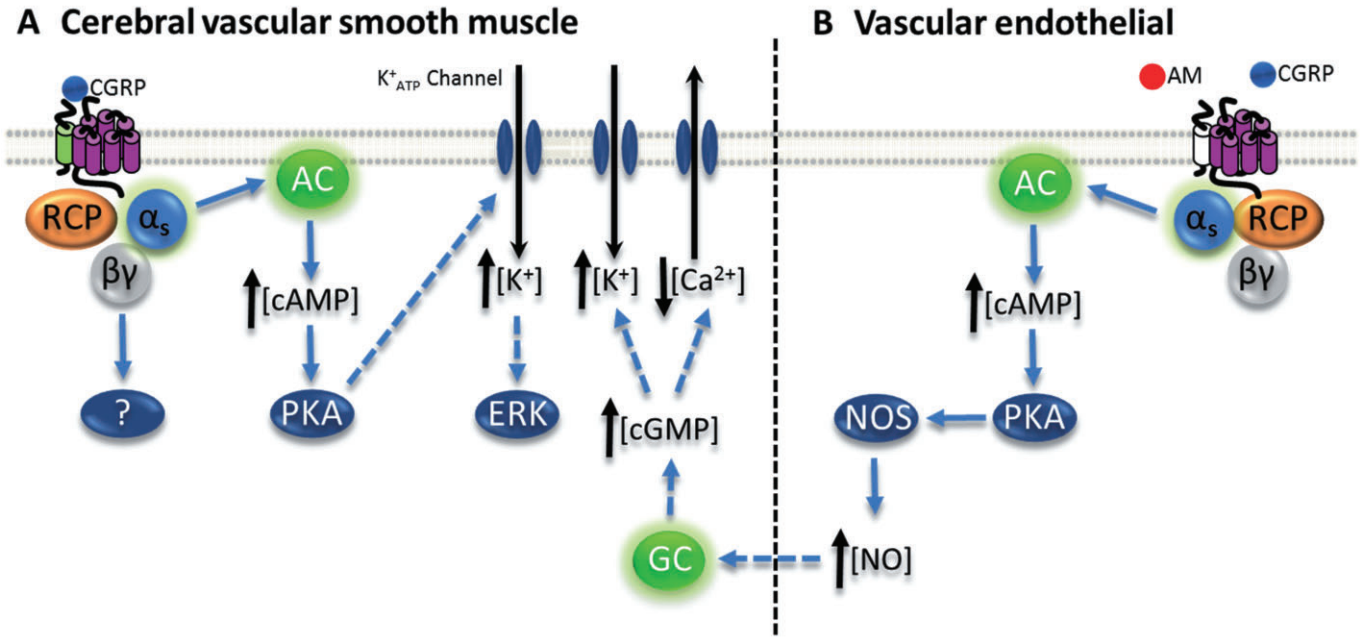

Figure 3

Proposed cranial CGRP-responsive receptor signalling in arteries. (A) In vascular smooth muscle cells the CGRP receptor activates a $G\alpha_s$-coupled signalling cascade. (B) In endothelium, activation of the CGRP receptor or an AM receptor (RAMP shown in white) activates $G\alpha_s$ and subsequently increases NO production, which can then act on vascular smooth muscle cells. Full arrows represent well-defined interactions, dashed arrows represent poorly defined or multistep pathways.

CGRP mediated signal transduction in the trigeminovascular system: the trigeminal ganglia

The TG contains at least two distinct cell types which express CGRP receptor components; neurons and satellite glia (Eftekhari *et al.*, 2010). Despite extensive research describing the mechanisms regulating CGRP expression and release in the TG (Raddant and Russo, 2011; Messlinger *et al.*, 2011), the effects of CGRP on the TG neurons and glia are far less well defined (Figure 4A and B).

In accordance with cell line studies, CGRP induced a concentration-dependent elevation in cAMP content in neuron-enriched TG cultures (Zhang *et al.*, 2007; Walker and Hay, 2011), similar to those observed in DRG cultures (Anderson and Seybold, 2004; Ng *et al.*, 2012). In one study in TG neurons, amylin and CGRP were equipotent in elevating cAMP, suggesting that functional CGRP and/or AMY receptors are present in these cells (Walker and Hay, 2011). Downstream of cAMP accumulation, CGRP potentially regulates the activity of PKA and cAMP response element binding protein (CREB); activation of both has been reported in TG neurons (Zhang *et al.*, 2007; Simonetti *et al.*, 2008; Walker and Hay, 2011). However, CREB phosphorylation was reportedly dependent on Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activation in TG neurons (Simonetti *et al.*, 2008). Interestingly, CGRP did not affect intracellular Ca^{2+} content in either cultured TG or DRG neurons (Anderson and Seybold, 2004; Cady *et al.*, 2011) indicating that CaMKII may be activated by a Ca^{2+}-independent mechanism. β-arrestin, which is known to interact with the CGRP receptor (Hilairet *et al.*, 2001), can activate CaMKII in conjunction with exchange proteins activated by cAMP (Mangmool *et al.*, 2010). Given the prevalence of reports indicating the involvement of mitogen-activated PK activation in CGRP-mediated signalling (Walker *et al.*, 2010), it is unsurprising that CGRP administration into the temporomandibular joint increases p38 and ERK1/2 activation in TG neurons (Cady *et al.*, 2011). Furthermore, capsaicin administration (which stimulates the release of CGRP and other factors from sensory neurons) was associated with ERK1/2 activation. However, this was not blocked by 900 μg kg^{-1} olcegepant in TG neurons, although the activation of c-fos in the STN was blocked by the antagonist (Sixt *et al.*, 2009), implying that TG ERK1/2 could be activated by a less olcegepant sensitive CGRP-responsive receptor, such as AMY_1 or AM_2 receptor or that capsaicin-induced ERK1/2 activation, was not due to CGRP. ERK activation could potentially be a direct result of capsaicin-induced transient receptor potential vanilloid 1 and neuron activation, or caused indirectly by other molecules co-released with CGRP, such as substance P (Helke *et al.*, 1981). Interestingly, SK-N-MC cells, a widely used CGRP receptor model, do not display ERK1 phosphorylation in response to CGRP (Disa *et al.*, 2000). It should be noted that signalling responses attributed directly to CGRP, such as ERK activation, could sometimes be controlled indirectly by secondary factors released upon CGRP stimulation. For example, CGRP may induce the release of vasoactive factors from mast cells (Theoharides *et al.*, 2005) or brain-derived neurotrophic

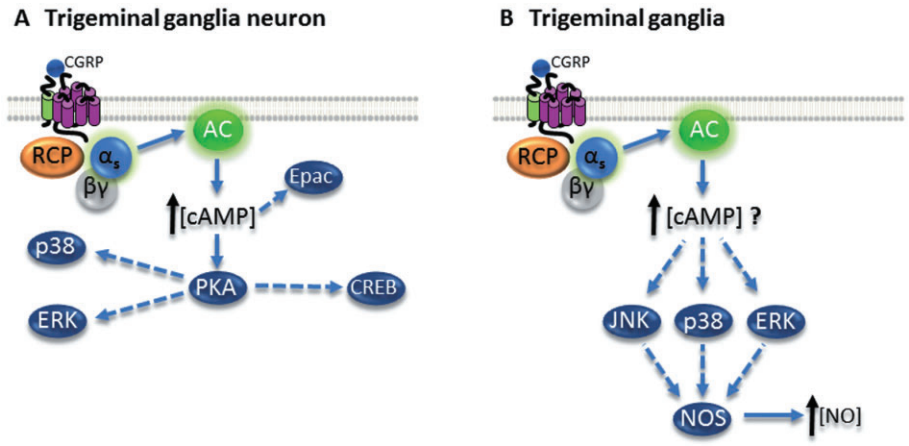

Figure 4

Proposed CGRP-responsive receptor signalling in the trigeminal ganglia. (A) In trigeminal ganglia neurons the CGRP receptor activates a $G\alpha_s$-coupled signalling cascade. (B) In satellite glia, activation of the CGRP receptor likely activates $G\alpha_s$ and subsequently increases NO production. Full arrows represent well-defined interactions. Dashed arrows represent poorly defined or multistep pathways.

factor from TG neurons (Buldyrev *et al.*, 2006), which could activate their own signalling cascades.

The mechanisms underlying CGRP signalling in TG glia probably involve coupling to $G\alpha_s$ and cAMP. Although no direct measurements of cAMP accumulation in response to CGRP have been reported in TG glia, cAMP or cAMP mimetics have been shown to mimic glial effects of CGRP in these cells (Li *et al.*, 2008; De Corato *et al.*, 2011). However, it is possible that cAMP-independent mechanisms are involved. The activation of MAPK cascades could represent major downstream components of CGRP signalling in TG glia. The phosphorylation of ERK1/2 in TG glia has been reported in both *in vivo* and *in vitro* models (Cady *et al.*, 2011; Ceruti *et al.*, 2011). Furthermore, activation of p38, ERK1/2 and c -jun N-terminal kinase in response to CGRP increased iNOS expression, leading to a longer-term elevation in NO production (Li *et al.*, 2008; Vause and Durham, 2009). CGRP has also been shown to elevate intracellular Ca^{2+} in TG glia (Ceruti *et al.*, 2011).

CGRP mediated signal transduction in the trigeminovascular system: the brainstem

The signals that convey cranial pain have been hypothesized to involve CGRP release from the trigeminal nerve in the STN of the brainstem (Messlinger *et al.*, 2011). However, it has also been suggested that CGRP, released in the STN, could act on trigeminal neurons which express CGRP receptors (Lennerz *et al.*, 2008). This region is particularly important in migraine, which is associated with increased activity of the STN (Stankewitz and May, 2011). The activation of neurons in the STN by capsaicin and NO-donors is blocked by olcegepant (Koulchitsky *et al.*, 2009; Sixt *et al.*, 2009). However, the significance of these findings is unclear. Furthermore, brainstem membranes did not respond to CGRP by producing cAMP (Stangl *et al.*, 1993). This observation is not in intact cells or tissue and should be confirmed using more sensitive methods. Despite the potential abundance and importance of CGRP in the brainstem, the underlying signalling mechanisms remain unstudied.

Conclusions and implications

The unique molecular identity of CGRP and related receptors, coupled with the complex nature of CGRP action, makes understanding its underlying biology and signalling mechanisms a distinct challenge. Despite these hurdles, considerable progress has been made in the development of CGRP receptor antagonists for the treatment of migraine. Although effective, there has been some controversy surrounding the efficacy of these candidate anti-migraine drugs (Tfelt-hansen, 2011). This discrepancy has been rationalized by a possible CNS site of action for these drugs (Tfelt-Hansen and Olesen, 2011).

In this review, we highlight another possible contributing factor. Potentially, the pathological effects of CGRP in migraine could be mediated through more than one distinct CGRP-responsive receptor. This is because components of other CGRP-responsive receptors are present in the trigeminovascular system and pharmacological phenotypes indicate receptor heterogeneity. The AMY_1 receptor is a likely candidate as it displays high affinity for CGRP but is only weakly antagonized by both olcegepant and telcagepant (Hay *et al.*, 2006; Moore and Salvatore, 2012). A great deal of work is required to determine if this is the case through careful and thorough analysis of receptor expression and pharmacological profiles in cells and tissues of interest. Once the precise receptor(s) involved are confirmed, in both healthy and diseased states, the development of signalling pathway-selective or biased drugs can be considered. These could have greater efficacy and/or fewer side effects. For furthering CGRP as a target in migraine and other conditions, it is important to

define which receptors and signalling pathways are the most relevant in appropriate disease models.

Conflict of interest

There are no conflicts of interest to declare.

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