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Susan D. Brain
Pierangelo Geppetti *Editors*

Calcitonin Gene-Related Peptide (CGRP) Mechanisms

Focus on Migraine

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Preface

We have enjoyed creating this book at this timepoint where we are starting to see the therapeutic benefits of the many years of CGRP research, since its discovery nearly 40 years ago. Our aim has been to cover the major issues relating to CGRP in keeping with the long-standing focus of the *Handbook of Experimental Pharmacology* series. Our aim has also been to describe the development of knowledge concerning the action and potential of CGRP ligands, leading to the proof of its role in migraine and other headaches. There are some fascinating twists and turns in this story.

The first was the realization that this potent vasodilator peptide CGRP, discovered in the thyroid tissue of elderly rats, is primarily found in sensory nerves. Indeed, those very nerves had been investigated due to their links with another neuropeptide substance P. Early studies involved their comparison, but it became clear in time that CGRP had a very distinct profile, with CGRP being the most prominent in the pathophysiology of migraine. Not only was CGRP found in the trigeminovascular system but it was realized by Goadsby and Edvinsson that it is released into the jugular vein during migraine. Of importance its inhibition was observed with treatment with sumatriptan and as migraine resolved. Finally, the injection of CGRP can induce migraine.

The second concerned the discovery of the unique receptor for CGRP; this caused a few false starts, but the manuscript of McLatchie and co-workers still makes interesting reading. This volume has recent information on this intriguing receptor family that we still are only just beginning to understand. Finally, after the development of the first non-peptide antagonist by Doods and colleagues at Boehringer, it was realized that small molecule antagonists of the CGRP receptor benefitted migraine in a manner that does not implicate the worrisome vasoconstriction of the triptans. However, this positivity was soon dashed when it was realized that treatment with such small molecules was associated with a rise in the plasma levels of liver enzymes in certain of the patients.

At some point during this journey of discovery, it was realized that antibody therapy that was already in use for arthritis (e.g. the anti-TNF biologics) could be useful for migraine. There was intense debate in that it may be difficult for antibodies whose action is restricted to the periphery to target the migraine-provoking CGRP. Notwithstanding, several companies started to develop antibodies. Rumours started

to surface in about 2012 that these antibodies were beneficial in migraine, with substantial evidence of benefit and negligible side effects by the time we started to plan this book. During its preparation we have seen their legal and ethical clearance for use in the treatment of migraine. There are now many reports of patients benefitting from this new class of therapy for migraine.

This has been a fantastic journey to date and we now look forward to the next stages and key questions, including the following: Will these antibodies be of use in other pain-related ailments? Can the prolonged use of such antibodies be considered as disease-modifying treatments? Will the orally available CGRP antagonists that remain in development 'push' the antibodies into a less prominent therapeutic place, due to their easier mode of administration?

We have assembled in this volume chapters from experts who have contributed to the development of our knowledge concerning CGRP. Many have been pivotal in the CGRP field. We thank everyone for their hard work in producing these manuscripts, which enables such a timely and complete volume. We would like to thank everyone, including those at Springer, who have worked so carefully to ensure the completed work for publication.

London, UK — Susan Brain
Florence, Italy — Pierangelo Geppetti

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CGRP Discovery and Timeline

Kate Arkless, Fulye Argunhan, and Susan D. Brain

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Abstract

Calcitonin gene-related peptide (CGRP) was discovered over about 35 years ago through molecular biological techniques. Its activity as a vasodilator and the proposal that it was involved in pain processing were then soon established. Today, we are in the interesting situation of having the approval for the clinical use of antagonists and antibodies that have proved to block CGRP activities and benefit migraine. Despite all, there is still much to learn concerning the relevance of the vasodilator and other activities as well as further potential applications of CGRP agonists and blockers in disease. This review aims to discuss the history and present knowledge and to act as an introductory chapter in this volume.

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Cardiovascular · CGRP · Migraine · Review

1 Discovery of the Sensory Neuropeptide Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide that was discovered in 1982 (Amara et al. 1982). It was realised that alternative processing of the RNA transcripts of the calcitonin gene leads to the generation of mRNA that encodes for CGRP instead of calcitonin. This discovery also revealed that calcitonin is the major product in healthy thyroid tissue, but a distinct mRNA precursor to CGRP predominates in the hypothalamus (Amara et al. 1982). This group, using recombinant DNA and molecular biology techniques, then quickly realised that CGRP is a neuropeptide. They also revealed its sensory neuronal localisation, in the rat trigeminal ganglia as well as sensory ganglia of the spine and in 'small beaded fibres' that innervated a range of organs and including the smooth muscle of blood vessels (Rosenfeld et al. 1983). They correctly suggested that it was involved in mediating functions that involved nociception, cardiovascular regulation and gastric-intestinal regulation (Rosenfeld et al. 1983). Indeed, they demonstrated that CGRP has potent effects on blood pressure regulation and catecholamine levels, primarily concentrating on the concept that CGRP acts on the central nervous system to stimulate sympathetic outflow of noradrenergic transmitters mediating increased blood pressure. They did, however, also show (but did not mention in the abstract) that CGRP caused a reduction in blood pressure. This was not further investigated except to point out that they observed a generalised vasodilation (Fisher et al. 1983). This manuscript emphasised their realisation that 'whilst this report is the first to describe biological actions of CGRP, future investigations are required to define its potential physiological roles as an intercellular transmitter'. This was swiftly followed by the publication of the structure of human CGRP, which had a high structural homology to rat CGRP (Morris et al. 1984) and its presence in the plasma of patients with medullary thyroid carcinoma (Morris et al. 1984). At the time, there was also confirmation that CGRP is localised to the spinal cord of humans and in a wide range of species (Gibson et al. 1984). It was then realised that human CGRP is a potent vasodilator, especially in the microcirculation (Brain et al. 1985). Indeed, it was suggested to be the most potent microvascular vasodilator known as the intradermal injection of femtomolar doses that induced a local erythema, due to increased blood flow that lasted for several hours in human skin (Brain et al. 1985). By comparison, substance P, prostaglandins and other microvascular vasodilators appeared substantially weaker (Brain et al. 1985). Later that year, it was shown that CGRP is a hypotensive agent in humans and associated with facial flushing in keeping with its potent microvascular effects (Gennari and Fischer 1985; Struthers et al. 1986). The examination of the effect of CGRP in human skin revealed that it was not only a more potent vasodilator than other known agents but also had a sustained duration of action (Brain et al. 1986b). Since the peripheral vasodilator activity of CGRP was

discovered, there has been an understanding that CGRP can mediate vasodilation via endothelial-dependent and endothelial-independent mechanisms (Brain and Grant 2004). A range of signalling mechanisms by which CGRP may act in both vascular and non-vascular signalling have been reported (Russell et al. 2014) and are further examined in this book (Fig. 1).

Moreover, it was realised at an early stage that CGRP coexists with substance P in sensory nerves, which is of potential functional relevance, in terms of modulating the effects of inflammatory mediators and responses (Brain and Williams 1985; Lundberg et al. 1985). However, CGRP may also exist in nerves alone, especially Aδ fibres and in the CNS and has been suggested to be produced via several non-vascular cells in addition (Russell et al. 2014).

2 Family and Structure

It was soon discovered that the 37-amino acid peptide CGRP exists as two isoforms (α and β). These peptides are coded by separate regions of chromosome 11. They differ minimally in structure and species, possessing a disulphide bridge between the Cys2 and the Cys7 residues of the peptide. The β form is synthesised via a separate gene and originally considered to be found in the gut and brain (Steenbergh et al. 1986). These α and β forms (also known as I and II) have very similar activities, in terms of their vasodilator activity (Brain et al. 1986a; Franco-Cereceda et al. 1987). The CGRP peptides were realised, over the next few years, to be the members of a family that not only includes calcitonin but also the structurally related peptides amylin (AMY), adrenomedullin (AM) and intermedin, also called adrenomedullin-2 (AM2) and sometimes called adrenomedullin-2/intermedin (Russell et al. 2014). Whilst calcitonin plays an important role in calcium regulation, the other members of the family all possess cardiovascular properties as well as inflammatory/metabolic biological properties. All have more recently become the targets for potential therapeutic approaches and are heavily investigated. Of note, an amylin analogue (Pramlintide, marketed as Symilin in the USA) is administered by subcutaneous injection (Wysham et al. 2008). It is effective but has a short half-life.

3 Receptors

It took over a decade of intense research to discover the unique nature of the CGRP receptors. Whilst a truncated CGRP peptide ($CGRP_{8\text{-}37}$) was shown to have antagonist activity at an early stage in cardiovascular tissue, it did not have this activity in certain other tissues, leading to the suggestion that two receptors existed (Chiba et al. 1989). Independently, Kapas and co-workers suggested that two structurally related receptors RDC1 for CGRP and L1 for adrenomedullin existed (Kapas et al. 1995; Kapas and Clark 1995). However, these receptors could not be confirmed by other workers. Eventually in 1998, a key publication revealed that CGRP acts through a receptor complex (McLatchie et al. 1998). The main subunit of the complex is a

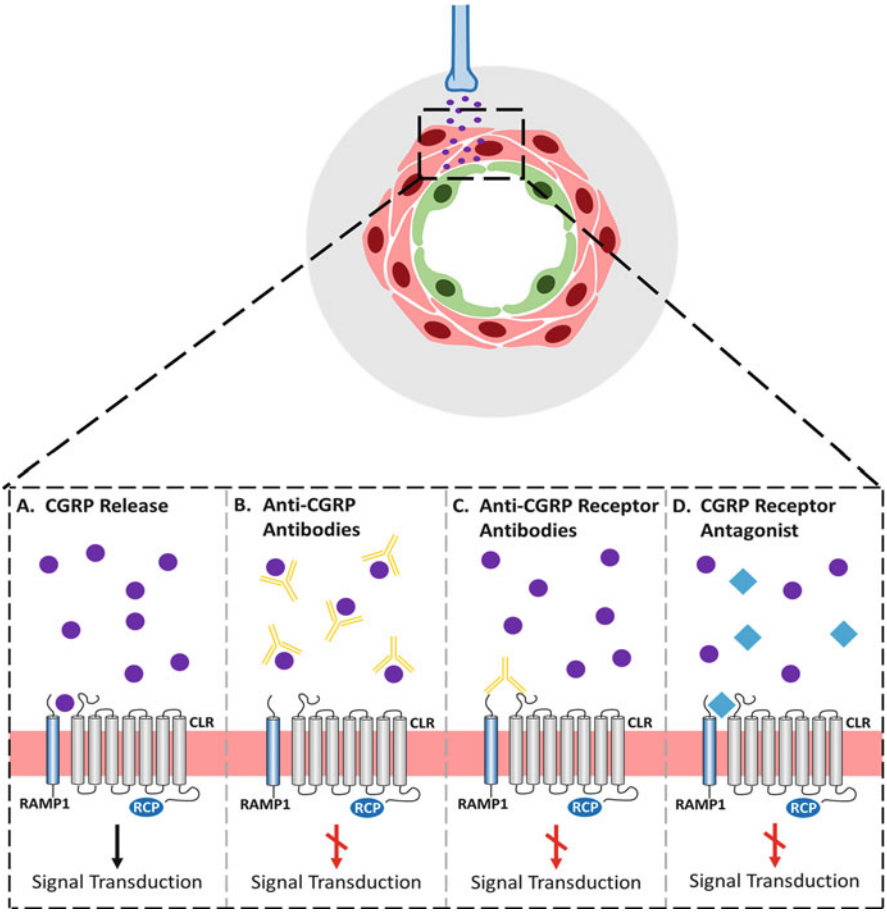

Fig. 1 Possible mechanisms to block calcitonin gene-related peptide (CGRP) signalling. (**a**) The neuropeptide, CGRP, is released from sensory nerves and acts through a heterodimeric receptor complex, composed of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein (RAMP1), along with an intracellular receptor component protein (RCP). Upon binding, signal transduction occurs, leading to vasodilation and other cardioprotective mechanisms. (**b**) One possible method of blocking CGRP is the use of anti-CGRP antibodies, such as LY2951742. Here, free CGRP is no longer available to bind its receptor complex, thus blocking signal transduction. In this scenario, however, other molecules with affinity for the CGRP receptor complex, such as adrenomedullin, may bind instead. (**c**) Antibodies can also be raised against the CGRP receptor complex to block signalling. For example, the human monoclonal antibody, AMG 334 (Erenumab) is currently in Phase III clinical trials for treatment of migraine. Blocking of this complex may cause CGRP to bind other receptors for which it has affinity, such as the amylin 1 receptor complex. (**d**) CGRP receptor antagonists, such as the small molecule inhibitor, BIBN4096BBS may also be used to block CGRP signalling but, again, any remaining free CGRP may still be able to bind other receptors

G-protein calcitonin receptor-like receptor (CLR) of the family B of G-protein coupled receptors. However, CLR could only act when linked to a single transmembrane protein that became known as receptor activity-modifying protein (RAMP). It was realised from cell studies that there are three RAMPs (RAMP1, RAMP2 and

RAMP3) and that the co-localisation at the cell membrane of RAMP1 with CLR revealed a functional CGRP receptor (McLatchie et al. 1998). Moreover, it soon became established that CLR and RAMP1 comprise a receptor with high affinity for CGRP, CLR and RAMP2 comprise a receptor with high affinity for adrenomedullin and CLR and RAMP3 comprise a receptor with various agonists of the CGRP family (McLatchie et al. 1998). Unlike CGRP and adrenomedullin, intermedin does not appear to preferentially stimulate a receptor and is described as a non-selective agonist of the CGRP receptors (Roh et al. 2004). The RAMP proteins are important for aiding the movement of CLR to the cell membrane and through interaction with CLR provide a binding site for ligands (McLatchie et al. 1998). This is a somewhat unique system allowing different pharmacology to be exhibited by each receptor, which will be further discussed in this book. The receptor is further complicated by the presence of a receptor component protein (RCP). RCP enhances the efficiency of the signalling of the CGRP receptor (Evans et al. 2000), most probably via Gs, although a range of other pathways exist (Walker et al. 2010).

One highly significant finding was that of the realisation that the amino acid residue 74, a basic tryptophan in non-primates, is a tryptophan in humans, leading to species selectively with respect to antagonists (Mallee et al. 2002). Meanwhile, amylin associates with the calcitonin receptor CTR and RAMPs, thus providing a family of receptors that are less well understood and will be discussed further in this book. Intriguingly, CGRP has a similar affinity to one of the amylin receptors (AMY1(a), comprised of the CTR and RAMP1 as for the CGRP receptor); the in vivo relevance is unclear, but further discussed (Walker et al. 2015; Hay et al. 2018).

4 Migraine

An early indicator that CGRP could act in the cerebral circulation was given by Hanko and co-workers studying the pial vessels of animals and humans in vitro (Hanko et al. 1985). It was found to be present in the trigeminal ganglion in rat, in more neurons than substance P (Lee et al. 1985). Edvinsson and colleagues revealed the ability of CGRP to relax cerebral vessels via cAMP as well as via other endothelial-dependent mechanisms (Edvinsson et al. 1985) and that it was more potent than substance P, indicating its potential physiological significance in the trigeminocerebral circulation, although migraine was not mentioned (McCulloch et al. 1986). The possibility that CGRP was involved in extracranial pain was raised (Uddman et al. 1986). A key step forward was the realisation that CGRP could be released into the jugular vein of cats after electrical stimulation of the trigeminovascular system, a model associated with migraine and in humans, during an operation involving thermo-coagulation stimulation, where facial flushing was also observed in some individuals (Goadsby et al. 1988). The use of a novel intravital microscopy rodent model involving a closed cranial window revealed that CGRP, rather than substance P, was responsible for the vasodilation (Williamson et al. 1997). The CGRP-induced dural vasodilation was suggested to mediate trigeminal sensitisation that was reduced in the presence of a 5HT1B/1D agonist (Cumberbatch et al. 1999); thus facilitating development of the hypothesis that migraine is a neurovascular disease.

It was shown using similar techniques in 1993 that in the animal model, the stimulation of the trigeminal ganglion was associated with increased blood flow and release of CGRP that was inhibited when either sumatriptan or dihydroergotamine were administered (Goadsby and Edvinsson 1993). A link between the therapeutic benefit and the effect of these drugs on reducing the release and action of CGRP was important. The human study revealed that sumatriptan, in addition to relieving the migraine, was highly associated with reduced levels of CGRP measured in the jugular vein (Goadsby and Edvinsson 1993).

CGRP has also been shown to induce migraine-like symptoms after intravenous infusion (Lassen et al. 2002) and this is further discussed in the book. This was perhaps some of the first key evidence that CGRP may act peripherally to affect the cerebral circulation in terms of enhancing pain. However, by comparison CGRP injected into the human forearm stimulates a sustained local increased blood flow, but not nociception in terms of either pain or itch (Brain et al. 1986b; Pedersen-Bjergaard et al. 1991).

5 Antagonists

The first non-peptide CGRP antagonist Olcegepant (BIBN4096BS) was developed by Doods and co-workers. It exhibited an affinity for CGRP receptors in the pM range and inhibited the facial flushing after stimulation of the trigeminal ganglion, in primates (Doods et al. 2000), as previously shown with CGRP. It was found to be effective as an anti-migraine treatment in humans for up to 6 h after onset (Olesen et al. 2004). This book will further chart the development of antagonists and antibodies since then.

Olcegepant has been described as the first potential anti-migraine therapy that is not a vasoconstrictor and indeed this has been supported by more recent studies. Telcagepant (MK-0974) which was available orally confirmed this promise. However, hepatic adverse indications were revealed through elevated plasma transaminase during phase III clinical trials (Ho et al. 2016). There remains a question over whether this adverse effect is due to the effects of inhibiting endogenous CGRP, or related to the chemistry of this specific type of CGRP antagonist (Gottschalk 2016). Importantly, supporting the latter suggestion, recently ubrogepant (MK-1602) has been shown to be effective in the acute treatment of migraine, without effects on the liver (Voss et al. 2016). The overall high tolerability of the CGRP antagonists, apart from the hepatic adverse events, is good and now well documented (Bigal et al. 2013; Gottschalk 2016). Furthermore, there is good evidence that CGRP antagonists do not affect blood pressure, under circumstances examined in humans to date.

6 Antibodies

Clinical trials for monoclonal antibodies with CGRP have been equally successful, when compared with the antagonists in benefitting migraine, and have been substantially reviewed elsewhere (Bigal et al. 2013; Deen et al. 2017). Positive clinical trials have been published for four antibodies to date. These are Galcanezumab, LY2951742 (Lilly), Eptinezumab, ALD403 (Alder) and Fremanezumab, LBR-101 (Teva), which

all target the peptide and finally Erenumab AMG334 (Amgen), which targets the CGRP receptor. The positive results with AMG334, in a phase 2 trial, are indicative that the CGRP receptor plays a primary role in migraine (Sun et al. 2016). These compounds may both prevent and benefit migraine. To date, there have been no hepatic adverse effects observed. These will be further discussed in this book. The trials with some of these compounds have now progressed to a stage where we have approval for some of these compounds for clinical use.

There is substantial excitement concerning the antibodies as the clinical trials have shown benefit in several types of migraine, with a low incidence of mild adverse effects. It is unlikely that antibodies cross the blood–brain barrier, thus it is widely thought that agents act via peripheral mechanisms. This understanding has led to a range of hypotheses being developed for the site of action that will be further explored in this book.

7 Other Indications for Calcitonin Gene-Related Peptide Antagonists and Antibodies

It has proved difficult to fully understand the physiological roles of CGRP, due to its broad localisation that encompasses practically all tissues of the body. It is therefore not surprising that it has been suggested to play a large role in a number of syndromes [recent review is given by Russell et al. (2014)] and in the neuro-immune axis (Assas et al. 2014). CGRP has also been suggested to play a central role in skin blushing and in various situations associated with menopause (Sharma et al. 2010; Hay and Poyner 2009; Russell et al. 2014). Potentially, CGRP antagonists may be of use in these syndromes. CGRP is suggested to be involved in painful arthritis from rodent studies (Nieto et al. 2015; Bullock et al. 2014); however, there is no evidence of a beneficial role of CGRP antagonists/antibodies to date. A clinical trial was carried out involving patients with osteo-arthritis knee pain, and the monoclonal antibody LY2951742, but unlike treatment with the positive control celecoxib, LY2951742 did not provide benefits under the conditions examined (Jin et al. 2016). Further information is given in a systematic review covering the subject of 'CGRP and non-headache pain' (Schou et al. 2017) and in this book.

8 Role of Calcitonin Gene-Related Peptide in the Cardiovascular System

The development of CGRP antagonists and antibodies that are beneficial in clinical trials involving migraines allows us to consider what other primary roles CGRP may have. Perhaps surprisingly, neither CGRP antagonists nor antibodies have significant effects on the physiological regulation of blood pressure or in influencing tendencies to peripheral vasoconstriction [reviewed in Bigal et al. (2013) and Russell et al. (2014)]. This indicates that CGRP is not a functionally important regulator of vascular tone. This would seem surprising considering its wide distribution within the cardiovascular system, potent vasodilator activity and evident presence of CGRP

receptors. One possibility is that CGRP is only protective when the cardiovascular system is stressed and this is the subject of ongoing debate (MaassenVanDenBrink et al. 2016). This is possible as clinical studies to date have involved humans those in the majority have a healthy cardiovascular system. However, the studies carried out to date include exercising on a treadmill with patients suffering from angina (Chaitman et al. 2012). These authors suggested that CGRP was redundant in their model, where other endogenous vasodilators presumably take a more vital role (Chaitman et al. 2012). The concept that wiping out CGRP may be detrimental to cardiovascular function is further explored in this book.

9 The Therapeutic Potential of Calcitonin Gene-Related Peptide Agonists

The previous sections outline scenarios that lead to the possibility that there may be insufficient endogenous CGRP, released in humans for its blockade, to markedly affect the cardiovascular system. This may perhaps, in part due to evolutionary progress. Alternatively, it has been shown that endogenous CGRP levels can decrease with disease, for example, plasma levels in chronic congestive heart failure (Taquet et al. 1992). This may be due to increased sensory activity during the disease subsequently leading to a loss of sensory nerve function due to depletion/desensitisation. This is despite receptors for CGRP being present in the vasculature in an active form and able to respond to endogenous ligands. If this is the case, then CGRP agonists may potentially have a beneficial role in protecting against the harm and severity of cardiovascular diseases such as heart failure. Indeed, there is some historical evidence that this may occur in humans. Firstly, Gennari and colleagues gave intravenous CGRP to five patients with heart failure. Whilst there were small effects on blood pressure and heart rate, an improved contractility was observed in all (Gennari et al. 1990). In a separate study, Shekhar and colleague studied an 8-h infusion time in nine patients with congestive heart failure, without evidence of tolerance. CGRP was only effective whilst being infused and acted to increase cardiac output, stroke volume and, interestingly, renal blood flow. However, there was little effect on blood pressure (Shekhar et al. 1991).

Finally, CGRP can dampen the immune responses in a variety of situations (Russell et al. 2014; Assas et al. 2014); but it is difficult to determine where and whether these effects may be pivotal in humans. Three interesting possibilities that are under current investigation are that firstly, CGRP has been shown to reduce Langerhans cell-induced HIV-1 transmission and to enhance proteasomal degradation (Ganor et al. 2013; Bomsel and Ganor 2017); secondly, CGRP has been suggested to mediate immuno-suppression in lung infections (Baral et al. 2018); and thirdly, CGRP may play a primary sensing role, that is protective and includes the parabrachial nerves in the CNS (Campos et al. 2018).

10 Conclusion

This introductory chapter acts as a review of current knowledge concerning CGRP in physiology and disease and the results from clinical trials using CGRP antagonists and antibodies to date. The potential of these concepts will be further discussed in this volume.

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CGRP Receptor Biology: Is There More Than One Receptor?

Debbie L. Hay

Contents

Abstract

Calcitonin gene-related peptide (CGRP) has many reported pharmacological actions. Can a single receptor explain all of these? This chapter outlines the molecular nature of reported CGRP binding proteins and their pharmacology. Consideration of whether CGRP has only one or has more receptors is important because of the key role that this peptide plays in migraine. It is widely thought that the calcitonin receptor-like receptor together with receptor activity-modifying protein 1 (RAMP1) is the only relevant receptor for CGRP. However, some closely related receptors also have high affinity for CGRP and it is still plausible that these play a role in CGRP biology, and in migraine. The calcitonin receptor/RAMP1 complex, which is currently called the AMY_1 receptor, seems to be the most likely candidate but more investigation is needed to determine its role.

Keywords

AMY_1 · CGRP · CGRP receptor · Migraine · RAMP1

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1 Introduction

Calcitonin (CT), amylin, CT gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin 2/intermedin (AM2) comprise the major members of the CT family of peptides. Although the members of this family share only low amino acid sequence homology, they have common structural features. Each peptide has two cysteine residues close to its N-terminus which form a disulphide bridge; this region is critical for biological activity in all cases. An α-helical region is present towards the N-terminus of each of the peptides and an amidated residue at the carboxy terminus is also a family trait.

CGRP has long been known to trigger receptor-dependent cellular signalling. However, the pharmacology and molecular identity of the receptor(s) is not "clean". This chapter will describe the molecular composition of known CGRP binding proteins, the activity of CGRP in relation to related peptides within the same family and in broad terms how this information reconciles with CGRP actions in cells and tissues. An important consideration is the behaviour of receptors from different model species. In order to understand CGRP receptors, it is also important to consider related peptides and their receptors because of the substantial functional overlap between their actions.

2 Proteins with Affinity for Calcitonin Gene-Related Peptide

The first molecular candidates for AM and CGRP receptors were proposed in 1995. One of these was a rat receptor that was reportedly responsive to AM. This G protein-coupled receptor (GPCR), receptor named L1 (or G10d), had a similar pattern of mRNA expression to that of ^{125}I-AM binding in rat tissues and when transfected into Cos-7 cells, elevated cAMP with an approximate EC_{50} of 7 nM in response to AM (Kapas et al. 1995). Also in 1995, the same group reported that a then orphan canine GPCR, RDC-1 (which shares approximately 30% sequence identity with L1), was a CGRP receptor as transfection into Cos-7 cells yielded cAMP production in response to CGRP, which could be blocked by $CGRP_{8-37}$. AM could also stimulate cAMP levels in cells transfected with RDC-1 with potency that might be expected for a CGRP receptor (Kapas and Clark 1995). In 1997, a potential human AM receptor with 73% amino acid homology to L1 was reported but the receptor had a different expression pattern to the rat receptor (Hanze et al. 1997). Attempts to further characterise these receptors as AM and CGRP receptors, respectively, have been unsuccessful (Kennedy et al. 1998; McLatchie et al. 1998). No binding of rat or human ^{125}I-AM could be found following transfection of either the putative human or rat AM receptor sequences. Furthermore, expression of RDC-1 in *Xenopus* oocytes and human embryonic kidney (HEK) 293 cells did not change the cellular response to CGRP (McLatchie et al. 1998). A report describing correlations between AM and CGRP receptor binding and their proposed molecular counterparts found no correlation between CGRP binding and RDC-1 mRNA in rat tissues (Chakravarty et al. 2000). According to the guidelines produced by the relevant

nomenclature committee of the International Union of Pharmacology, these receptors are not considered to be candidate AM or CGRP receptors (Hay et al. 2018; Poyner et al. 2002). The human receptor corresponding to RDC-1 is now known as atypical chemokine receptor 3 and thought to be a decoy receptor; it was formerly called GPR159 or CXCR7 (see www.guidetopharmacology.org). However, another link between AM and this receptor has been found, whereby this receptor has been proposed as a decoy receptor for this peptide (Klein et al. 2014). More work is needed to determine how significant this finding is to AM biology. L1 is known as GPR182 and is considered to be a class A orphan GPCR.

In addition to L1 and RDC-1, a further GPCR, belonging to the class B sub-group and sharing no significant amino acid sequence identity to those receptors, was cloned from rat pulmonary blood vessels (Njuki et al. 1993). This receptor has ~50% overall amino acid sequence identity with the CT receptor (CTR) and was thus named the CT receptor-like receptor (Njuki et al. 1993). The abbreviation CLR is now used for simplicity (Hay et al. 2018). The human isoform of CLR was identified in 1995 from cerebellum and is closely related to rat CLR (Fluhmann et al. 1995). CLR or CLR-like receptors have been cloned from many other species, including porcine, bovine, mouse and flounder (Aiyar et al. 2002; Elshourbagy et al. 1998; Miyauchi et al. 2002; Sekiguchi et al. 2016; Suzuki et al. 2000). Early attempts to find a ligand for CLR were unsuccessful (Fluhmann et al. 1995). However, when human CLR was transfected into HEK293 cells a clear CGRP receptor pharmacology was observed (Aiyar et al. 1996). These studies differed from previous ones in the cell type used for transfection. Initially, Cos-7 cells had been used. The results of Aiyar and colleagues were confirmed with rat CLR and later porcine CLR was also identified as a CGRP receptor in HEK293 cells (Elshourbagy et al. 1998; Han et al. 1997). These studies led to the pertinent question: what was the factor in HEK293 cells that allowed CLR to act as a fully functional receptor for CGRP (Han et al. 1997)?

In 1998, Foord and colleagues provided new insight into the way that GPCRs and their pharmacology can be regulated (McLatchie et al. 1998). A new family of single transmembrane domain proteins termed receptor activity-modifying proteins (RAMPs) were discovered. It was found that these proteins were required for functional expression of CLR at the cell surface, explaining why it had been so difficult to find binding or function when this receptor was transfected into cells not expressing RAMPs (Fluhmann et al. 1995; Han et al. 1997; McLatchie et al. 1998).

Three human RAMPs have been cloned; RAMP1, RAMP2 (both cloned from SK-N-MC cells) and RAMP3 (cloned from human spleen) (McLatchie et al. 1998). Their amino acid sequences are 31% identical and 56% similar to one another and the respective rat and mouse (and other species) RAMPs have been cloned in addition to human (Husmann et al. 2000; McLatchie et al. 1998; Nagae et al. 2000; Oliver et al. 2001; Miyauchi et al. 2002). The structures of the extracellular domain region of CLR in complex with RAMP1 and RAMP2 are now known, providing useful insights into their mechanism of action (Booe et al. 2015; Kusano et al. 2012; ter Haar et al. 2010).

The discovery that RAMPs are essential for the functionality of CLR explains why this receptor was an orphan until this time. Of considerable interest was the discovery that the receptors generated by CLR/RAMP complexes recapitulated CGRP and AM pharmacology that had previously been described in various tissues and cell lines (Foord et al. 1999; Fraser et al. 1999; McLatchie et al. 1998). The discovery of RAMPs finally assigned firm molecular entities as CGRP and AM receptors, laying the foundation of all subsequent work. Co-expression of human CLR with RAMP1 constituted a CGRP receptor and co-expression of CLR with RAMP2 or RAMP3 yielded AM receptors (McLatchie et al. 1998). The AM receptors formed by CLR with RAMP2 or RAMP3 are defined as AM_1 and AM_2 subtypes, respectively (Hay et al. 2018); see Table 1. However, the precise nature of the pharmacology observed can be dependent on the species of the RAMP/CLR proteins. For example, rodent RAMP3-based receptors have a more mixed AM/CGRP receptor phenotype than the human equivalents (Hay et al. 2003; Husmann et al. 2000; McLatchie et al. 1998). This is covered further below. Table 1 summarises the receptors that are formed from CLR/RAMP complex formation.

In 1996, another protein that conferred CGRP responsiveness in *Xenopus* oocytes was described. This 146-amino acid protein was cloned from guinea pig organ of Corti and does not share the classical seven transmembrane domains of the other receptors described for the CT family of peptides (Luebke et al. 1996). This protein, called "receptor component protein" (RCP) does not form a direct binding site for CGRP but appears to be a key component of the CGRP receptor signalling system (Luebke et al. 1996). RCP acts as a peripheral membrane protein that facilitates receptor signalling. The physiological significance of this protein is still unclear, and compared to the CLR/RAMP1 complex itself, it is poorly studied.

Table 1 Summary of the molecular components and agonist pharmacology for the CT family of peptides (Bailey et al. 2012; Halim and Hay 2012; Hay et al. 2002, 2003, 2018; Husmann et al. 2000; Miyauchi et al. 2002; Oliver et al. 2001)

Receptor name	Molecular components	Human agonist pharmacology	Rodent agonist pharmacology[a]
CGRP	CLR + RAMP1	CGRP>AM = AM2	CGRP>AM = AM2
AM_1	CLR + RAMP2	AM>AM2 ≥ CGRP	AM> > CGRP
AM_2	CLR + RAMP3	AM = AM2 > CGRP	AM≥AM2 ≥ CGRP
CT	Calcitonin receptor	CT > Amy ≥ CGRP	CT > Amy = CGRP
AMY_1	Calcitonin receptor + RAMP1	Amy = CGRP	Amy = CGRP
AMY_2	Calcitonin receptor + RAMP2	Poorly defined	Poorly defined
AMY_3	Calcitonin receptor + RAMP3	Amy > CGRP	Amy = CGRP

[a]Some of these studies use a mixture of rat and mouse receptor components, making the pharmacology of discrete rodent receptors difficult to define

Soon after the discovery of RAMPs, it was identified that these proteins could interact with CTR, in addition to CLR. RAMPs also change CTR pharmacology (Hay et al. 2018). Here, there is an alteration in CGRP and amylin pharmacology, depending on whether RAMP1, RAMP2 or RAMP3 is expressed. Human CTR with human RAMP1 (the AMY_1 receptor) forms a dual high affinity receptor for both amylin and CGRP, whereas the human RAMP2 and RAMP3 complexes (AMY_2 and AMY_3, respectively) are high affinity amylin receptors. In a similar manner to CLR complexes, however, species plays a role in pharmacology.

3 Pharmacology of Calcitonin Gene-Related Peptide-Responsive Receptors

CLR/RAMP1 is now considered to be the canonical CGRP receptor and has high affinity for CGRP and around 10- to 20-fold lower affinity for AM and AM2 (Hay et al. 2018). Amylin and CT are weaker agonists at this complex. Amylin can activate this receptor but high nanomolar concentrations of the peptide are required to do this at the human receptor. At the rat receptor, amylin is more potent (Walker et al. 2015). Several antagonists have been developed that act at this receptor. These include fragments of CGRP itself, such as $CGRP_{8\text{-}37}$, or shorter C-terminal fragments (Watkins et al. 2013). A number of small molecules with high affinity for this receptor have also been identified as part of migraine drug development programmes. Examples of these are olcegepant, telcagepant, atogepant, ubrogepant and rimagepant. The pharmacology of several but not all of these is in the public domain. In general, these have high affinity for CLR/RAMP1 and lower but appreciable affinity for CTR/RAMP1, and very low or not measurable affinity for CTR alone or RAMP2- or RAMP3-based complexes with CLR or CTR (Moore and Salvatore 2012). However, it is important to note that cross-receptor pharmacology is only readily available for olcegepant and telcagepant, and it is not known for certain how rimagepant, atogepant and ubrogepant behave at all the different receptor complexes. Thus, it is only assumed that their pharmacology will be similar to structurally related compounds where the data are fully reported.

CTR/RAMP1 has also long been recognised to have high affinity for CGRP. This was first reported in the earliest studies of this receptor complex but has since been replicated many times (Christopoulos et al. 1999; Hay et al. 2005, 2018; Hay and Walker 2017; Kuwasako et al. 2004; Leuthauser et al. 2000; Tilakaratne et al. 2000). Although current nomenclature calls this complex the AMY_1 receptor due to its high affinity for amylin, CGRP equals amylin in potency/affinity and on this basis alone could be considered as a dual amylin/CGRP receptor (Hay et al. 2018). This naming convention has probably caused this receptor to be less studied as a CGRP receptor. It is important that this complex is studied both in terms of CGRP and amylin biology to determine its cognate ligand(s) in vivo because it is not known how

important this receptor is for amylin biology either (Hay et al. 2015). Recent data has shown that CTR/RAMP1 may be activated by CGRP endogenously because it has been found in the trigeminovascular system where CGRP is abundant (Walker et al. 2015). There are some antagonists of CTR/RAMP complexes (e.g. AC187, AC413 and salmon CT_{8-32}) but none that are selective for CTR/RAMP1; hence, these are only of limited use in characterising this receptor (Hay et al. 2005, 2015). As with this whole family because there is significant cross-reactivity of most agonist and antagonist ligands across two or more receptors, it is recommended that multiple tools are used to probe the identity and function of the receptors (Hay et al. 2018).

It is generally thought that CGRP has little activity at other CLR and CTR complexes. However, this is not necessarily true. Rodent CLR/RAMP3 (AM_2) and CTR/RAMP3 (AMY_3) receptors have higher affinity for CGRP than their human counterparts (Bailey et al. 2012; Halim and Hay 2012; Hay et al. 2003; Husmann et al. 2000). CGRP potency/affinity can almost equal that of AM at the rat AM_2 receptor (Hay et al. 2003). It is not known whether this is something that only occurs in isolated cells because more work is needed in this area. Hence, in terms of in vitro receptor pharmacology CGRP has high affinity at CLR/RAMP1 and CTR/RAMP1 across species and can have high affinity for RAMP3-based CLR and CTR receptors in a species-dependent manner. Thus, at the AMY_3 and AM_2 receptors, CGRP has higher affinity at rodent than human receptors (Bailey et al. 2012; Halim and Hay 2012; Hay et al. 2003). CGRP also has affinity at RAMP2-based receptors (Hay et al. 2018; Husmann et al. 2000). However, it is important to point out that there are not many studies of non-human receptors and many of these use mixed-species components. Further work is needed to more deeply characterise CGRP responses across these receptor complexes in different species, including other model species that are important for drug development such as dog and other primates. When considering what receptor CGRP may be acting through, there are actually quite a few possibilities and it should not be forgotten that complex CGRP pharmacology has been known for several decades (Dennis et al. 1990; Juaneda et al. 2000; Poyner 1995). The CLR/RAMP1 complex is clearly an important CGRP receptor but there is much evidence suggesting that this may not be the only relevant molecular entity responsible for the actions of CGRP.

A further factor to consider for the pharmacology of CGRP receptors is the potential differences that may be found when considering different signalling pathways. Most work is done measuring cAMP and to a more limited extent radioligand binding, which has given the pharmacological nomenclature that we have to date. However, distinct profiles are certainly possible when considering the plethora of other signalling pathways that CGRP can activate and there is emerging evidence that this may need to be considered to a greater extent both for agonists and antagonists (Walker et al. 2010, 2017; Weston et al. 2016). Moreover, for CTR there are also splice variants to consider which differ in expression, signalling and pharmacological profiles with or without RAMPs (Furness et al. 2012; Qi et al. 2013).

4 Calcitonin Gene-Related Peptide Receptors in Migraine

As will be evident from other chapters in this book, the CLR/RAMP1 complex is widely considered to be the relevant complex for migraine. This is the receptor that small molecule drugs have the highest affinity for and the complex to which an antibody drug has been targeted. This is also the receptor that most work has been done on to identify its localisation in migraine-relevant tissues. Does this mean that this is the only relevant receptor to CGRP in general or more specifically to CGRP in migraine? At this time, the answer has to be "no" because there is insufficient evidence to rule out a role for other complexes that bind CGRP with high affinity. As examples, this is because many small molecule antagonists that have been designed to target CLR/RAMP1 can also block CGRP activity at CTR/RAMP1 (Hay et al. 2006; Moore and Salvatore 2012; Salvatore et al. 2010; Walker et al. 2017), and because CTR/RAMP1 expression has hardly been studied in tissues so it is not known whether this receptor is present or absent at sites relevant to CGRP action. The monoclonal antibodies in clinical development bind either to the CGRP peptide itself or to the CLR/RAMP1 complex. In time, the clinical data may show some separation either in efficacy or differences in adverse events between these mechanisms. At this stage, it is too early to say whether the targeting of only CLR/RAMP1 by the Amgen/Novartis antibody, assuming it has no ability to bind to CTR/RAMP1 [there is limited data in the public domain (Shi et al. 2016)], shows any benefit over targeting the ligand, which presumably impedes activity at all CGRP-responsive receptors. A molecule that selectively targets CTR/RAMP1 is needed to firmly establish whether or not this receptor plays a role in migraine.

In conclusion, because the human CTR/RAMP1 AMY_1 receptor has high affinity for CGRP and has been reported in migraine-relevant tissue, this complex should certainly be considered as a possible player in CGRP action in migraine in addition to the canonical CLR/RAMP1 CGRP receptor.

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The Structure of the CGRP and Related Receptors

John Simms, Sarah Routledge, Romez Uddin, and David Poyner

Contents

Abstract

The canonical CGRP receptor is a complex between calcitonin receptor-like receptor (CLR), a family B G-protein-coupled receptor (GPCR) and receptor activity-modifying protein 1 (RAMP1). A third protein, receptor component protein (RCP) is needed for coupling to Gs. CGRP can interact with other RAMP–receptor complexes, particularly the AMY1 receptor formed between the calcitonin receptor (CTR) and RAMP1. Crystal structures are available for the binding of $CGRP_{27–37}$ [D^{31},P^{34},F^{35}] to the extracellular domain (ECD) of CLR and RAMP1; these show that extreme C-terminal amide of CGRP interacts with W84 of RAMP1 but the rest of the analogue interacts with CLR. Comparison with the crystal structure of a fragment of the allied peptide adrenomedullin

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bound to the ECD of CLR/RAMP2 confirms the importance of the interaction of the ligand C-terminus and the RAMP in determining pharmacology specificity, although the RAMPs probably also have allosteric actions. A cryo-electron microscope structure of calcitonin bound to the full-length CTR associated with Gs gives important clues as to the structure of the complete receptor and suggests that the N-terminus of CGRP makes contact with $His^{5.40b}$, high on TM5 of CLR. However, it is currently not known how the RAMPs interact with the TM bundle of any GPCR. Major challenges remain in understanding how the ECD and TM domains work together to determine ligand specificity, and how G-proteins influence this and the role of RCP. It seems likely that allosteric mechanisms are particularly important as are the dynamics of the receptors.

Keywords
Allostery · Amylin · Calcitonin · Cryo-electron microscopy · Crystallography · Family B G-protein-coupled receptor · Molecular dynamics

1 Introduction to the CGRP Receptors

The CGRP receptor as defined by IUPHAR is the complex between calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1) (Hay et al. 2017). It was the first receptor to be identified as a complex between a G-protein-coupled receptor (GPCR) and a RAMP (McLatchie et al. 1998). However, the peptide also has a high affinity at the AMY1 receptor formed between the calcitonin receptor (CTR) and RAMP1; its potency at this receptor is equal to that of amylin and it may be an endogenous agonist here (Walker et al. 2015). It has modest affinity at the AM2 and AMY3 receptors (CLR/RAMP2 and CTR/RAMP3, respectively) and can stimulate the AM1 and AMY2 receptors at pharmacological concentrations. In the older literature, there was much talk of CGRP1 and CGRP2 receptors; the CGRP1 receptor corresponds to the CLR/RAMP1 complex whereas it is likely that much of the pharmacology ascribed to the CGRP2 receptor in fact corresponds to the AMY1 receptor, perhaps with some contribution from the AM2 and AMY3 receptors (Hay et al. 2008). Most analysis has been done on the CGRP receptor and this will be the focus of the current chapter.

Both CLR and CTR are class B GPCRs. The three RAMPs each have an N-terminus of around 100–120 amino acids, a single transmembrane domain (TMD) and a C-terminus of around ten residues; their structures have been recently reviewed elsewhere (Hay and Pioszak 2016), as has the structure–activity relationship for CGRP (Watkins et al. 2013). In brief, for CLR the RAMPs have two main functions; they translocate CLR to the cell surface and also create the binding pocket for the endogenous peptides and also some non-peptide antagonists (McLatchie et al. 1998; Hay and Pioszak 2016).

Activation of CLR follows the two-domain model found in other class B GPCRs, where this is achieved by binding of the peptide N-terminus to the TMD of the receptor. The C-terminus of the peptide binds to the extracellular domain (ECD) of the receptor, contributing to the overall affinity of the peptide. CLR couples

Table 1 Summary of crystal structures of the extracellular domains of CTR and CLR/RAMP complexes with bound ligands

RAMP	GPCR	Ligand	RSCB ID/Reference
$RAMP1_{26–117}$	$CLR_{22–133}$	Telcagepant	3N7R, ter Haar et al. (2010)
$RAMP1_{26–117}$	$CLR_{22–133}$	Olcegepant	3N7S, ter Haar et al. (2010)
MBP-$RAMP1_{24–111}$-$(GSA)_3$-$CLR_{29–144}$-$(H)_6$		$CGRP_{27–37}$ [D^{31},P^{34},F^{35}]	4RWG, Booe et al. (2015)
MBP-RAMP2[L106R]$_{55–140}$-$(GSA)_3$-$CLR_{29–144}$-$(H)_6$		$AM_{25–52}$	4RWF, Booe et al. (2015)
–	H_6-$CTR_{25–144}$	[$BrPhe^{22}$]$sCT_{8–32}$	5II0, Johansson et al. (2016)

predominantly to Gs, although it can couple to other G-proteins and there is some evidence that this results in changes of the pharmacology of the receptor, depending on the RAMP (Weston et al. 2016a).

There is no crystal structure for an entire CLR/RAMP1 heterodimer, but a number of structures are available showing the ECD of this complex, in combination with both the non-peptide antagonists (Olcegepant and Telcagepant) and a modified C-terminal CGRP fragment (Table 1). There is also a structure of $AM_{22–52}$ bound to the ECDs of CLR and RAMP2 and of an analogue of $sCT_{8–32}$ bound to the ECD of the CTR. In addition, there are cryo-em structures for the full-length CTR and glucagon receptors (although the N-terminus of the CTR is not properly resolved) and a crystal structure of the full-length GLP-1 receptor (Liang et al. 2017; Jazayeri et al. 2017; Zhang et al. 2017). Thus, it is possible to model the TMD of CLR with some confidence, although the interaction between this part of the molecule and the juxtamembrane region of RAMP1 remains speculative. Figure 1 shows one possible structure for the complex between full-length CLR and RAMP1, but it should be considered as illustrative only. In this chapter, the structure of each component of the receptor will be considered, starting at the ECD. The human receptors will be considered, unless otherwise stated. There is well over 90% identity between the components of the human receptors and those from mammalian species normally used as models, although, particularly in RAMP1, there are some differences that are pharmacologically relevant. These will be discussed in the text.

2 The Extracellular Domain of Calcitonin Receptor-Like Receptor/Receptor Activity-Modifying Protein 1; Peptide Binding

The basic structure of the ECD of CLR/RAMP1 is shown in Fig. 2. The first 22 amino acids of CLR form a signal peptide and it is assumed that this is cleaved. The next 15 or so amino acids are not resolved in existing crystal structures but beyond this is a clear section of alpha helix; the unresolved extreme N-terminus may at least partly form a continuation of this in the full-length receptor. One side of the

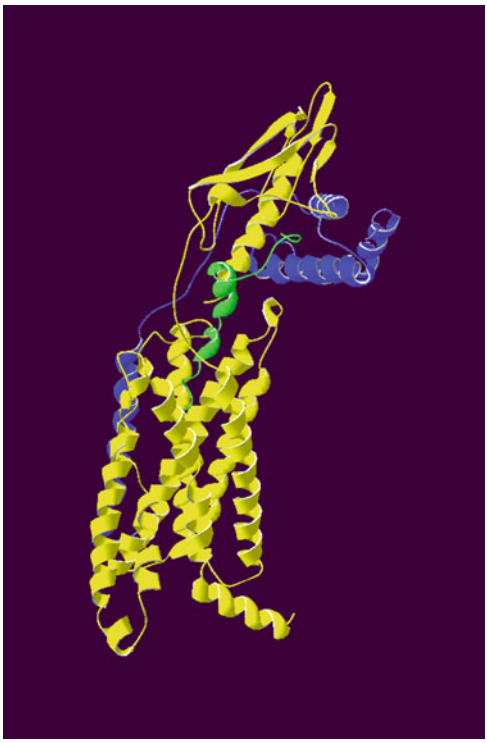

Fig. 1 Speculative structure of the full-length calcitonin receptor-like receptor (CLR)/receptor activity-modifying protein 1 (RAMP1) complex with bound CGRP. Yellow, CLR; blue, RAMP1; green, CGRP

N-terminus faces the bound CGRP and the other RAMP1. Beyond the alpha helix are a three sets of beta sheets connected by loops with a final section of helix, which probably leads into the "stalk", a helix that connects TM helix 1 (TM1) with the ECD. RAMP1 consists of three helices, with helices 2 and 3 facing CLR.

The analogues of calcitonin, CGRP and AM used for crystallisation all bind in an extended form to the ECDs of CTR or CLR, unlike other class B GPCRs which have a largely alpha-helical conformation (Booe et al. 2015; Johansson et al. 2016). Examination of the structures of these bound to their cognate receptors gives some insights into the specificity of the CLR/RAMP1 complex for CGRP. The ligands all terminate in beta turns, explaining why analogues where this structure is strengthened have high affinity (reviewed in Watkins et al. 2013); however, for CGRP and AM this is a beta I-turn whereas for the calcitonin analogue it is a beta II-turn. Importantly, the C-terminal Pro of calcitonin is directed towards CTR, close to W79 and Y131. By contrast for CGRP and AM, the terminal Phe or Tyr faces towards the RAMP (Fig. 2). F37 of CGRP contacts W84 of RAMP1. In RAMP2, the equivalent of W84 is F111 which cannot contact AM. Instead, E101 contacts Y52 of AM. In RAMP1, the equivalent residue of E101 is W74 but this is not in contact with CGRP. There are no further direct contacts between either peptide and the RAMPs; instead, there are multiple contacts between the peptides and CLR or CTR as appropriate (Booe et al. 2015; Lee et al. 2016). There is evidence for some small but potentially

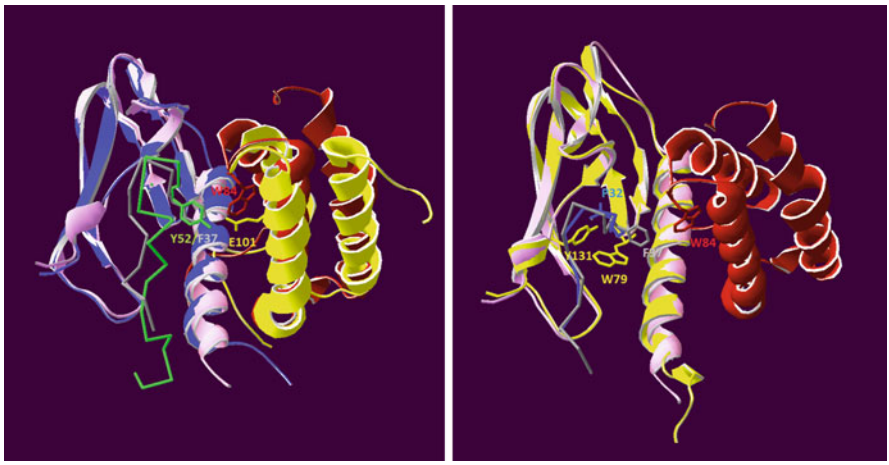

Fig. 2 Structure of the ECDs of CLR/RAMP1 and RAMP2 and calcitonin receptor (CTR). Left: CLR (pink) with RAMP1 (red) and bound $CGRP_{27–37}$ [D^{31},P^{34},F^{35}] (grey) superimposed on CLR (blue) with RAMP2 (yellow) and $AM_{22–52}$ (green). F37 ($CGRP_{27–37}$ [D^{31},P^{34},F^{35}]), Y52 ($AM_{22–52}$), W84 (RAMP1) and E101 (RAMP2) are shown in line form. Right: CLR with RAMP1 and bound $CGRP_{27–37}$ [D^{31},P^{34},F^{35}] (colours as previously) superimposed on CTR (yellow) and [$BrPhe^{22}$]$sCT_{8–32}$ (blue). F37 ($CGRP_{27–37}$ [D^{31},P^{34},F^{35}]), P32 ([$BrPhe^{22}$]$sCT_{8–32}$), W84 (RAMP1 and W79/Y131 (CTR) are shown in line form

significant RAMP-dependant shifts in the conformation of the contact residues on CLR, suggesting that the RAMPs act in part by allostery (Booe et al. 2015). This effect is probably mediated via changes in the β1–β2 loop of CLR which forms part of the peptide-binding pocket (Booe et al. 2018). The detailed knowledge of how peptides interact with the C-termini of the different receptors has already resulted in the rationale design of analogues with enhanced affinity or changed selectivity (Booe et al. 2018).

Whilst the crystal structure of [$BrPhe^{22}$]$sCT_{8–32}$ bound to the ECD of the CTR receptor provides important insights into the selectivity of CTR for calcitonin, there remain other subtleties. There is a report that *N*-glycosylation of the CTR enhances affinity for salmon calcitonin (Ho et al. 1999). Studies of the isolated ECD of the CTR have shown that this is due to the presence of a single *N*-acetyl glucosamine attached to N130 and it enhances the binding of FITC-AC413(6–25) Y25P (an amylin analogue modified to bind to the CTR) and $sCT_{8–32}$ to the ECD of the CTR and FITC-AC413(6–25) to a fusion of the ECDs of CTR and RAMP2 (i.e. the ECD of the AMY_2 receptor) (Lee et al. 2017). On the basis of modelling, the authors suggest that the glycan acts allosterically to enhance ligand affinity; as N130 faces away from the bound CT analogue in the crystal structure, it is difficult to envisage any direct mechanism. It is not known if any similar mechanism exists for CLR-based receptors.

A major puzzle with amylin receptors is that C-terminal residue, Y37-amide, plays very little role in its binding; for the AM and CGRP, the C-terminus makes crucial contacts with the RAMPs. This implies that at amylin receptors, the RAMPs

act largely through allosteric mechanisms (Gingell et al. 2016; Lee et al. 2017). Molecular modelling has suggested that the RAMPs might influence the dynamics of loop 5 and residues immediately C-terminal of the CTR (Gingell et al. 2016). On the basis of the structure of the salmon calcitonin analogue bound to the ECD of the CTR, it has been suggested that the RAMPs change the orientation of R126 in loop 5 of CTR to enhance the affinity of the receptor for amylin (Johansson et al. 2016). RAMPs enhance the affinity of a CTR/CLR orthologue from *Branchiostoma floridae* to bind its calcitonin/CGRP orthologues (Sekiguchi et al. 2016). The authors of this study consider that this arises from the RAMPs enhancing cell surface expression of the CTR/CLR orthologue. However, C-termini of the calcitonin/CGRP orthologues which this receptor binds appear much closer to calcitonin than CGRP (a shared terminal Pro-amide) and so it is not clear that they make direct contact with the RAMPs. If this is correct, it would further strengthen the case for an allosteric role of RAMPs. In turn, this raises the possibility that allosteric modulators acting on the ECDs of the RAMP/GPCR complexes might produce useful therapeutic effects, if suitable drug-binding pockets can be targeted.

3 The Extracellular Domain of Calcitonin Receptor-Like Receptor/Receptor Activity-Modifying Protein 1; Non-peptide Antagonist Binding

Crystal structures are available showing the interactions of ligands with CLR/RAMP1 as well as the CLR/RAMP1 structure by itself (Fig. 3). The two non-peptide antagonists of the CGRP receptor, olcegepant and telcagepant, bind to the ECD of the receptor and sit between RAMP1 and CLR in the groove that is used by the peptides for binding, making contacts with amino acids in both subunits. A set of tryptophans (W84 and W74 of RAMP1, W72 and W121 of CLR) are of particular importance in the interactions with the antagonists; W72 and W84 produce a predominantly hydrophobic pocket for the docking of the antagonists (ter Haar et al. 2010). Residue 74 is normally a basic amino acid in non-primates, explaining the species dependency of these antagonists (Mallee et al. 2002). It can readily be appreciated from these structures that the endogenous peptides will not be able to interact with the ECD with the antagonists in place. Although the affinity of olcegepant is higher than that of telcagepant, the latter is the more efficient ligand when considering the ratio of molecular weight to affinity. This arises from an additional hydrogen bond formed with T122 of CLR and the ability of the diflurophenyl group to fit further into the RAMP1-binding pocket compared to the larger dibromotyrosyl group of olcegepant (ter Haar et al. 2010).

Compared to the unliganded receptor, both antagonists cause some rearrangement of residue side chains when they bind. There is movement of R119 and a rotation of W72 of CLR as well as a slight movement of W74 of RAMP1. The rotation of W72 is of particular significance as this forms a "shelf" on which the piperidine group of the antagonists can sit (ter Haar et al. 2010). Olcegepant but not telcagepant shows pathway-selective antagonism for the $AMY_{1(a)}$ receptor; this suggests that at least at this receptor, there are further conformational changes produced when it binds to

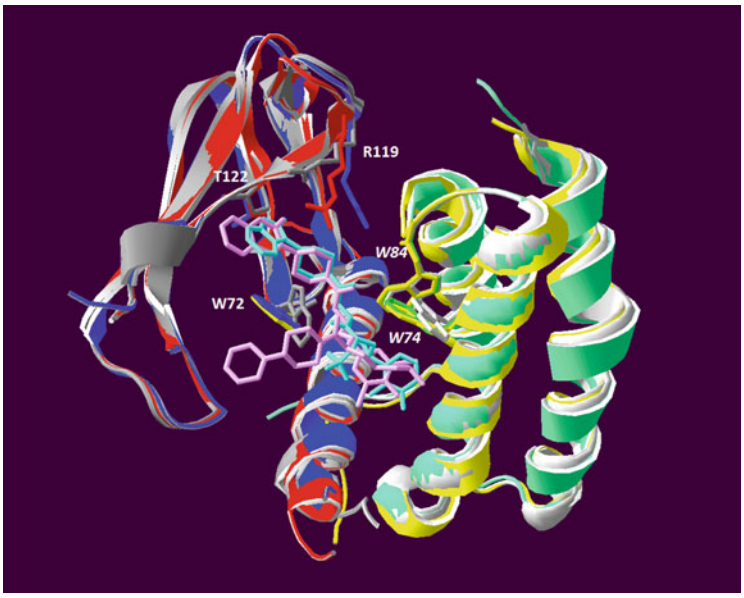

Fig. 3 Interaction of non-peptide antagonists with the ECD of CLR/RAMP1. The overlay compares CLR (grey)/RAMP1 (white) in the absence of bound ligand with CLR (red)/RAMP1 (yellow) with olcegepant (pink) and CLR (blue)/RAMP1 (green) with telcagepant (light blue). W74 and W84 are shown on the RAMP1 structures, with W72, R119 and T122 on CLR

the ECD (Walker et al. 2017). The mechanism of action of pathway-specific effects of agents that bind exclusively to the ECDs of the receptors is unclear. There is evidence for some family B GPCRs that changes in the ECD conformation are important in receptor activation; effects on the ECDs could be transmitted to the TM domain of the receptor via the extracellular loops (see below, Sect. 4). It is possible that a similar mechanism is at work with CLR- and CTR-based receptors, although the RAMP ECD–TM interfaces introduce additional complexity (but perhaps also new opportunities for drug discovery).

4 The TM Domains of Calcitonin Receptor and Calcitonin Receptor-Like Receptor

There is a mass of mutagenesis data on the ECLs and TMDs of CLR, but a clear interpretation of this remains elusive (Barwell et al. 2012; Woolley et al. 2013; Vohra et al. 2013; Watkins et al. 2016). The recent availability of a cryo-em structure of CT docked to the CTR is an important development, despite its relatively low resolution (Liang et al. 2017). A major barrier to applying this structure to the binding of CGRP to its receptors is a lack of certainty as the role of the RAMPs at this level of the receptor. Experimental evidence has been produced for a RAMP-binding interface on the receptor involving mainly TMs 6 and 7 or 1–5 (see Barwell et al. 2012 for review).

Alanine scans of the ECLs of CLR show that residues in all three are important, but ECL2 is of particular significance. Residues at the top of this loop influence the binding of $CGRP_{8–37}$, suggesting that residues 1–7 are located at the base of the loop. This is consistent with the cryo-em structure of CT bound to the CTR, where the authors suggest that S5 and T6 of CT are in contact with $His^{5.40b}$ (residue 302 in CTR, 295 in CLR), high on TM5 with N14 of CT making contacts with the backbone of ECL2 (Liang et al. 2017). Whilst it has been customary to consider agonist activity as being determined just by the disulphide-bonded loops of these receptors, it has recently been shown that $CGRP_{8–37}$ has partial agonist activity at the $AMY_{1(a)}$ receptor (Walker et al. 2017); it is tempting to suggest that this involves an interaction of residues 8 to around 14 with ECL2 or 3 of CTR. The pathway-selective antagonism of olcegepant (which binds exclusively to the ECD) at the $AMY_{1(a)}$ receptor further points to probably interactions between the ECDs and the ECL of this receptor (Walker et al. 2017). Structural data suggests that there is considerable movement of the ECD on activation of the glucagon receptor (Zhang et al. 2017); perhaps something similar happens with the $AMY_{1(a)}$ receptor.

Whilst ECL2 is the main contact for bound CT and is also the most significant loop for CGRP binding, the peptide-binding pocket is shaped by interactions with the other two ECLs. Mutagenesis has shown that the RAMPs influence the extracellular loops of CLR in their ability to determine CGRP potency (Watkins et al. 2016) and it is easy to envisage how by changing the orientation of these loops, peptide selectivity might be changed. ECL3 has also been shown to be important in AM receptors (Kuwasako et al. 2012). It is also interesting to note how mutation of a few residues at the base of ECLs can change specificity for receptors; thus, $H374A^{7.47b}$ increases AM potency 100-fold at the CGRP receptor (Woolley et al. 2017). This demonstrates that peptide selectivity is determined both by interactions at the N- and C-termini; a favourable interaction between the N-terminus of the peptide and juxtamembrane region can outweigh an unfavourable interaction at the C-terminus with the RAMP. It may be possible to design drugs that act on the TM domain of CLR but which can retain some selectivity in terms of receptor activation.

Beyond the ECLs, an extensive network of both hydrophobic and hydrophilic TM residues are important in controlling CLR activation, as judged by mutagenesis (Fig. 4). Many of these residues are also implicated in the activation of other family B GPCRs, suggesting that there are common themes to receptor activation (Wootten et al. 2013, 2016; Cordomi et al. 2017; Singh et al. 2015). Furthermore, RAMP influences rapidly diminish away from the base of the ECLs. Structure–activity studies show that Thr6 of CGRP is conserved in all members of the CGRP/AM/CT family and is essential for receptor activation (Watkins et al. 2013). It is tempting to see this interacting with $H^{5.40b}$ in both CTR and CLR. Combined with other interactions at the ECLs, this may be sufficient to cause a rearrangement of contacts at the top of the receptor, leading to the pivoting of TMs 5 and 6 around TM3. There is a pronounced proline kink in TM6, so any movement of this helix will cause the opening of a G-protein-binding pocket on the cytoplasmic face of the receptor.

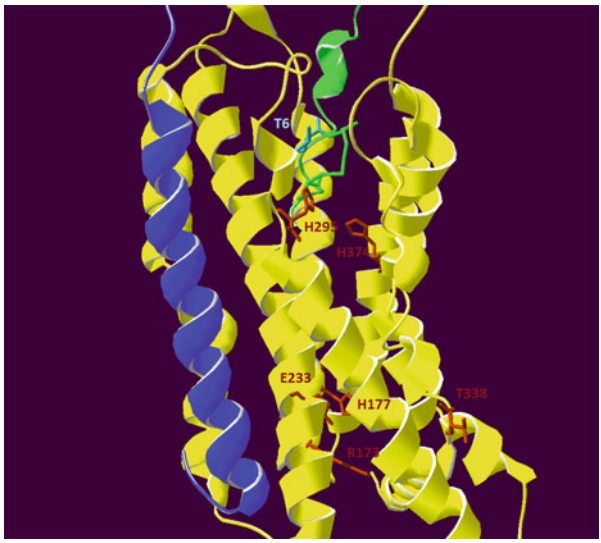

Fig. 4 Important TM residues in CLR. Yellow, CLR; blue, RAMP1 (speculative); green, CGRP. T6 on CGRP is in blue, R173, R177, E233, H295, T338 and H374 of CLR are shown in brown

This requires the rupture of a hydrogen bond between $T388^{6.42b}$ at the base of TM6 and $E233^{3.50b}$, which in turn instead interacts with $H177^{2.50b}$. $R173^{2.46b}$ drops down, allowing contact with Gs (Barwell et al. 2013). These movements are also linked to a shift in H8 and the base of TM7 (Vohra et al. 2013).

An overall model of receptor activation is that the peptide agonists at CLR- and CTR-based receptors make a variety of contacts from the top of the ECLs to the beginning of the TM domain. These cause reorientation of the ECLs, with residues either moving towards or away from the bound peptide. These changes get funnelled to the amino acids that control the orientation of TMs 3, 5 and 6 and so trigger changes in the TM bundle leading to the opening of the binding pocket for G-proteins or arrestins.

Significant work has demonstrated how RAMPs can influence ligand bias and affinity at amylin receptors (Morfis et al. 2008; Udawela et al. 2006a, b, 2008). This suggests that the C-terminus of the RAMP influences G-protein coupling. As noted above, RAMPs also change peptide G-protein-coupling preferences and hence receptor pharmacology of CLR; modelling indicates that the C-terminus of the RAMP can directly interact with the probable G-protein-binding pocket of CLR (Weston et al. 2016b). The different G-proteins themselves will be expected to act as allosteric modulators of the GPCRs, potentially changing their ligand-binding properties. This may explain why it has been reported that at the CGRP receptor, AM is more potent than CGRP at Gi coupling (Weston et al. 2016a); it would be useful to have further confirmation of this observation as it has important implications for ligand selectivity and receptor pharmacology.

5 The C-Terminus

The C-terminus of CLR remains little explored as regard its role in the CGRP receptor, although it has been studied in the AM1 receptor. Deletion of the entire C-terminus including H8 greatly impairs CLR expression, although the receptor that is able to reach the cell surface can still couple to Gs. There is a serine/threonine rich region distal to H8 and deletion of these residues impairs receptor internalisation, presumably by disrupting phosphorylation and interaction with β-arrestin (Conner et al. 2008). For the AM1 receptor, determinants influencing Gs coupling were also observed in this region and H8 (Kuwasako et al. 2006, 2010, 2011), but this was not seen in the single study done on the CGRP receptor. It is not clear if this reflects a difference between AM1 and CGRP receptors or is a consequence of the different cell lines used to express the receptors.

6 Receptor Component Protein

Receptor component protein (RCP) is a 148-amino acid, 17-kDa peripheral membrane protein. It is required for efficient coupling of the CGRP receptor to Gαs and AM acting at the AM1 receptor, as shown by knockdown of RCP expression (Evans et al. 2000). There is no information on whether it is plays a similar role in AM2 receptors. RCP appears to physically associate with the receptor, interacting with its second intracellular loop (ICL2) (Dickerson 2013). Loss of RCP does not affect the affinity of CGRP for its receptor, or significantly alter trafficking to the cell surface and so is not required in order for CLR and RAMP1 to interact. Decreases in RCP expression have been correlated with reduced sensitivity to CGRP under a number of physiological and pathological conditions (Dickerson 2013). Interestingly, a *Drosophila* class B GPCR, CG17415, also appears to interact with a homologue of RCP and human RCP can enhance coupling of this receptor to Gαs (Johnson et al. 2005). This indicates that RCP–receptor interactions appear to have co-evolved with the emergence of class B GPCRs. RCP may be an important target for allosteric modulators.

In addition to its role as part of CGRP and AM1 receptors, RCP is a component of human RNA polymerase 3 where it is known as rpc9; homologues of this protein are found in organisms as distant as yeast. In RNA polymerase III, it forms a dimer with rpc8. It may be involved in the binding of RNA transcripts as they exit the polymerase (Hu et al. 2002). Interestingly, nuclear translocation of RCP has been observed in NIH3T3 cells following challenge with CGRP, perhaps suggesting a role in nuclear signalling in addition to facilitating Gαs coupling (Sardi et al. 2014).

7 Conclusion

We currently have clear evidence for how the C-terminus of CGRP interacts with the ECD of its receptor and there are good clues as to how the N-terminus interacts with the TM domain of CLR. It is likely that we will shortly be in possession of structures

of many CLR– and CTR–RAMP complexes that are relevant to the understanding of CGRP pharmacology and these will give definite information on the structure of the entire GPCR–RAMP complex. Even with this information, some significant challenges may remain. We need to understand the interactions between the ECD and TM portions of the receptor and how they work together in determining ligand binding and receptor activation. More knowledge is needed on how G-proteins may act to influence ligand binding and the roles of RCP are still poorly understood. It may be that the CLR– and CTR–RAMP complexes are particularly finely tuned allosteric machines, showing long-range interactions and that control of their dynamics is of particular significance. Knowledge of the structure of CGRP and related receptors will help rationalise the mode of action and selectivity of existing therapeutic agents and should point the way to the design of new drugs.

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CGRP Receptor Signalling Pathways

Graeme S. Cottrell

Contents

Abstract

Calcitonin gene-related peptide (CGRP) is a promiscuous peptide, similar to many other members of the calcitonin family of peptides. The potential of CGRP to act on many different receptors with differing affinities and efficacies makes deciphering the signalling from the CGRP receptor a challenging task for researchers.

Although it is not a typical G protein-coupled receptor (GPCR), in that it is composed not just of a GPCR, the CGRP receptor activates many of the same signalling pathways common for other GPCRs. This includes the family of G proteins and a variety of protein kinases and transcription factors. It is now also

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clear that in addition to the initiation of cell-surface signalling, GPCRs, including the CGRP receptor, also activate distinct signalling pathways as the receptor is trafficking along the endocytic conduit.

Given CGRP's characteristic of activating multiple GPCRs, we will first consider the complex of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1) as the CGRP receptor. We will discuss the discovery of the CGRP receptor components, the molecular mechanisms controlling its internalization and post-endocytic trafficking (recycling and degradation) and the diverse signalling cascades that are elicited by this receptor in model cell lines. We will then discuss CGRP-mediated signalling pathways in primary cells pertinent to migraine including neurons, glial cells and vascular smooth muscle cells.

Investigation of all the CGRP- and CGRP receptor-mediated signalling cascades is vital if we are to fully understand CGRP's role in migraine and will no doubt unearth new targets for the treatment of migraine and other CGRP-driven diseases.

Keywords
Calcitonin gene-related peptide · Calcitonin receptor-like receptor · G protein · Protein kinase · Receptor activity-modifying protein · Signalling · Trafficking

Abbreviations

ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
ECE1	Endothelin-converting enzyme 1
ERK	Extracellular-regulated protein kinase
ET_A	Endothelin A receptor
GPCR	G protein-coupled receptor
IL	Interleukin
JNK	c-Jun N-terminal kinase
PKA	Protein kinase A
PKC	Protein kinase C
NO	Nitric oxide
NOS	Nitric oxide synthase
RAMP	Receptor activity-modifying protein
RCP	Receptor component protein

1 The Discovery of the Calcitonin Gene-Related Peptide Receptor

Although Amara et al. (1982) discovered calcitonin gene-related peptide (CGRP) in 1982, it was not until many years later that the identity of the receptor for CGRP was confirmed (McLatchie et al. 1998). A crucial development in the discovery of the CGRP receptor was the identification of a new family of single transmembrane proteins called receptor activity-modifying proteins (RAMPs). It had long been suspected that the G protein-coupled receptor (GPCR), calcitonin receptor-like receptor (CLR) was the receptor for CGRP. However, when this protein was expressed in model cell lines, CLR did not traffic to the cell-surface and CGRP was unable to elicit cellular responses typical of CGRP.

The human neuroblastoma cell line, SK-N-MC, was well-known to bind radiolabelled CGRP and CGRP promoted signalling following incubation with CGRP (Van Valen et al. 1990). McLatchie et al. (1998) used an expression cloning strategy in *Xenopus* oocytes that relied on a CGRP-mediated signalling pathway to activate a co-injected cystic fibrosis transmembrane regulator as a physiological read-out. Repeated subdivision of a positive pool of cDNA clones led to the isolation of a gene encoding a 148-amino acid protein that the authors named RAMP1. Initially, the authors were surprised as they expected the CGRP receptor to be a GPCR. However, further experimentation revealed that co-expression of RAMP1 with CLR was required in order to yield a high affinity CGRP receptor.

Although it was initially proposed that the role of RAMP1 was only to transport the CGRP receptor (CLR) to the cell-surface (McLatchie et al. 1998), it is now known that both CLR and RAMP1 play a vital role on the recognition of CGRP (Banerjee et al. 2006; Kuwasako et al. 2003). Indeed, RAMP1 fulfils multiple roles in the formation of a mature CGRP receptor. RAMP1 co-expression promotes the trafficking of CLR (together with RAMP1) to the cell-surface, increases the molecular mass of CLR by promoting the glycosylation process, actively participates in the binding of CGRP at the cell-surface (Fraser et al. 1999; McLatchie et al. 1998) and plays an important role in the post-endocytic sorting of CLR [reviewed in Klein et al. (2016)].

Since the discovery of RAMP1, we now know that this protein family has two other members, that also heterodimerize with CLR, forming receptors for adrenomedullin (also a member of the calcitonin family of peptides) (McLatchie et al. 1998). In addition, RAMPs also modify other GPCRs to generate receptors for other members of the calcitonin family of peptides [reviewed in Hay and Pioszak (2016)].

2 Calcitonin Gene-Related Peptide Receptors Mediate G Protein-Dependent Signalling

The first report of a signalling pathway evoked by CGRP was the cAMP-dependent effect of CGRP on amylase release from guinea pig pancreatic acinar cells (Seifert et al. 1985). Further evidence of the involvement of adenylate cyclase in signalling

by CGRP was subsequently observed in cat cerebral arteries (Edvinsson et al. 1985), rat striated muscle (Kobayashi et al. 1987), chick skeletal muscle (Laufer and Changeux 1987) and mouse diaphragm (Takami et al. 1986). The first discovery that CGRP signalled through G proteins was reported by Takamori and Yoshikawa (1989). The experimental evidence was obtained by measuring twitch force in curarized rat skeletal muscle and this early study provided evidence that CGRP-mediated signalling occurred through $G\alpha_s$, but not through $G\alpha_i$ (Takamori and Yoshikawa 1989). Cholera toxin enhanced the effect of CGRP on twitch, whereas pertussis toxin had no effect (Takamori and Yoshikawa 1989).

It is now well established that the CGRP receptor couples to G proteins to initiate signalling (Fig. 1). During the cloning of the RAMPs, signalling through the CGRP receptor was examined by recording increases in intracellular cAMP (McLatchie et al. 1998). Thus, CGRP receptors couple to $G\alpha_s$-type G proteins to stimulate the activity of cell-surface enzyme adenylate cyclase, which subsequently converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Typically, increases in cAMP then stimulate the activation of protein kinase A (PKA). Another G protein-mediated effect is the mobilization of intracellular calcium from the endoplasmic reticulum. Activation of $G\alpha_{q/11}$ proteins promotes the activity of phospholipase Cβ, which cleaves the cell-surface located lipid, phosphatidylinositol 4,5-bisphosphate, leaving diacylglycerol anchored to the cell-surface and inositol 1,4,5-trisphosphate free to activate inositol 1,4,5-trisphosphate receptors present on the endoplasmic reticulum, which in turn causes an efflux of Ca^{2+} into the cytoplasm. Diacylglycerol promotes the activity of protein kinase C (PKC). Although, the CGRP receptor is mainly considered a $G\alpha_s$-coupled GPCR, it is becoming clear that it is not as simple as a GPCR being either $G\alpha_s$-, $G\alpha_i$- or $G\alpha_{q/11}$-coupled. After the cloning of RAMPs, the same laboratory investigated the G protein coupling of CGRP receptors in Swiss 3T3 cells and *Xenopus* oocytes and reported coupling to both pertussis toxin-sensitive and toxin-insensitive G proteins, inferring that the CGRP receptor can couple to both $G\alpha_s$ and $G\alpha_i$ proteins (Main et al. 1998). However, another interpretation could be that the pertussis toxin affected the balance of the stimulatory and inhibitory processes responsible for cAMP production.

The first evidence that CGRP promotes coupling of CLR to $G\alpha_{q/11}$ proteins was observed in 293 cells (Aiyar et al. 1999). In agreement with this and using a fura-2/AM-based Ca^{2+}-assay, another group reported increases in intracellular calcium levels following exposure of 293 cells expressing CLR and RAMP1 to CGRP (Kuwasako et al. 2000). In the study by Aiyar et al. (1999), the calcium released in response to a CGRP challenge was derived from a thapsigargin-sensitive store, indicating an endoplasmic reticulum-dependent release. Further studies examining the post-endocytic sorting and signalling of the CGRP receptor in 293 cells also confirmed that CGRP promotes the mobilization of intracellular calcium (Cottrell et al. 2007; Padilla et al. 2007).

The issue of CGRP receptor G protein coupling, as with many GPCRs, is now much more complex than first thought. A study using both yeast and mammalian cells (293 cells) demonstrated that CGRP receptors couple to $G\alpha_s$-, $G\alpha_i$- and $G\alpha_{q/11}$

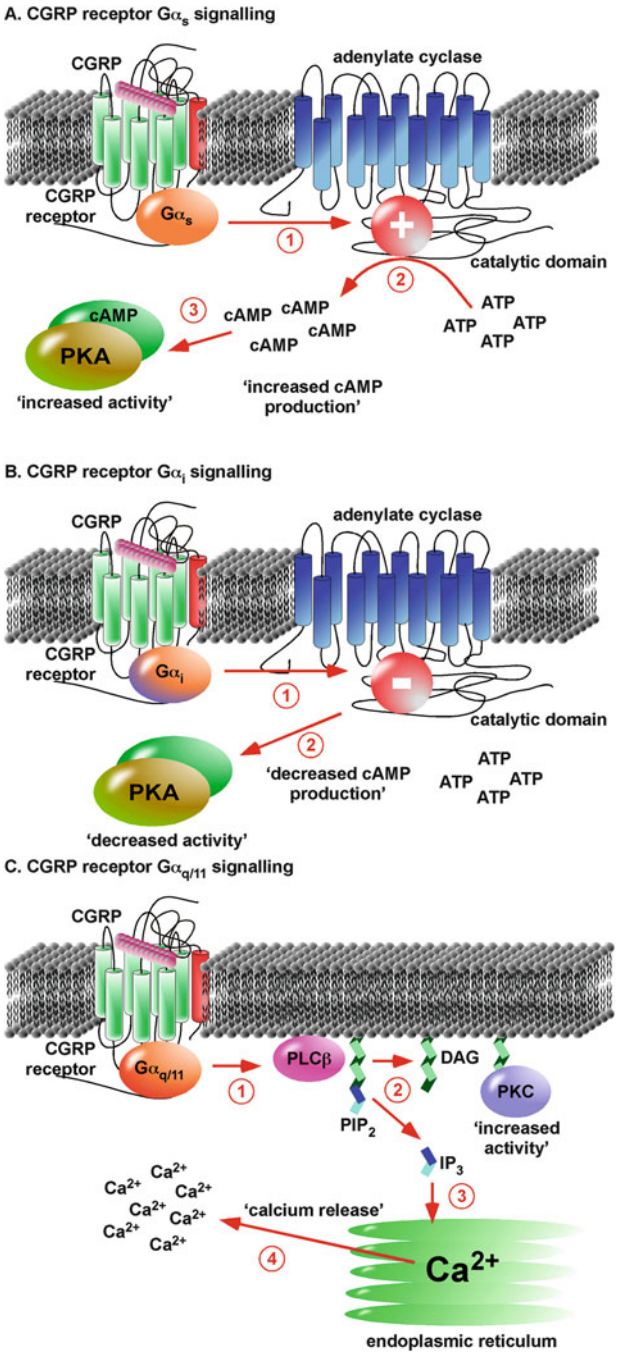

Fig. 1 Cell-surface Gα protein signalling from CGRP receptors. Following of exposure of CGRP receptors to CGRP, CGRP receptors couple to (**a**) $G\alpha_s$ proteins, (**b**) $G\alpha_i$ proteins and (**c**) $G\alpha_{q/11}$ proteins. (**a**, **step 1**) $G\alpha_s$ proteins promote the activity of adenylate cyclase, which converts ATP to

proteins (Weston et al. 2016), with the resultant signalling dependent on the relative expression levels of the G proteins. This study highlights the importance of choosing an appropriate model system to study CGRP-induced signalling pathways.

3 Calcitonin Gene-Related Peptide Receptor Component Protein

Before the cloning of RAMPs (McLatchie et al. 1998), a cytosolic protein named receptor component protein (RCP) that conferred CGRP responsiveness in *Xenopus* oocytes was identified by expression cloning using a cochlear hair cell cDNA library, using activation of the cystic fibrosis transmembrane conductance regulator as a signalling read-out (Luebke et al. 1996). RCP is a 16-kDa protein that lacks any predicted membrane spanning domains that would explain how it could be a receptor for CGRP; the authors surmised that it potentiated signals generated by an endogenous CGRP receptor in the oocytes. Supporting this hypothesis, both CLR and RAMP1 have subsequently been identified in *Xenopus* oocytes (Guillemare et al. 1994; Klein et al. 2002; Kline et al. 1988).

After the cloning of CLR and RAMP1, it was thought that these two proteins alone were sufficient to form a high affinity CGRP receptor. However, much of the work examining CGRP receptor signalling was performed in cell lines that endogenously expressed RCP. A study further examining the role of RCP in CGRP-induced signalling identified a major confounding issue when examining its role on CGRP-mediated signalling. All the model cell lines they screened, including many cell lines previously used in studies on CGRP receptor, contained endogenous RCP (Evans et al. 2000). In order to study the role of RCP in CGRP receptor signalling, the authors silenced RCP expression using RCP antisense RNA, confirming much reduced expression by western blotting. Although the levels of CGRP receptor present at the cell-surface were unaffected by RCP knockdown, as confirmed by ligand binding, CGRP-mediated cAMP production was a third of that compared to the same cells expressing RCP (Evans et al. 2000). Thus, indicating an important role for RCP in CGRP-induced cAMP production.

It is now clear that CLR, RAMP1 and RCP form a complex at the cell-surface yielding a high affinity CGRP receptor. RCP co-immunoprecipitates with CLR (Evans et al. 2000) and RAMP1 (Prado et al. 2001). Furthermore, it is now clear

Fig. 1 (continued) cyclic AMP (cAMP, a second messenger) promoting accumulation in the cell. (**a**, **step 2**) cAMP binds to the regulatory subunit of protein kinase A (PKA) stimulating its kinase activity. (**b**, **step 1**) $G\alpha_i$ proteins inhibit the activity of the catalytic domain of adenylate cyclase, reducing the levels of cyclic AMP in the cell. (**b**, **step 2**) Thus, there is less cAMP available to bind to the regulatory subunit of PKA and its activity is diminished. (**C**, **step 1**) $G\alpha_{q/11}$ proteins increase the activity of phospholipase Cβ which (**c**, **step 2**) processes phosphatidylinositol 4,5-bisphosphate leaving diacylgycerol anchored to the cell-surface which recruits protein kinase C (PKC). (**c**, **step 3**) The released inositol 1,4,5-trisphosphate (IP_3) diffuses and promotes the opening of inositol trisphosphate receptors on the endoplasmic reticulum which act as calcium channels and (**c**, **step 4**) intracellular levels of calcium ions increase

that RCP interacts with CLR via its second intracellular cytoplasmic loop (Egea and Dickerson 2012). The interaction between RCP and the second intracellular cytoplasmic loop was first confirmed using a yeast two-hybrid system. No such interactions using the other cytoplasmic loops or the C-terminal tail were observed. Co-immunoprecipitation experiments confirmed the interaction or lack of interaction between these intracellular portions of CLR and RCP (Egea and Dickerson 2012). Furthermore, expression of the second intracellular cytoplasmic loop of CLR acted as a dominant-negative for CGRP-induced signalling, reducing cAMP production by 74%, without significantly affecting the EC_{50} (Egea and Dickerson 2012). Interestingly, the C-terminal tail of CLR was also inhibitory towards CGRP-induced signalling, suggesting a role for this portion of the GPCR in activating cAMP production. The loss of maximal effect on cAMP production without loss of efficacy at the CGRP receptor could have been explained in two ways, by a reduction in the number of CGRP receptors at the cell-surface or a loss of signalling from a constant number of cell-surface CGRP receptors. Radioligand binding and ELISA experiments determined that expression of the second intracellular cytoplasmic loop of CLR had no effect on the trafficking of CLR and RAMP1 to the cell-surface, indicating that the loss of the RCP–CLR interaction accounted for the reduction in CGRP-induced cAMP accumulation (Egea and Dickerson 2012). Thus, confirming a role for RCP in CGRP receptor signalling either by direct coupling of the membrane traversing components of the CGRP receptor to intracellular signalling proteins (G proteins) or by altering the microdomain localization of CLR and RAMP1 at the cell surface. The exact mechanism by which RCP exerts its effects on CGRP receptor-mediated cell signalling remains unknown.

4 Calcitonin Gene-Related Peptide Receptor Internalization and Trafficking

Stimulation of most GPCRs promotes their removal from the cell-surface to intracellular compartments and the CGRP receptor is no exception (Kuwasako et al. 2000) (Fig. 2). A green fluorescent protein-tagged CLR was used to visualize the internalization of the CGRP receptor after exposure to CGRP (Kuwasako et al. 2000). Typically, upon activation GPCRs undergo a conformational change that leads to the phosphorylation of serine and threonine residues on the receptor by protein kinases belonging to the G protein-coupled receptor kinase (GRK) family. Using 293 cells metabolically labelled with $^{32}P[P_i]$, it was discovered that CLR, but not RAMP1, was phosphorylated within 5 min after exposure to CGRP (Hilairet et al. 2001). Although the GRKs responsible for CGRP receptor phosphorylation have yet to be definitively identified, a study by Aiyar et al. proposed the involvement of GRK6 (Aiyar et al. 2000). However, it is unclear from this study whether CLR was co-expressed with RAMP1 and as such, care must be taken when considering the validity of the observations. The post-translational phosphorylation increases the affinity of the CGRP receptor for cytosolic proteins called β-arrestins

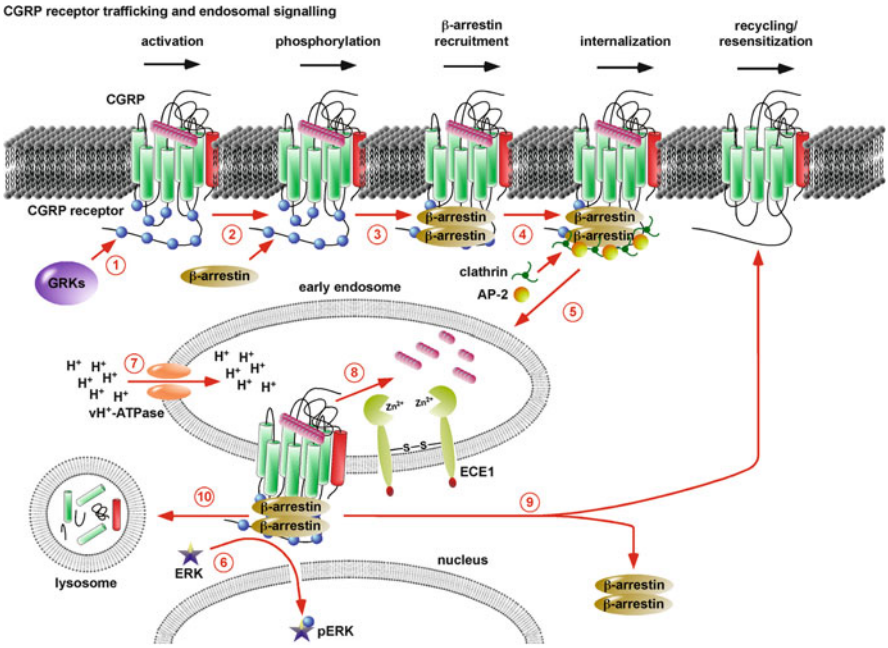

Fig. 2 Trafficking, sorting and endosomal signalling of CGRP receptors. (1) CGRP binds to the CGRP receptor promoting a conformational change and G protein-coupled receptor kinases (GRKs) phosphorylate the CGRP receptor. (2) The phosphorylated CGRP receptor has an increased affinity for β-arrestins and (3) β-arrestins translocate from the cytosol to interact with the CGRP receptor at the cell-surface. (4) β-arrestins act as a molecular scaffold recruiting clathrin and AP-2 to (5) facilitate internalization to early endosomes. (6) In the early endosomes, activated CGRP receptors promote the phosphorylation of extracellular-regulated protein kinase (ERK), which then translocates to the nucleus. (7) Vacuolar H^+-ATPase (vH^+-ATPase) pumps in protons acidifying the vesicle and (8) the changing pH reduces the affinity of CGRP for its receptor, CGRP dissociates and is degraded by endothelin-converting enzyme 1 (ECE1). (9) β-arrestins are released from the CGRP receptor which is presumably dephosphorylated and then trafficked back the cell-surface (recycling) to mediate resensitization. (10) Alternatively, if exposed to CCGRP for sustained periods, the CGRP receptor is trafficked to lysosomes for degradation

which translocate to the cell-surface to interact with the CGRP receptor (Hilairet et al. 2001). It is well established that β-arrestins perform at least three conserved functions on GPCRs [reviewed in Gurevich and Gurevich (2015), Jean-Charles et al. (2017) and Peterson and Luttrell (2017)]. First, they uncouple GPCRs from G protein via steric hindrance to terminate cell-surface G protein-dependent signalling. Second, β-arrestins act as a molecular scaffold recruiting proteins such as clathrin and adapter proteins (e.g. AP-1), that are essential for the internalization of the GPCR in a process termed clathrin-dependent endocytosis. Finally, many GPCRs remain bound to β-arrestins for long periods in intracellular vesicles called endosomes (Oakley et al. 2000). In endosomes, β-arrestins act as a hub for the

recruitment of signalling molecules to initiate a wave of signalling that is distinct from that initiated at the cell-surface. Internalization of the CGRP receptor is prevented by incubation of cells in hypertonic medium, indicative of a clathrin-mediated process (Kuwasako et al. 2000). A subsequent study showed that overexpression of a dominant-negative form of β-arrestin prevented internalization of CGRP receptors and that a yellow fluorescent protein-tagged β-arrestin translocated to the cell-surface after exposure to CGRP, indicating the dependence of CGRP receptor internalization on β-arrestins (Hilairet et al. 2001). Furthermore, a GTPase-defective mutant of the protein dynamin (Herskovits et al. 1993) also prevented CGRP receptor internalization (Hilairet et al. 2001). Both dynamin and β-arrestins participate in clathrin-coated vesicle-mediated endocytosis of GPCRs (Zhang et al. 1996).

Following sequestration and internalization, many GPCRs are trafficked to intracellular vesicles called endosomes. Activated CGRP receptors traffic together with CGRP to endosomes positive for early endosome-associated protein 1 (Cottrell et al. 2005, 2007; Padilla et al. 2007). The fate of the activated CGRP receptors is dependent on the duration of this exposure to CGRP (Cottrell et al. 2007). If cells expressing CGRP receptors are transiently exposed to CGRP, then CGRP receptors are efficiently recycled back to the cell-surface to mediate resensitization. In contrast, chronic exposure to CGRP shunts the receptors to a degradative pathway and the CGRP receptors are broken down by peptidases in lysosomes (Cottrell et al. 2007). The recycling of CGRP receptors is regulated by acidification of the endosomal system by H^+-ATPase and by the proteolytic breakdown of CGRP by an endosomal-located peptidase and can be prevented by inhibitors of both processes (Padilla et al. 2007). In a mechanism similar to the regulation of bioactive peptides at the cell-surface, whereby peptidases regulate the functional availability of peptides such as angiotensin II and substance P by proteolysis, endothelin-converting enzyme 1 (ECE1) regulates the availability of CGRP within endosomes. As CGRP receptors pass through the endosomal system and the vesicles mature, protons are pumped into the vesicles lowering the pH. ECE1 is inactive towards CGRP at neutral pH; however, ECE1 has an optimal pH for the proteolytic cleavage of CGRP of pH 5–5.5 (Fahnoe et al. 2000; Padilla et al. 2007). As the pH is lowered, CGRP has a lower affinity for the CGRP receptor and becomes the substrate for ECE1 in the endosome. Cleaved by ECE1, CGRP can no longer bind to the CGRP receptor. The unbinding of CGRP presumably results in a conformational change of the CGRP receptor and β-arrestins bound to the receptor in endosomes are released, translocating back to the cytosol. The CGRP receptor, freed from β-arrestins, is then presumably dephosphorylated and free to recycle back to the cell-surface in a Rab4- and Rab11-dependent mechanism (Cottrell et al. 2007; Padilla et al. 2007). This ECE1-dependent mechanism has also been shown to regulate CGRP receptors in intact arteries (McNeish et al. 2012). However, the mechanism by which a cell switches from recycling to lysosomal degradation of CGRP receptors in the presence of continued exposure to CGRP remains undetermined.

There is also the question of the molecular mechanism that targets CGRP receptors for degradation following exposure to CGRP. Degradation of receptors is an important mechanism by which cells can control the responsiveness of a cell to a particular biological signal. Degradation of receptors allows a cell to permanently reduce its ability to respond to a particular stimulus, at least until new receptors are synthesized and trafficked to the cell-surface.

Ubiquitin is a 76-amino acid protein that is covalently attached prevalently to lysine residues (Hershko et al. 1980; Schlesinger et al. 1975; Wilkinson 2005). This post-translational modification, termed ubiquitination, serves as a signal to the cell to breakdown the protein. Ubiquitin molecules themselves are efficiently cleaved off intact and recycled. The majority of cytosolic proteins are degraded by the proteolytic activity of the proteasome, large multi-protein complex with threonine residues at the heart of its active site [reviewed in Budenholzer et al. (2017)]. In contrast, membrane proteins such as GPCRs are typically degraded by peptidases in lysosomes. As described above, the CGRP receptor is no exception and in the continued presence of CGRP, both CLR and RAMP1 are degraded in lysosomes (Cottrell et al. 2007). The first study examining the mechanism that controls the targeting of a mammalian GPCR to lysosomes involved the β_2-adrenoceptor (Shenoy et al. 2001). Following agonist-induced internalization, the β_2-adrenoceptor was ubiquitinated on lysine residues and trafficked to lysosomes. Mutation of all the intracellular facing lysine residues to arginine (which cannot be ubiquitinated) resulted in a GPCR that was not ubiquitinated and instead of trafficking to lysosomes, recycled back to the cell-surface, indicating that the agonist-induced ubiquitination served as a signal to the cell to traffic the GPCR to lysosomes. This mechanism also serves as a target for other GPCRs (Jacob et al. 2005; Marchese and Benovic 2001). However, ubiquitination of GPCRs can also regulate different phases of trafficking, affecting internalization and the rate of degradation. Furthermore, not all GPCRs are ubiquitinated following agonist-induced activation. The CGRP receptor is not ubiquitinated following stimulation with CGRP, yet still traffics to lysosomes (Cottrell et al. 2007). Thus, a different molecular mechanism must exist that indicates to the cell that CLR and RAMP1 must be targeted to lysosomes. After internalization to early endosomes, receptor cargo is sorted within the multivesicular body, with multiple 'sorting proteins' with potential roles in deciding the fate of the receptor [reviewed in Szymanska et al. (2018)]. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is a component of multi-protein complex found on endosomal membranes (Bache et al. 2003; Komada et al. 1997). HRS is a multi-domain protein and contains a ubiquitin-interacting motif that has an essential role in endosomal sorting processes (Shih et al. 2002; Urbe et al. 2003). HRS plays a role in the trafficking of many GPCRs and indeed, the CGRP receptor (Hanyaloglu et al. 2005; Hasdemir et al. 2007; Hislop et al. 2004). However, as the CGRP receptor is not ubiquitinated, it is unlikely that this is through a direct interaction with the HRS ubiquitin-interacting motif. The exact mechanisms by which CGRP receptors are sorted and targeted to lysosomes are still unknown and warrant further investigation.

5 Calcitonin Gene-Related Peptide Receptor Signalling Pathways in Model Cell Lines

5.1 Activation of Calcitonin Gene-Related Peptide Receptors Promotes Activation of Protein Kinases

In common with many GPCRs, the CGRP receptor activates many different protein kinase cascades. From the work in model cell lines, it is well established that activated CGRP receptors can couple to $G\alpha_s$, $G\alpha_i$ or $G\alpha_{q/11}$ proteins, with the overall resulting effect dependent upon the cell type used to study the mechanism (Weston et al. 2016). If we take these results at face value, the balance between $G\alpha_s$ and $G\alpha_i$ will lead to accumulation or reduction in intracellular levels of cAMP. Alterations in cAMP levels will have a direct effect on cAMP-dependent protein kinase, commonly known as PKA. Composed of four subunits, PKA comprises two regulatory subunits and two catalytic subunits. When cAMP levels become elevated, cAMP binds to the regulatory subunits, promoting conformational change between the regulatory and catalytic subunits, unleashing the catalytic activity of PKA. Considering the $G\alpha_{q/11}$-dependent pathway, two signalling molecules in the form of diacylglycerol and inositol 1,4,5-trisphosphate are generated from the phospholipase Cβ-dependent cleavage of phosphatidylinositol 4,5-bisphosphate at the cell-surface. Diacylglycerol serves to both activate and anchor PKC to the inner leaflet of the plasma membrane. Inositol 1,4,5-trisphosphate diffuses to the endoplasmic reticulum activating inositol 1,4,5-trisphosphate receptors which open and release calcium in the cytoplasm.

As CLR and RAMP1 are commonly expressed with RAMP2, RAMP3 and calcitonin receptors and CGRP has been observed to activate adrenomedullin and amylin receptors, it is often difficult to ascertain which signalling cascades are activated by direct interaction of CGRP with CGRP receptors. In addition to this caveat, it is also clear that CGRP receptors may also couple to intracellular signalling cascades in a cell type-specific fashion. For this reason, we will first consider what is known about the protein kinases activated by CGRP receptors in model cell lines, before examining what should be considered CGRP-induced, not CGRP receptor-induced signalling pathways.

The first reported study analysing the protein kinase pathways elicited by the CGRP receptor showed that activation of the porcine CGRP receptor coupled to extracellular-regulated protein kinase (ERK) and p38 with no significant effect on the c-jun N-terminal kinase (JNK) pathway (Parameswaran et al. 2000). Both the ERK and p38 activities were dependent on time and the concentration of CGRP. Furthermore, the activation of these pathways was decreased by preincubation with the CGRP receptor antagonist, $CGRP_{8\text{-}37}$ and the PKA inhibitor, H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Parameswaran et al. 2000). In contrast, wortmannin, an inhibitor of phosphatidylinositol 3-kinase, only attenuated ERK activation.

The mouse CGRP receptor components CLR and RAMP1 were cloned, expressed and the CGRP receptor characterized in COS-7 cells (Miyauchi et al. 2002). The study confirmed CGRP-induced cAMP accumulation and examined

subsequent kinase activation using a variety of reporter assays (Miyauchi et al. 2002). The results from this study indicated that activation of the CGRP receptor promoted the activities of PKA and ERK but had no effect on JNK or nuclear factor-kappaB signalling (Miyauchi et al. 2002). This early work has subsequently been confirmed using the human CGRP receptor in COS-7 cells (Walker et al. 2017). Here, the authors examined CGRP-mediated activation of ERK, p38 and PKA and observed PKA and ERK activation, but in contrast to the earlier study (Parameswaran et al. 2000), no CGRP receptor-dependent activation of the stress-regulated protein kinase, p38, was reported (Walker et al. 2017).

5.2 Calcitonin Gene-Related Peptide Receptor Activation of Endosomal Signalling Pathways

For a long time, it was considered that the primary function of GPCRs was to signal from the cell surface via G proteins. In particular, activation of heterotrimeric G proteins promotes the production of second messengers such as cAMP and the release of intracellular calcium. However, it is now clear that following internalization to early endosomes, β-arrestins act as molecular hubs, recruiting signalling molecules to GPCRs in endosomes promoting a second wave of GPCR signalling that is distinct from cell-surface initiated signalling [reviewed in Eichel and von Zastrow (2018), Irannejad and von Zastrow (2014) and Sposini and Hanyaloglu (2017)]. This recruitment of signalling molecules to endosomes allows GPCR to generate compartmentalized signals that still remain largely unexplored. The first report of an endosomal signalling complex was for the β_2-adrenoceptor (Luttrell et al. 1999). Subsequently, DeFea et al. (2000) highlighted that β-arrestin-dependent signalling from endosomal neurokinin 1 receptors regulated the proliferative and anti-apoptotic effects of substance P. Since then, a number of studies have shown the importance of endosomes as signalling platforms for pain (Cottrell et al. 2009; Jensen et al. 2017; Yarwood et al. 2017). It was clear from studies examining the trafficking of the CGRP receptor that β-arrestins are recruited by this receptor and remain associated with the receptor for long periods in endosomes (Padilla et al. 2007). It is now also clear that GPCRs can also signal from endosomes via $G\alpha_s$ proteins (Feinstein et al. 2013; Ferrandon et al. 2009; Irannejad et al. 2013; Thomsen et al. 2016; Tsvetanova et al. 2015; Van Dyke 2004). Endosomal $G\alpha_s$ signalling has also been reported for other GPCRs including the parathyroid hormone receptor (Ferrandon et al. 2009), β_2-adrenoceptor (Irannejad et al. 2013) and vasopressin type 2 receptor (Feinstein et al. 2013). Furthermore, super resolution microscopy revealed that β-arrestins and $G\alpha_s$ proteins remain physically associated with the GPCR in endosomes providing a platform to regulate cAMP formation (Feinstein et al. 2013). Additional interactions with the endosomal sorting proteins, GPCR-associated binding protein 1 and dysbindin, highlight a role for $G\alpha_s$ proteins in the post-endocytic sorting of receptors (Rosciglione et al. 2014).

A recent study has highlighted the importance of CGRP receptor-dependent endosomal signalling in generating distinct signals that are important for the transmission of pain (Yarwood et al. 2017). This study reaffirmed that the CGRP receptor

is internalized in clathrin- and dynamin-dependent process and that it activates both PKA and PKC. In order to investigate the distinct signals generated from endosomes by the CGRP receptor, this study used a conjugation technique to target the CGRP receptor antagonist, $CGRP_{8-37}$, to endosomes (Jensen et al. 2017; Rajendran et al. 2008). $CGRP_{8-37}$ was conjugated to cholestenol using a polyethylene glycol 12 linker, promoting accumulation of the antagonist in endosomes and thus, providing a mechanism to antagonize CGRP receptor endosomal signalling. Incubation of CGRP receptor expressing cells with the cholesterol conjugated antagonist prevented activation of ERK in the nucleus, indicating a specific role for endosomal CGRP receptor signalling in generating ERK signals in the nucleus (Yarwood et al. 2017). The importance of this endosomal signalling for the transmission of pain was further investigated using capsaicin-, formalin- and complete Freund's adjuvant-induced model of mechanical allodynia. The study compared the effectiveness of $CGRP_{8-37}$ and endosomally targeted $CGRP_{8-37}$ in protecting against CGRP-induced pain and found that the unconjugated $CGRP_{8-37}$ afforded less protection than cholestenol-conjugated $CGRP_{8-37}$, supporting a role for CGRP receptor signalling from endosomes in the nociceptive process (Yarwood et al. 2017). It is interesting to note that this study also showed that the $G\alpha_s$ protein inhibitor, NF449 (Hulsmann et al. 2003), also suppressed nuclear ERK activation and thus, it is not clear if β-arrestin plays a role in this CGRP receptor endosomal signalling. It could be that the $G\alpha_s$ protein subunit remains associated with the active receptor in endosomes as has been shown for other receptors (Feinstein et al. 2013; Ferrandon et al. 2009; Irannejad et al. 2013) and that β-arrestin could mediate distinct and yet undiscovered endosomal signalling cascades.

6 Calcitonin Gene-Related Peptide-Mediated Signalling in Primary Cells

Although difficult to distinguish if all the effects of CGRP are mediated by CGRP receptors in primary cells and intact tissues due to the promiscuity of CGRP with other GPCRs, we will now examine the signalling cascades activated by CGRP in cells and tissues relevant in migraine. There are multiple sites where CGRP receptor may influence migraine: CGRP receptors in the cerebrovasculature (Edvinsson et al. 2002; Moreno et al. 1999; Oliver et al. 2002), CGRP receptors on dural mast cells (Ottosson and Edvinsson 1997; Theoharides et al. 2005), postsynaptic CGRP receptors on second-order sensory neurons (Fischer et al. 2005; Levy et al. 2005; Storer et al. 2004) and CGRP receptors in the trigeminal ganglion (Lennerz et al. 2008).

6.1 Calcitonin Gene-Related Peptide Signalling in Neuronal Cells

Exposure of cultured mouse trigeminal ganglion neurons cultures to CGRP promotes concentration-dependent increases in cAMP and also promotes up-regulation of CGRP itself (Zhang et al. 2007). Two PKA inhibitors, H89 and

8-Br-RP-cAMPS (Schafer et al. 1994), prevented CGRP-mediated effects on the CGRP promoter, providing consistent evidence with that observed in cell lines that CGRP couples to $G\alpha_s$ proteins, and promotes cAMP accumulation and activation of PKA. Supporting the activation of cAMP-dependent signalling, another study also in rat trigeminal ganglion neurons reported concentration-dependent increases in cAMP production in response to CGRP (Walker et al. 2015).

Further evidence for CGRP-dependent activation of protein kinases arises from a series of studies examining the regulation of $P2X_3$ receptors in mouse trigeminal ganglion cultures. $P2X_3$ receptors are activated by ATP and important in the transmission of pain signals [reviewed in Fabbretti (2013)]. Exposure of trigeminal ganglion cell cultures to GCRP promoted a delayed up-regulation of $P2X_3$ receptors at the cell-surface (Fabbretti et al. 2006). The cell-surface up-regulation of $P2X_3$ receptors was prevented by the CGRP receptor antagonist, $CGRP_{8\text{-}37}$, and by the protein kinase inhibitors, PKA inhibitor fragment 14–22 and chelerythrine chloride, implying a role for PKA and PKC, respectively (Fabbretti et al. 2006). In addition, CGRP treatment of mouse trigeminal ganglion cultures also enhances gene transcription of $P2X_3$ receptors (Simonetti et al. 2008), an observation supported by an independent study (Cady et al. 2011). In the former study, the authors observed CGRP-induced activation of Ca^{2+}-calmodulin-dependent kinase and phosphorylation of the cAMP-response element-binding protein, the latter presumably via a PKA-dependent mechanism. The effect of CGRP on gene transcription was only partially prevented by the inhibitors of PKA and PKC and co-incubation of the inhibitors did not have an additive effect, whereas KN93, an inhibitor of Ca^{2+}-calmodulin-dependent kinase, completely abolished the increase in transcription (Simonetti et al. 2008). Together, these studies provide evidence that CGRP may contribute to persistent pain through up-regulation of $P2X_3$ receptors and by redistribution of existing receptors to the cell surface. Brain-derived neurotrophic factor (BDNF) is known to play a role in processing pain through activity-dependent plastic changes in synaptic transmission [reviewed in Pezet and McMahon (2006)] and CGRP can promote the release of BDNF from trigeminal ganglion neurons (Buldyrev et al. 2006). A study showed that there are two distinct subpopulations of trigeminal ganglion neurons, expressing either BDNF receptors or CGRP receptors (32% overlap) (Simonetti et al. 2008). The distinct distribution of CGRP receptors and BNDF receptors in neurons raises the possibility that the BDNF released in response to CGRP can act in either an autocrine or paracrine manner. Similar to CGRP, BDNF promoted up-regulation of $P2X_3$ receptors is dependent on Ca^{2+}-calmodulin-dependent kinase (Simonetti et al. 2008).

Elevated actions of ERK and p38 are reported to be associated with neuronal sensitization and pain [reviewed in Ramesh (2014)]. To investigate the effect of CGRP on mitogen-activated protein kinases in neurons, rats were injected with CGRP in the temporomandibular joint and trigeminal ganglion isolated after 2 and 24 h (Cady et al. 2011). Examination of the isolated tissues by immunohistochemistry revealed that CGRP promoted activation of ERK, p38 and PKA both in neurons and satellite glial cells within the mandibular (V3) region of the ganglion, 24 h post-injection. Activation of sensory neurons has long been associated with

expression of c-fos, a member of the intermediate family of transcription factors (Hunt et al. 1987). After injection of CGRP, the examination of rat spinal trigeminal nucleus revealed that CGRP promoted a significant up-regulation of c-fos expression both 2 and 24 h post-injection (Cady et al. 2011). There are multiple signalling mechanisms that promote the expression and activation of c-fos [reviewed in Gao and Ji (2009)]. However, ERK activity is known to promote the activation of cAMP-response element-binding protein, which in turn may bind to the promoter regions of many transcription factors including c-fos to induce their expression (Gille et al. 1995; Hodge et al. 1998; Sassone-Corsi et al. 1988). It is interesting to speculate that the CGRP-dependent expression of c-fos could be dependent on ERK activity, but this was not examined in this study.

Multiple CGRP-induced kinase signalling pathways have been postulated to provide neuroprotection of sensory, cortical and cerebellar neurons (Abushik et al. 2016). Cultured rat trigeminal, cortical and cerebellar neurons were exposed to homocysteine as a model of neurotoxicity [reviewed in Obeid and Herrmann (2006)]. Preincubation of neurons with CGRP for 20 min protected the neurons from the neurotoxic effect of homocysteine. The potential contributions of PKA (PKA inhibitor fragment 14–22), PKC (chelerythrine chloride) and Ca^{2+}-calmodulin-dependent kinase (KN93) pathways in this CGRP-mediated effect were examined using specific inhibitors of each pathway (Abushik et al. 2016). Incubation of neurons with KN93 and the PKA inhibitor, not the PKC inhibitor, prevented the protection afforded by CGRP. However, inhibition of PKC had no effect on CGRP-mediated neuronal protection. To provide proof that CGRP also protects neurons in vivo, mice were subjected to permanent middle cerebral artery occlusion as an ischaemic insult. Magnetic resonance images revealed significant reductions in the size of the lesion in mice treated with CGRP compared to control (Abushik et al. 2016). Unfortunately, the authors did not confirm the participation of the kinase pathways in the in vivo model.

6.2 Calcitonin Gene-Related Peptide Signalling in Glial Cells

Satellite glial cells surround the neuronal cell body and participate in signal processing and transmission in sensory ganglia [reviewed in Lecca et al. (2012)]. Nitric oxide (NO) promotes the relaxation of blood vessels, a phenomenon mimicked by CGRP and linked to the pathophysiology of migraine. CGRP receptors are also expressed on the satellite glial cells resident within the trigeminal ganglion (Eftekhari et al. 2010; Lennerz et al. 2008; Miller et al. 2016). Activation of glial cell cultures with CGRP promoted a concentration-dependent increase in the expression of inducible nitric oxide synthase (iNOS) and NO release (Li et al. 2008). A subsequent study by the same group showed that CGRP significantly increased the mitogen-activated protein kinases regulated transcription factors Elk, ATF-2 and CHOP (Vause and Durham 2009). In particular, the study highlighted increased CGRP-dependent activity of ERK, JNK and p38. Inhibition of each of the kinase pathway activities with specific kinase inhibitors [ERK, U0126 (inhibits an upstream

kinase, mitogen-activated protein kinase kinase); JNK, SP600125 and p38, SB239063] prevented CGRP-induced up-regulation of iNOS and cellular production of NO (Vause and Durham 2009). Microarray analysis of CGRP-stimulated trigeminal glial cells also supports an important role for CGRP in regulating kinase-related signalling pathways (Vause and Durham 2010). In addition to kinase-related proteins, the microarray analysis also identified cytokines as a major target for CGRP-dependent induction. Stimulatory cytokines such as cytokine-induced neutrophil chemoattractant-3, fractalkine, granulocyte-macrophage colony-stimulating factor, interleukin (IL)-1α, leptin and macrophage inflammatory protein 3α having the largest up-regulation 8 h post-CGRP stimulation and induction of the inhibitory cytokines IL-10 and IL-4 was greatest after 24 h (Vause and Durham 2010).

Schwann cells support neuronal function by associating with nerve fibres and promote myelin production [reviewed in Miron (2017)]. The cell-surface components of the CGRP receptor, CLR and RAMP1, have been observed in Schwann cells in close proximity to both myelinated and unmyelinated nerves (Lennerz et al. 2008). A rat Schwann cell line (RT4-D6P2T) was stimulated with CGRP and a concentration-dependent induction of IL-1β was observed at protein level, by western blotting (Permpoonputtana et al. 2016). The production of IL-1β and IL-6 in response to CGRP increased over time, whereas there was no detectable increase in the production of tumour necrosis factor-α. The same study examined CGRP-induced kinase activation by western blotting, showing that CGRP promoted the phosphorylation of ERK. The phosphorylation of ERK was prevented by a CGRP receptor antagonist, $CGRP_{8\text{-}37}$, the PKA inhibitor, H89 and SQ22536, an inhibitor of adenylate cyclase (Permpoonputtana et al. 2016). Similarly, CGRP-induced expression of IL-1β was prevented by H89, SQ22536 and PD98059, an inhibitor of MEK-1. Together, this study showed the importance of the cAMP-PKA/ERK signalling cascade in the up-regulation of the important inflammatory mediator, IL-1β. Interestingly, unlike IL-1α which is expressed in cells under normal homeostasis, IL-1β is only expressed in response to inflammatory stimuli [reviewed in Dinarello (2011)], suggesting that CGRP is perceived as a danger signal by Schwann cells.

In an alternative approach to examining CGRP-induced signalling pathways in the trigeminal ganglion, the expression levels of inflammatory cytokines both at the mRNA level (using RT-PCR arrays and qPCR) and protein level (by immunohistochemistry) were analysed in an organ culture (Kristiansen and Edvinsson 2010). In this study, the authors observed that the morphological structure of the culture was well preserved after 24 h in culture and even in the absence of any stimulus there was a strong induction of pro-inflammatory cytokines including IL-6 and leukaemia inhibiting factor. Incubation of the organ culture with CGRP further enhanced the expression of a number of cytokines. Although this study did examine the effect of kinase inhibitors on expression of cytokines, they did not examine if they altered CGRP-dependent changes in cytokine expression (Kristiansen and Edvinsson 2010). Thus, it remains to be determined if CGRP activates kinase pathways to affect cytokine expression in an intact trigeminal ganglion culture.

Although it has been commonly reported that CGRP receptors promote mobilization of intracellular calcium, there is limited information as to whether CGRP promotes calcium mobilization in cells of the trigeminal ganglion. Cultures of mouse trigeminal ganglion were examined at the single cell level following exposure to CGRP using a fura2/AM-based assay (Ceruti et al. 2011). This study reported that although the satellite glial cells mobilized calcium in response to CGRP, this phenomenon was not observed in the trigeminal neurons and is supported by a previous study (Fabbretti et al. 2006). This evidence supports the notion that CGRP receptors are predominantly $G\alpha_s$-coupled in the neurons, but CGRP receptors expressed in the satellite glial cells are also coupled to $G\alpha_{q/11}$.

6.3 Calcitonin Gene-Related Peptide Signalling in Vascular Smooth Muscle Cells

CGRP receptors are present in the blood vessels that supply the brain. There have been many reports of expression of CGRP receptor components in vascular smooth muscle cells from different species (Cottrell et al. 2005; Lennerz et al. 2008; Miller et al. 2016). To date, there have not been any reports of expression of CGRP receptors on endothelial cells at sites relating to migraine. In agreement with this, CGRP-dependent relaxation of pial vessels from rabbit, cat and human was shown to be endothelium-independent (Hanko et al. 1985). The vascular smooth muscle cells are in close proximity to nerve endings, which contain CGRP. Therefore, nerves that release CGRP will activate smooth muscle CGRP receptors and thereby promote vasodilation via a neurogenic-dependent mechanism. In the periphery, CGRP and its receptors are present in endothelial cells (Doi et al. 2001; Hagner et al. 2001; Nikitenko et al. 2006) and an endothelium- and CGRP-dependent vasodilation has been reported in isolated rat aortic rings (Grace et al. 1987). In addition, CGRP promotes the proliferation of endothelial cells (Haegerstrand et al. 1990) and endothelial progenitor cells (Zhou et al. 2010). Although CGRP does not have a role on the regulation of blood pressure in normal individuals (Ho et al. 2010; Olesen et al. 2004), it does have a protective effect in individuals with cardiovascular disease [reviewed in Smillie and Brain (2011)]. In the absence of any studies on smooth muscle cells from migraine-related regions of the brain, we will discuss the studies investigating CGRP receptor signalling in vascular smooth muscle cells from other vascular beds.

The main blood supply to the cochlea is the spiral modiolar artery and altered blood flow in the cochlea was proposed to be involved in the development of hearing loss and tinnitus [reviewed in Hesse (2016)]. Thus, the activation of CGRP receptors in this artery was thought to be potentially beneficial. An investigation of the effect of CGRP on gerbil spiral modiolar artery responses measuring changes in vascular diameter also examined the potential signalling pathways involved (Herzog et al. 2002). Arteries were loaded with the calcium indicator dye, Fluo-4, and changes in the calcium levels in response to a challenge with CGRP were monitored by microscopy. CGRP was found to cause a transient decrease in intracellular calcium levels of the smooth muscle cells. Furthermore,

CGRP also stimulated cAMP production (Herzog et al. 2002), suggesting that CGRP receptors in smooth muscle cells are coupled to $G\alpha_s$ proteins. The decrease in calcium concentration was speculated to be attributable to the opening of K^+ channels causing hyperpolarization and closure of L-type Ca^{2+} channels and a cAMP-dependent effect on the contractile apparatus (Herzog et al. 2002).

Scherer et al. reported the reversal of endothelin-1 induced spasms of spiral modiolar artery by CGRP (Scherer et al. 2002). The molecular basis for this CGRP-specific reversal has since been investigated in mesenteric arteries (Meens et al. 2009, 2010, 2011, 2012), that also express CGRP receptors with activation causing vessel relaxation (Cottrell et al. 2005; Lei et al. 1994). Endothelin-1 is potent vasoconstrictor (O'Brien et al. 1987; Yanagisawa et al. 1988) and can activate both endothelin A (ET_A) receptors and endothelin B receptors (Arai et al. 1990; Sakurai et al. 1990) present on vascular smooth muscle cells (Hori et al. 1992; Nakamichi et al. 1992; Russell et al. 1997; Wendel-Wellner et al. 2002). In the rat mesenteric arteries, endothelin-1 promoted a potent concentration-dependent contraction that was prevented by the ET_A receptor antagonist, BQ123, but not by the endothelin B receptor antagonist, BQ788, indicating that only ET_A receptors were involved in the endothelin-1-dependent effect (Meens et al. 2009). Capsaicin acts on transient potential vanilloid 1 receptors to release CGRP from nerve endings, promoting the relaxation of mesenteric arteries (Fujimori et al. 1990). Stimulation of mesenteric arteries with capsaicin promoted a significantly greater relaxation of mesenteric arteries that were pre-contracted with endothelin-1, compared to arteries pre-contracted with phenylephrine with the authors speculating that endothelin-1-promoted contractions are hypersensitive to CGRP-mediated reversal (Meens et al. 2009). In contrast, other relaxing agents including isoproterenol (a β_2-adrenoceptor agonist and activator of cAMP production), forskolin (a direct activator of adenylate cyclase), sodium nitroprusside (an NO donor) and pinacidil (an activator of ATP-sensitive K^+-channels) all caused similar concentration-dependent relaxations irrespective of whether endothelin-1 or phenylephrine were used to cause the initial contraction (Meens et al. 2009). Investigation into the mechanism by which activation of CGRP receptors selectively reverses the effects of endothelin-1 on ET_A receptors identified a previously unidentified CGRP receptor-dependent signalling pathway (Meens et al. 2012). The long-lasting effect of endothelin-1 on the contractile responses of mesenteric arteries is attributed to the tight binding and slow dissociation kinetics of endothelin-1 on ET_A receptors [reviewed in De Mey et al. (2009, 2011)]. Using fluorescently labelled endothelin-1 as an agonist of ET_A receptor and two-photon laser scanning microscopy, CGRP was observed to promote the dissociation of endothelin-1 from the smooth muscle layer of mesenteric arteries (Meens et al. 2010). As other activators of cAMP production did not mimic this ligand/receptor dissociation, the authors investigated the role of $G\beta\gamma$ protein subunits (Meens et al. 2012). The study showed that the CGRP-specific effect on endothelin-1 contractions was independent of cAMP, as inhibitors of adenylate cyclase (SQ22536) and PKA (H89 and KT5720) were ineffective at blocking the CGRP-dependent reversal of endothelin-promoted contractions (Meens et al. 2012). However, gallein, a low molecular mass inhibitor of $G\beta\gamma$ protein function (Lehmann

et al. 2008), not only enhanced CGRP-induced cAMP production in cultured rat mesenteric vascular smooth muscle cells, it also prevented the CGRP-specific effect of relaxing endothelin-1-dependent contractions (Meens et al. 2012). To date, this remains the only report of a function of CGRP-activated Gβγ protein subunits, although if any Gβγ proteins are involved remains to be determined.

Migraines have been proposed as a reaction to the higher levels of oxidative stress that occur between attacks in migraineurs [reviewed in Borkum (2018)]. This raises the question of whether CGRP is released as a consequence of and a reaction to oxidative stress. Sensory nerves express a plethora of transient receptor potential ion channels including transient receptor potential ankyrin 1 receptor (TRPA1) (Kobayashi et al. 2005; Story et al. 2003). TRPA1 is activated by a variety of endogenous and exogenous agents including mustard oil (McNamara et al. 2007), 4-hydroxynonenal (Macpherson et al. 2007; Trevisani et al. 2007) and allicin (Bautista et al. 2005; Macpherson et al. 2005). In addition, agents that generate oxidative stress also promote the activation of TRPA1 (Hill and Schaefer 2009). Thus, as activation of TRPA1 channels promotes the release of CGRP (Trevisani et al. 2007), the release of CGRP in migraineurs may be a consequence of the increased oxidative stress. However, a protective role for CGRP against oxidative stress has been proposed (Schaeffer et al. 2003a, b). Vascular smooth muscle cells were exposed to oxidative stress using a hydrogen peroxide generating system (glucose/glucose oxidase system) for 1 h and the viability of cells then examined using an MTT assay and apoptosis examined using Hoechst staining and annexin-V labelling (Schaeffer et al. 2003b). The viability was diminished in a concentration-dependent manner and cells were rescued by pretreatment of cell with CGRP. Investigation of the signalling pathways involved observed that although the generation of oxidative stress itself increased the activity of ERK, pretreatment of the cells with CGRP significantly enhanced the ERK activity (Schaeffer et al. 2003b). No role for JNK in CGRP-dependent prevention of the hydrogen peroxide-induced reduction in cell viability was observed (Schaeffer et al. 2003b). In contrast, inhibitors of ERK (PD98059) and p38 (SB203580) both reversed the effect of CGRP protection (Schaeffer et al. 2003b). In support of the protective effect of CGRP against oxidative stress, lentivirus-mediated overexpression of CGRP in the Schwann cell line, RSC96, transiently protected against high levels of glucose in the growth medium (Wu et al. 2015). The CGRP-induced signalling pathway in this cell line remains unidentified. Therefore, during high levels of oxidative stress CGRP may be released to maintain cell viability, but is it other signalling events that contribute the sensitization of sensory neurons and increased vasodilation, that are deleterious and contribute to the development of migraine.

7 Conclusions

It is clear that we have discovered a wealth of information regarding the signalling mediated by both CGRP and CGRP receptors and that it is complex, yet compared with other GPCRs much less of known about these signalling pathways. We have delineated some of the CGRP and CGRP receptor pathways and it is now known

that they involve multiple types of G protein, protein kinases and transcription factors. Understanding the role of CGRP is complicated in that some of the effects of CGRP have been shown to be protective, whereas others have deleterious effects and contribute to the development of migraine pathophysiology. Preventing the activation of CGRP receptor (with small molecule inhibitors and monoclonal antibodies) and the actions of CGRP (with monoclonal antibodies) have proved that CGRP and its receptor are valid targets for the prevention of migraine. The relatively new avenue of CGRP receptor signalling along the endocytic pathway and the involvement of these distinct signalling pathways in the transmission of pain is an exciting development. This phenomenon requires further investigation and will no doubt prove vital for identifying new potential targets. Whether these targets will be known or novel proteins, or whether they are allosteric modulators or biased agonists or antagonists of CGRP receptors is an unknown commodity. Only further knowledge and scientific understanding of CGRP- and CGRP receptor-mediated signalling pathways will help in the quest to make migraine pain a problem of the past.

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Pathways of CGRP Release from Primary Sensory Neurons

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Contents

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Abstract

The benefit reported in a variety of clinical trials by a series of small molecule antagonists for the calcitonin gene-related peptide (CGRP) receptor, or four monoclonal antibodies against the neuropeptide or its receptor, has underscored the release of CGRP from terminals of primary sensory neurons, including trigeminal neurons, as one of the major mechanisms of migraine headaches. A large variety of excitatory ion channels and receptors have been reported to elicit CGRP release, thus proposing these agonists as migraine-provoking agents. On the other side, activators of inhibitory channels and receptors may be regarded as potential antimigraine agents. The knowledge of the intracellular pathways underlying the exocytotic process that results in CGRP secretion or its inhibition is, therefore, of importance for understanding how migraine pain originates and how to treat the disease.

Keywords

CGRP · Migraine · Neurogenic inflammation · Primary Sensory Neurons

1 Sources of CGRP Release

1.1 Primary Sensory Neurons

The seminal findings that increased levels of calcitonin gene-related peptide (CGRP), but not of other neuropeptides, were found in plasma from the jugular veins that collect intra- and extracranial blood during migraine or cluster headache attacks (Fanciullacci et al. 1995; Goadsby et al. 1990) have underlined the role of CGRP in the mechanisms of primary headaches (Edvinsson and Warfvinge 2017). Main anatomical structures where the precursor protein of the pre-pro-CGRP is produced and processed are a subpopulation of primary sensory neurons and a subpopulation of intrinsic neurons of the gut (Russell et al. 2014). The α-CGRP is the final product of the CGRP precursor that, from the soma of pseudo-unipolar peptidergic sensory neurons, is transported to their central and peripheral endings. It is stored at both sites in large-core vesicles. These neurons include dorsal root ganglion (DRG), vagal ganglion (VG), and trigeminal ganglion (TG) neurons. CGRP-expressing sensory neurons encompass heterogeneous subpopulations of DRG neurons in terms of anatomy, electrophysiology, neurochemistry, and function. Thus, CGRP has been found in most of the small-sized and some of the intermediate-sized neurons (Gibson et al. 1984), with myelinated slow-conduction C fibers or thinly myelinated Aδ fibers with a higher conduction velocity.

The tachykinins, substance P (SP) and neurokinin A (NKA), are present as peptide neurotransmitters in a proportion of small-sized DRG/VG/TG neurons and are often colocalized with CGRP (Gibson et al. 1984). Usually, SP and NKA are co-released with CGRP from peripheral and central endings of DRG/VG/TG neurons that express the capsaicin-sensitive member of the transient receptor potential (TRP) family of channels, the vanilloid 1 subtype (TRPV1). Notably, in human peripheral tissues

innervated by the trigeminal nerve, the release of CGRP, but not that of SP, has been reported (Geppetti et al. 1992).

1.2 Intrinsic Gut Neurons

While α-CGRP expression is confined to DRG/VG/TG primary sensory neurons, the β-isoform, which differs from α-CGRP by only three amino acids in humans, is mainly produced by intrinsic neurons of the enteric nervous system (Russell et al. 2014). Although the dichotomy in the localization of the two CGRP isoforms has been challenged (Li et al. 2009), β-CGRP is usually found in the intestines, with concentrations up to seven times more than α-CGRP (Mulderry et al. 1988). β-CGRP expressed by intrinsic neurons of the gut, which are TRPV1-negative and are therefore capsaicin-insensitive, is spared by the depleting action that results from exposure to high doses of capsaicin (Mulderry et al. 1988). Nevertheless, possible co-expression and co-release of the two isoforms in different areas of the peripheral nervous system, depending on specific circumstances, cannot be excluded. It should be further noted that CGRP-positive fibers originating from extrinsic TRPV1-positive spinal sensory neurons are present in mammals (Tan et al. 2010). In principle, both CGRP isoforms may be released from the gastrointestinal tissues, by non-specific stimuli, whereas capsaicin should solely release α-CGRP.

1.3 Central Neurons

Although CGRP and CGRP receptors have been found in a variety of brain regions (Edvinsson and Warfvinge 2017), their function in structures located inside the blood-brain barrier (BBB) is still the object of much uncertainty. Modulation of CGRP release by a repertoire of ion channels and receptors from peripheral terminals of nociceptors has been reproduced in release experiments from central fibers terminating within the dorsal spinal cord. However, conclusive evidence that such release is associated with nociceptive transmission is lacking. Additional localization of CGRP has been found in neurons of the ventral horns, apparently identified as motoneurons, whereas no CGRP-positive neurons have been reported in dorsal horn (Gibson et al. 1984).

1.4 Non-neuronal Cells

CGRP may be expressed in non-neuronal cells. β-CGRP mRNA and, to a lesser extent, α-CGRP (Hou et al. 2011) have been found in human and rodent-cultured keratinocytes. Both α-CGRP and β-CGRP have also been reported in endothelial progenitor cells that accumulate in damaged endothelium to repair injury and influence vascular remodeling (Zhao et al. 2007). CGRP expression seems to be more abundant in early rather than late endothelial progenitor cells (Fang et al.

2011). There is also some evidence that CGRP is produced by several types of immune cells, including lymphocytes, and peripheral blood mononuclear cells, including activated but not resting B lymphocytes (Bracci-Laudiero et al. 2002), monocytes, and macrophage-activated adipocytes (Linscheid et al. 2004). However, it should be underlined that the proof that CGRP is released from these non-neuronal cells, thus exerting a function, is still lacking.

2 Excitatory Receptors in Primary Sensory Neurons

Although various neuronal and non-neuronal cells may express, and potentially release, CGRP, the neuropeptide important in migraine should derive from primary sensory neurons of the trigeminal ganglion and, possibly, from the upper cervical DRGs. In order to elicit a biologically meaningful discharge of CGRP, or for its attenuation, the neurons should be enriched by excitatory or inhibitory receptors/channels, respectively (Fig. 1). In this paper we will focus on the ones that appear to have a major pathophysiological or therapeutic relevance for migraine.

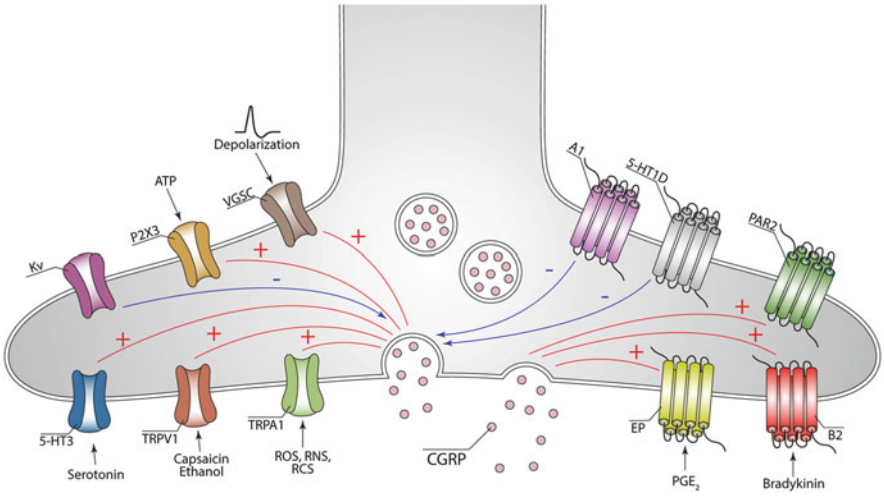

Fig. 1 Schematic representation of peptidergic, CGRP-containing peripheral nerve terminal. Excitatory (+) or inhibitory (−) pathways promote or attenuate, respectively, CGRP release. *Kv* potassium channel, *ATP* adenosine triphosphate, *P2X3* ligand-gated ion channel receptor, *VGSC* voltage-gated sodium channel, *A1* adenosine receptor 1, *5-HT$_{1D}$* 5-hydroxytryptamine (serotonin) receptor 1D subtype, *PAR2* protease-activated receptor 2, *B2* bradykinin 2 receptor, *PGE$_2$* prostaglandin E$_2$, *EP* prostaglandin E receptor, *CGRP* calcitonin gene-related peptide, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *RCS* reactive carbonyl species, *TRPA1* transient receptor potential ankyrin 1, *TRPV1* transient receptor potential vanilloid 1, *5-HT$_3$* 5-hydroxytryptamine (serotonin) receptor 3 subtype

2.1 Receptors for ATP

Receptors for adenosine triphosphate (ATP) are classified in ligand-gated P2X and metabotropic P2Y receptors, the first group encompassing ion channel receptors and the second G-protein-coupled receptors. P2X2 and P2X3 have been shown in TG neurons (Staikopoulos et al. 2007), where P2X receptors may sensitize sensory neuron functions, including the release of CGRP. P2X3 receptor stimulation caused sensitization of TG neurons, such that a subthreshold amount of KCl was sufficient to increase intracellular Ca^{2+} levels and CGRP secretion (Masterson and Durham 2010). Activation of P2Y receptors in rabbit esophagus mucosa potentiated HCl-evoked release of CGRP (Ma et al. 2011). However, minimal CGRP release was reported following ATP stimulation of P2Y receptors of isolated adult rat DRG neurons (Sanada et al. 2002). Furthermore, ATP did not cause any meaningful release of CGRP from sensory nerve terminals in areas of relevance for migraine mechanism, such as the rat dura mater (Zimmermann et al. 2002). ATP via PY2 receptor activation solely facilitated low pH-evoked release (Zimmermann et al. 2002). As for the action of CGRP on P2X-expressing neurons, it has been reported that, while a proportion of CGRP-binding TG neurons are P2X3-immunopositive, CGRP does not acutely affect the ATP receptor functioning (Fabbretti et al. 2006). Thus, while an indirect tissue-dependent action of ATP via PY2 receptors may favor the release of sensory CGRP, a direct action of ATP in the neurosecretory process is unlikely.

2.2 PAR Receptors

Protease-activated receptor 2 (PAR2) belongs to the PAR family, which encompasses four members. Although PAR1 is expressed by nociceptors and elicits neuropeptide-mediated responses (de Garavilla et al. 2001), more information has been obtained regarding PAR2 that coexists with TRPV1 in peptidergic spinal afferent neurons. Stimulation of PAR2 receptors has been shown to release SP and CGRP, thus producing neurogenic inflammatory responses (Steinhoff et al. 2000). Exposure to PAR2 agonists was found to enhance TRPV1 expression in sensory neurons and to potentiate capsaicin-evoked and TRPV1-dependent currents or Ca^{2+} response in isolated DRG neurons and CGRP release from dorsal spinal cord (Amadesi et al. 2004). In addition, PAR2 activation by stimulating the release of CGRP and tachykinins from capsaicin-sensitive sensory neurons was found to trigger the cytoprotective secretion of gastric mucus in rats (Kawabata et al. 2001). More importantly, supernatants from cultured human pancreatic cancer tissues induced CGRP release from DRG neurons, in a manner that was attenuated by selective PAR2 antagonists (Zhu et al. 2017).

2.3 Serotonin Receptors

Of the large number of serotonin receptors, only 5-$HT_{1B/D}$ and 5-HT_2 are involved in migraine mechanism as selective receptor agonists or partial agonists provide either acute or chronic benefit in migraine, respectively. The ligand-gated ionotropic 5-HT3 receptor is the only serotonin receptor with excitatory activity that, upon activation, has been associated with the release of CGRP (Hua and Yaksh 1993; Tramontana et al. 1993). However, there is no evidence that endogenously released serotonin acts on the 5-HT_3 receptor to release CGRP and, thus, contributes to the migraine mechanism.

2.4 Bradykinin and Prostaglandin Receptors

The proinflammatory and proalgesic peptide, bradykinin, has been reported to release SP and CGRP in vivo and from different isolated preparations in vitro, including the guinea pig heart and the rat dorsal spinal cord via activation of B2 receptors (Figini et al. 1995; Geppetti et al. 1988b). Although expression of B2 receptors has been documented in primary sensory neurons (Steranka et al. 1988), release of sensory neuropeptides does not seem to depend on a direct B2-mediated action of bradykinin on nerve terminals. Attenuation of bradykinin-evoked release of CGRP from isolated guinea pig atria (Geppetti et al. 1990). The reference is correct and rat spinal cord (Andreeva and Rang 1993) by indomethacin indicates the involvement of prostanoids. This conclusion was strengthened by the observation in the isolated guinea pig heart that both arachidonic acid- and bradykinin-evoked CGRP release were abated or markedly attenuated by indomethacin, respectively (Geppetti et al. 1991).

The proposal that prostanoids play a major role on the release of CGRP evoked by arachidonic acid (Geppetti et al. 1990) has been confirmed and amplified by subsequent studies. There are indications that prostaglandins are the final common pathway of several proinflammatory mediators to promote CGRP release, as cyclo-oxygenase inhibition attenuated the neuropeptide outflow from a rat skin preparation evoked by a combination of bradykinin, serotonin, and histamine (Averbeck and Reeh 2001). Of interest for migraine mechanisms and treatment, CGRP release evoked by bradykinin from the intracranial vessels of the guinea pig, similar to results obtained in the heart, was abolished by pretreatment with indomethacin (Geppetti et al. 1990). To strengthen the role of endogenously released prostanoids in CGRP release, direct exposure of cultured DRG neurons to PGE_2 and PGI_2 evoked CGRP release (Hingtgen et al. 1995). Efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) to abort migraine attacks may depend on their ability to inhibit prostaglandin synthesis and the ensuing release of the pro-migraine neuropeptide, CGRP.

3 Excitatory Channels in Trigeminal Primary Sensory Neurons

3.1 Sodium Channels

Nine different voltage-gated sodium channel (VGSC) isoforms (Nav1.1–Nav1.9) that share a common overall structural motif, but with different amino acid sequences, have been described (Catterall 2000). Genetic and pharmacological findings in experimental animals and humans have implicated some of them in the mechanism of different types of pain, especially the tetrodotoxin (TTX)-sensitive Nav1.3 and Nav1.7 and the TTX-resistant Nav1.8 and Nav1.9 (Catterall 2000). Antidromic invasion of terminal fibers of nociceptors by propagated action potential (Bayliss 1901) has been hypothesized to account for the activation paradigm that results in the liberation of a chemical mediator that promotes vasodilatation and sensitization of neighboring sensory fibers (Lewis 1936). There is now conclusive evidence that indicates CGRP as the substance released locally from cutaneous sensory nerve terminals that mediates neurogenic vasodilatation (Sinclair et al. 2010).

Depolarization produced by electrical field stimulation or high K^+ concentrations results in the release of sensory neuropeptides via the opening of TTX-sensitive VGSCs and the ensuing influx of extracellular Ca^{2+}, usually through voltage-gated calcium channels (VGCC) of the *N*-type (Lundberg et al. 1992). If this is the typical way of releasing SP, NKA and CGRP from central and peripheral terminals of sensory neurons, it is by no means exclusive. For example, the prostaglandin-dependent release pathway activated by bradykinin is in large part TTX-resistant, whereas VGCCs are markedly involved (Geppetti 1993). Furthermore, additional and diverse mechanisms contributing to the release of sensory neuropeptides, particularly that one elicited by the hot spice, capsaicin, which is a selective TRPV1 agonist, are also entirely TTX-resistant (Maggi et al. 1988).

3.2 TRP Channels

TRPV1 belongs to a larger family of ligand-gated cation channels that in mammals encompasses 28 members grouped in 6 families distinguished on the basis of their sequence homology (Nilius and Szallasi 2014). Some of them are expressed in primary sensory neurons, thereby attracting interest for their possible roles in pain mechanisms. These include the vanilloid 2, 3, and 4 subtypes (TRPV2, TPV3, and TRPV4), the melastatin 3 and 8 subtypes (TRPM3 and TRPM8), and the ankyrin 1 subtype (TRPA1). They have been labeled as thermosensors, because of their ability to sense different temperatures from noxious cold (TRPA1) to very noxious heat (TRPV2). However, the coding for efficient detection of temperature appears more complex and integrated, as deletion of the three different channels, TRPV1, TRPM3 and TRPA1, is required to abolish heat sensation in mice (Vandewauw et al. 2018).

More importantly for the present discussion, in the last decade, a number of reports have shown that some TRP channels are particularly sensitive to the redox state of the milieu, the TRPA1 channel being the most sensitive (Mori et al. 2016). In fact, a large series of reactive oxygen (ROS), nitrogen (RNS), and carbonyl (RCS) species target TRPA1 by binding to specific cysteine residues (C415S, C422S, and C622S) of the intracellular domain (Macpherson et al. 2007). The unprecedented list of endogenous molecules that gate TRPA1 includes the ROS, H_2O_2 (Sawada et al. 2008); the RNS, peroxynitrite (Andersson et al. 2015); and the RCS, 4-hydroxynonenal (Trevisani et al. 2007). Nevertheless, seminal studies that have fully revealed the role of nociceptors in releasing proinflammatory neuropeptides from their peripheral terminals, to mediate neurogenic inflammatory responses, have been possible by using capsaicin that selectively targets TRPV1.

Long before the cloning of TRPV1 (Caterina et al. 1997), the understanding of the unique physiopharmacology of the "capsaicin receptor" was clear. Exposure to high capsaicin concentrations via a massive influx of extracellular Ca^{2+} into the nerve terminal elicits two distinct and time-related effects. First, capsaicin excites the nerve terminal, producing the classical burning pain sensation coupled to the release of sensory neuropeptides and the ensuing neurogenic inflammatory responses. Shortly after, probably due to the neurotoxic action of excessive cytoplasmic Ca^{2+} levels gained by prolonged exposure to high capsaicin concentrations, nerve terminals become insensitive to further stimulation by capsaicin or any other stimulus (Bevan and Szolcsanyi 1990). In vivo, systemic administration or topical application of capsaicin produces the same dual effects observed in vitro, which, however, are characterized by a time-dependent recovery (Geppetti et al. 1988a). The so-called capsaicin desensitization has been exploited in the use of topical preparations for the treatment of post-herpetic or HIV-related neuralgias (Katz et al. 2015).

The ability of capsaicin exposure to elicit and attenuate pain sensation parallels that to cause and inhibit neuropeptide release. Thus, tissues containing peptidergic sensory neurons after exposure to capsaicin undergo an initial release of neuropeptides, followed by refractoriness to any further release. An example of such a property has been reported in human tissues containing trigeminal sensory fibers, such as the iris and ciliary body. Capsaicin released CGRP at a first, but not a second, exposure to capsaicin (Geppetti et al. 1992). Notably, capsaicin failed to release SP from this trigeminal human preparation, underlying that CGRP, and not SP, mediates neurogenic inflammation in humans (Geppetti et al. 1992). The observation that telcagepant, one of the first small molecule antagonists of the CGRP receptor, abated the vasodilatation evoked by capsaicin application to the human forearm (Sinclair et al. 2010) demonstrated that CGRP is the compound released from sensory nerves to dilate arterioles and sensitize neighboring terminal fibers (Bayliss 1901; Lewis 1936). It also suggests that beneficial effects in cluster headache or migraine of repeated topical application of capsaicin to areas innervated by the trigeminal nerve is due to capsaicin desensitization (Fusco et al. 2003) and the ensuing reduced ability to release CGRP.

A series of pharmacological and genetic data point to TRPA1 as one of the major transducers that links CGRP release, local inflammation, neuronal sensitization, and pain. Indirect evidence has associated oxidants and reactive molecules with migraine, as indicated by increased nitrate/nitrite levels found in premenopausal women in association with their migraine (Tietjen et al. 2009). More generally, preclinical and clinical findings underscore the role of oxidative stress in migraine (Borkum 2018). However, the oxidant-dependent pathways that lead to oxidant generation that in turn promote the CGRP-dependent migraine pain remain to be investigated.

4 Inhibitory Receptors and Channels in Trigeminal Primary Sensory Neurons

4.1 Adenosine Receptors

There is evidence that drugs that increase the levels of cAMP or cGMP, such as cilostazol or sildenafil, respectively, are detrimental for migraine (Ashina et al. 2017). Thus, it is not surprising that activation of receptors coupled to a G-protein that inhibits the activity of adenylyl cyclase and reduces the intracellular levels of cAMP is beneficial in migraine. A selective agonist for the adenosine receptor 1 (A1), GR79236, inhibited the release of CGRP evoked by superior sagittal sinus stimulation in the cat (Goadsby et al. 1988). Initial enthusiasm for new therapeutic opportunities for migraine by activating such inhibitory receptors was attenuated by cardiovascular adverse effects, such as bradycardia, and the issue whether A1 stimulation may cause vasoconstriction rather than inhibition of neurotransmitter release (Arulmani et al. 2005).

4.2 Serotonin 5-HT_{1D} Receptors

More clinically relevant information has been obtained on the localization and function of the serotonin 5-HT_{1B} and 5-HT_{1D} subtypes, expressed by the vascular smooth muscle and the sensory nerve terminal, respectively. Their association with a Gi protein, which inhibits adenylyl cyclase, indicates that their stimulation causes vasoconstriction and inhibits the release of SP and CGRP, in vascular smooth muscle and sensory nerve terminals, respectively (Moskowitz 1992). Ergot derivatives, such as ergotamine or dihydroergotamine, target 5-$HT_{1B/D}$ receptors but also activate 5-HT_2, α-adrenergic, and dopamine receptors, which markedly contribute to their adverse reactions. As triptans selectively stimulate the 5-$HT_{1B/D}$ receptor, migraine amelioration should solely depend on these receptor subtypes. However, there is no final conclusion as to whether resolution of the migraine attack

by triptans or ergots is associated with 5-HT_{1B}-mediated vasoconstriction or 5-HT_{1D}-mediated attenuation of CGRP release or both. Positive clinical evidence in migraine treatment with lasmiditan, a ditan with a selective agonistic action on the 5-HT_{1F} subtype (Rubio-Beltran et al. 2018), whose expression is apparently confined to sensory nerve terminals, suggests that inhibition of CGRP release should be the major antimigraine mechanism of triptans/ergots. However, a firm conclusion on this long-lasting debate has not yet been reached.

4.3 Potassium Channels

Opening and closing of inhibitory K^+ channels mediate fine tuning of neuronal responses. A triple cysteine pocket within neuronal M-channel subunits (Kv7.2–7.5) is oxidatively modified by H_2O_2 (Gamper et al. 2006) and nitric oxide (NO) to control the release of CGRP (Ooi et al. 2013). The gaseotransmitter modulator, hydrogen sulfide (H_2S), may activate both the TRPV1 and TRPA1 channels, thereby evoking the release of CGRP (Pozsgai et al. 2012). However, H_2S produces mechanical allodynia and increases neuronal excitability in TG neurons, via an autocrine mechanism underlined by the colocalization of the H_2S-generating enzyme cystathionine beta-synthase (CBS) and H_2S-sensitive Kv1.1 and Kv1.4 channels (Feng et al. 2013).

5 Agents that Provoke Migraine Attacks and Release CGRP

5.1 Nitric Oxide Donors

Headaches can be provoked by occupational exposure to, or treatment with, organic nitrates (Thadani and Rodgers 2006; Trainor and Jones 1966). From these observations, the NO donor, glyceryl trinitrate (GTN), has been proposed and used as a provocation test for migraine attacks (Iversen et al. 1989; Sicuteri et al. 1987). GTN causes a mild, rapidly developing, and short-lived headache in most subjects, including healthy controls, which is followed, mainly in migraineurs, by a markedly delayed headache that fulfills the criteria of a typical migraine attack (Iversen et al. 1989; Sicuteri et al. 1987). Thus, the migraine is temporally dissociated from the immediate and short-lived (<10 min) release of NO (Persson et al. 1994) and the consequent cGMP-dependent (Guo et al. 2008) vasodilatation. Degranulation of meningeal mast cells (Ferrari et al. 2016; Reuter et al. 2001), phosphorylation of extracellular signal-regulated kinase (ERK) in meningeal arteries (Zhang et al. 2013), and delayed meningeal inflammation sustained by induction of NO synthase and prolonged NO generation have been proposed to explain the delayed GTN-evoked headache. The ability of NO generated by GTN to release CGRP

(Ramachandran et al. 2014; Strecker et al. 2002) has been also considered. However, unlike the delayed migraine headache, CGRP release after exposure to GTN/NO is rapid. Thus, further mechanisms must be explored to understand the timing and mechanism of migraine evoked by NO donors.

5.2 Ethanol and TRPV1

It is known that migraineurs report headaches after ingestion of alcoholic beverages more easily than healthy controls and that after a considerable delay they may experience real migraine attacks (Kelman 2007). Relatively elevated concentrations of ethanol sensitize TRPV1 by lowering the normal temperature for channel activation by 8°C (from 43 to 35°C), thus allowing normal body temperature to gate the channel (Trevisani et al. 2002). This activating mechanism elicits a Ca^{2+}-dependent secretion of CGRP from peripheral terminals of DRG neurons (Trevisani et al. 2002). By TRPV1 targeting and the ensuing CGRP release, ethanol produces dilatation of meningeal arteries of guinea pigs (Nicoletti et al. 2008). However, there is a temporal dissociation between the rapid and almost immediate TRPV1-dependent and CGRP-mediated vasodilatation and the much more delayed development of migraine attacks. *The International Classification of Headache Disorders, 3rd edition* (IHS 2018) distinguishes an immediate alcohol-induced headache, which develops within 3 h and resolves within 72 h from alcohol ingestion, and the delayed alcohol-induced headache (previously defined hangover headache), which develops within 5–12 h after alcohol ingestion and resolves within 72 h of onset. As after 12 h from ingestion no ethanol is found in plasma, it seems unlikely that TRPV1 and CGRP are the sole contributors of alcohol-related headaches.

5.3 TRPA1 Agonists

Umbellulone is a major constituent of the *Umbellularia californica*, also known as the headache tree, because seasonal exposure to the scent of the plant provokes severe headaches, including attacks of cluster headache (Benemei et al. 2010) in susceptible individuals. Umbellulone may quickly react with thiols of cysteine residues of TRPA1, thereby activating the trigeminovascular system to evoke a CGRP-dependent meningeal vasodilatation (Nassini et al. 2012), indicating that channel targeting may be the underlying mechanism by which the scent of *Umbellularia californica* evokes migraine attacks. Acrolein is contained in combustion exhausts or can be produced endogenously following peroxidation of plasma protein phospholipids. Its ability to target TRPA1 (Bautista et al. 2006) has also been shown in TG neurons where it can dilate meningeal vessels via a CGRP-mediated mechanism (Kunkler et al. 2011) (Fig. 2).

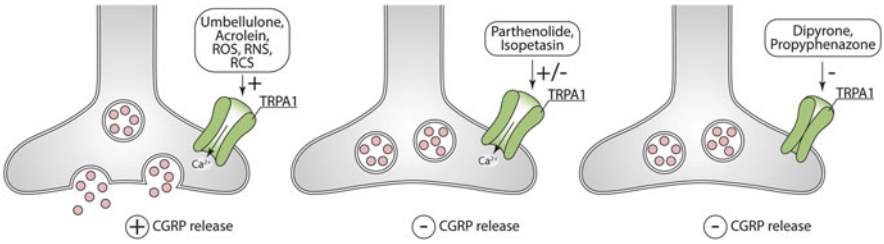

Fig. 2 Schematic representation of the different modes of stimulating or inhibiting CGRP release from peptidergic primary sensory neurons by drugs that target TRPA1, in relation to their ability to provoke or ameliorate migraine, respectively. Umbellulone, which provokes migraine, acrolein, ROS, RNS and RCS are full TRPA1 agonists that target TRPA1 and release CGRP. The prophylactic antimigraine agents, parthenolide and isopetasin, behave as partial TRPA1 agonists that desensitize the channel and the nerve terminal, thus attenuating CGRP release. Dipyrone and propyphenazone, effective drugs for the acute migraine treatment, are selective TRPA1 antagonists

6 Agents that Ameliorate Migraine Attacks and Inhibit CGRP Release

6.1 Nonsteroidal Anti-inflammatory Drugs

We have already addressed the role of triptans that attenuate the release of CGRP and the resultant proinflammatory and proalgesic effects by activating inhibitory 5-HT_{1D} receptors expressed by perivascular nerve terminals. NSAIDs are additional drugs commonly used for the acute treatment of migraine attacks. Their analgesic action relies on the blockade of cyclooxygenases, thereby inhibiting the production of proinflammatory and proalgesic prostaglandins. The ability of prostaglandins (see above) to release CGRP from terminals of peptidergic nociceptors and the ability of NSAIDs to attenuate arachidonic acid or bradykinin-evoked CGRP release (Geppetti 1993) support the view that at least part of the antimigraine action of NSAIDs is due to their ability to attenuate the prostaglandin-evoked (Hingtgen et al. 1995) release of the pro-migraine neuropeptide.

6.2 Herbal Medicines

Herbal medicines have been used for centuries for alleviating pain and headaches. From popular medicine, some of these preparations have gained a more robust position in the therapeutic antimigraine armamentarium because of positive clinical trials and recent acquisitions of their mechanisms of action. Petasin and isopetasin (Avula et al. 2012), contained in butterbur [*Petasites hybridus (L.) Gaertn.*], are considered responsible for the antimigraine effects of the herbal extract. In fact, a preparation containing standardized amounts (minimum 15%, corresponding to 7.5 mg) of petasin/isopetasin (Avula et al. 2012; Danesch and Rittinghausen 2003)

showed a beneficial action in migraine prevention (Grossmann and Schmidramsl 2000; Lipton et al. 2004; Pothmann and Danesch 2005). These studies led to the indication by the American Headache Society guidelines with a level A recommendation of butterbur for migraine prophylaxis (Holland et al. 2012). Several hypotheses, including antileukotriene or antimuscarinic activity (Ko et al. 2001; Thomet et al. 2001), have been proposed to explain the antimigraine action of petasin/isopetasin. Recently, by considering that petasin and its cross-conjugated isomer, isopetasin, are eremophilane sesquiterpene esters of petasol and angelic acid, which contain electrophilic double bonds and can potentially interact with bionucleophiles, it has been hypothesized that they could interact with TRPA1 (Benemei et al. 2017).

The observation that isopetasin targets TRPA1 to evoke Ca^{2+} response in TG neurons and to release CGRP from central terminals of TRPA1-expressing neurons (Benemei et al. 2017) was, however, conflicting with the beneficial action of butterbur in migraine. However, the excitatory action of isopetasin on sensory neurons is weak, as it behaves as a TRPA1 partial agonist. In addition, after in vitro or in vivo exposure to isopetasin, the TRPA1 channel and the TRPA1-expressing trigeminal neurons undergo concentration- and dose-dependent desensitization. Thus, after an initial moderate excitatory action, exposure to isopetasin results in prolonged inhibition of nociceptive responses and CGRP release from TRPA1-expressing neurons (Benemei et al. 2017). A similar moderate excitatory action followed by a prolonged desensitizing effect has been previously reported by parthenolide, a major constituent of *Tanacetum parthenium* (Materazzi et al. 2013). Preparations containing the herbal extract or parthenolide are marketed for migraine prophylaxis. Thus, attenuation of TRPA1 activity on trigeminal neurons, associated with a reduced CGRP release, may be a common mechanism of both butterbur and *Tanacetum parthenium* to ameliorate migraine (Fig. 2).

6.3 Pyrazolone Derivatives

A randomized double-blind clinical trial (Bigal et al. 2002) supported the pharmacoepidemiological observation that the pyrazolone derivative, dipyrone (metamizole), is effective for the acute relief of migraine attacks (Ramacciotti et al. 2007). Although it is not available in some countries (particularly the USA and the UK) because of its association with potentially life-threatening blood dyscrasias such as agranulocytosis, dipyrone remains a successful remedy for treating pain and migraine headaches in other countries. In Brazil, a much larger number of patients (almost 60%) treat their migraine attacks with medicines containing dipyrone alone or in combination, compared to patients who use paracetamol (16%), triptans (6%), or NSAIDs (12%) (Chagas et al. 2015).

Dipyrone and the associated pyrazolone derivative, propyphenazone, which is also successfully used for acute migraine treatment, are commonly included in the larger family of NSAIDs. However, previous (Lorenzetti and Ferreira 1985) and

more recent reports (Nassini et al. 2015) have argued against this conclusion. While indomethacin was equipotent in reducing edema and hyperalgesia evoked by carrageenan in rats, dipyrone showed a remarkable anti-hyperalgesic action but a poor anti-inflammatory effect (Lorenzetti and Ferreira 1985). More recently, it has been reported that carrageenan-evoked mechanical allodynia was attenuated by dipyrone and propyphenazone, without affecting local prostaglandin E_2 levels (Nassini et al. 2015). As dipyrone and propyphenazone selectively inhibited TRPA1-dependent Ca^{2+} responses and currents and attenuated TRPA1-mediated pain-like responses in models of inflammatory and neuropathic pain (formalin, carrageenan, partial sciatic nerve ligation, and bortezomib), it was proposed that analgesia by dipyrone and propyphenazone is due to their TRPA1 antagonistic effect. Finally, dipyrone and propyphenazone reduced the TRPA1-evoked release of CGRP from primary sensory neurons, a response that may account for the antimigraine action of these drugs (Nassini et al. 2015) (Fig. 2).

7 Conclusion

The release of neurotransmitters is usually initiated by propagated action potentials which invade nerve terminals through the opening VGSC. This event is followed by the opening of the VGCC, which, allowing Ca^{2+} influx, promotes the migration and fusion with the plasma membrane of the vesicles that store neurotransmitters, thus leading to neurosecretion. Regarding trigeminal primary sensory neurons, orthodromically propagated action potentials from the periphery invade central terminals to elicit the CGRP release in the lamina I and II of the dorsal horn of the brain stem inside the BBB. It is therefore unlikely that small molecules (olcegepant or telcagepant) which poorly penetrate the BBB (Hostetler et al. 2013; Tfelt-Hansen and Olesen 2011) exert their antimigraine activity (Ho et al. 2008; Olesen et al. 2004) at such a central site of action. Efficacy in migraine treatment of recently developed anti-CGRP receptor monoclonal antibodies (Goadsby et al. 2017; Silberstein et al. 2017), whose penetration of the BBB is limited to 0.1–0.5%, further points to a peripheral action of these new antimigraine medicines.

The observation that effective migraine treatment is associated with blockade of CGRP receptors outside the BBB implies that migraine mechanism is mediated by CGRP release not from central, but rather from peripheral endings of trigeminal primary sensory neurons. There are two neurophysiological pathways that result in peripheral CGRP release. The first is driven by antidromically propagated action potentials that, in a TTX-sensitive manner, invade very terminal fibers, mostly surrounding meningeal or pericranial arterial vessels, to promote a VGCC-dependent neurosecretion. The second is promoted by the activation of ligand-gated ion channels, including the acid-sensitive TRPV1 and the oxidative stress sensor TRPA1, as well as a number of excitatory receptors expressed on the cell membrane. This second pathway is TTX-insensitive and mostly independent from VGCC. Finally, we underline that we owe most of our current understanding of the subtle

mechanisms underlying the outflow of CGRP from TG neurons that have disclosed new avenues for the treatment of migraine to the pioneering studies on antidromic conduction in sensory neurons, which promote neurogenic inflammation and sensitization to pain (Bayliss 1901; Lewis 1936), and to the use of capsaicin (Bevan and Szolcsanyi 1990), the prototypical activator and desensitizing agent of TRPV1, and peptidergic nociceptors.

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CGRP in Animal Models of Migraine

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Abstract

With the approval of calcitonin gene-related peptide (CGRP) and CGRP receptor monoclonal antibodies by the Federal Drug Administration, a new era in the treatment of migraine patients is beginning. However, there are still many unknowns in terms of CGRP mechanisms of action that need to be elucidated to allow new advances in migraine therapies. CGRP has been studied both clinically and preclinically since its discovery. Here we review some of the preclinical data regarding CGRP in animal models of migraine.

Keywords

Animal model · Antibody · CGRP · Migraine

1 Introduction

Migraine is the third most common medical condition in the world (Vos et al. 2012) and a highly debilitating neurological disease (Lipton et al. 2007). Calcitonin gene-related peptide (CGRP) has been in the forefront of migraine research for years, both clinically and in animal models. With the arrival of CGRP monoclonal antibodies for the treatment of migraine headaches, patients are hoping to find better relief for a disorder that highly impairs their quality of life. Here we will review some of the preclinical evidence that led to the realization that CGRP is a key player in migraine pathophysiology (Edvinsson et al. 2018; Ong et al. 2018; Russo 2015a).

After the initial discovery of CGRP (Amara et al. 1982; Rosenfeld et al. 1983), it was suggested that the neuropeptide was linked to nociception and cardiovascular regulation due to its distribution in small trigeminal and spinal sensory ganglion cells and in sensory fibers surrounding the blood vessels (Rosenfeld et al. 1983). Functional studies soon demonstrated that CGRP is the most potent vasodilatory peptide (Brain et al. 1985, 1986), a record that still stands today (Brain and Grant 2004; Russell et al. 2014). In the following years, more systematic studies of CGRP distribution (Skofitsch and Jacobowitz 1985), as well as its colocalization with substance P (Lee et al. 1985; Uddman et al. 1985; Wiesenfeld-Hallin et al. 1984), provided hints that CGRP might play a role in migraine pathophysiology (Edvinsson 1985). Studies in humans and animal models soon afterward laid the foundation for the field as we know it today (Edvinsson 2017).

2 Involvement of CGRP in Animal Migraine Models

Over the years there have been a variety of animal models developed to study migraine. Many, which are described below, were shown to involve CGRP and its receptor in some way. Since CGRP has a multitude of actions in the body (Russell et al. 2014), it is hard to predict which of these may be key for migraine. Nonetheless, evidence from animal models suggests there are both peripheral and central sensitization mechanisms that may be relevant to migraine (Russo 2015b). In the periphery, CGRP is released from primary afferents of the trigeminal nerve into the perivascular space of the meninges, as well as within the ganglia. Receptors have been identified on arterioles, primary afferents that do not express CGRP, glia, and mast cells (Fig. 1). Actions at some or all of these sites can lead to sensitization of trigeminal nociceptive fibers that could contribute to the headache of migraine. In the central nervous system, CGRP released from neurons can act as a neuromodulator to increase glutamatergic signaling. This enhanced neurotransmission could in turn lead to central sensitization that could contribute to headache and other heightened sensory perceptions, such as photophobia. These models are outlined in Fig. 1 and are described below.

2.1 Trigeminal Ganglion Activation Model

The subjective nature of headaches often precludes proper diagnosis and treatment but also makes this pathology hard to model in animals, which cannot orally report their pain. Based on the idea that migraine involves the activation of the trigeminovascular system, one of the first animal models for migraine headache was stimulation of the trigeminal ganglion. This model helped improve our understanding of the anatomy and pharmacology of the trigeminovascular system (Akerman et al. 2013). In particular, Goadsby et al. showed that the electrical stimulation of the trigeminal ganglion in cats induced an elevation of CGRP-like immunoreactivity in blood samples taken from the external jugular vein (Goadsby et al. 1988) and increased the release of CGRP into the cranial circulation on the side of the stimulation (Goadsby and Edvinsson 1993). In rats, stimulation of the trigeminal ganglion caused an increase in blood flow ipsilateral to the side of stimulation that was reduced by intravenous injection of the CGRP antagonist $CGRP_{8–37}$ (Escott et al. 1995).

2.2 Meningeal Stimulation Model

One of the most widely used models of migraine headache to date is stimulation of the dura mater of the meninges that line the brain. Meningeal stimulation can be achieved by application of inflammatory compounds or by electrical stimulation. In anesthetized cats, electrical stimulation of the superior sagittal sinus increased the levels of CGRP in jugular vein blood by 85%, which provided the first evidence that

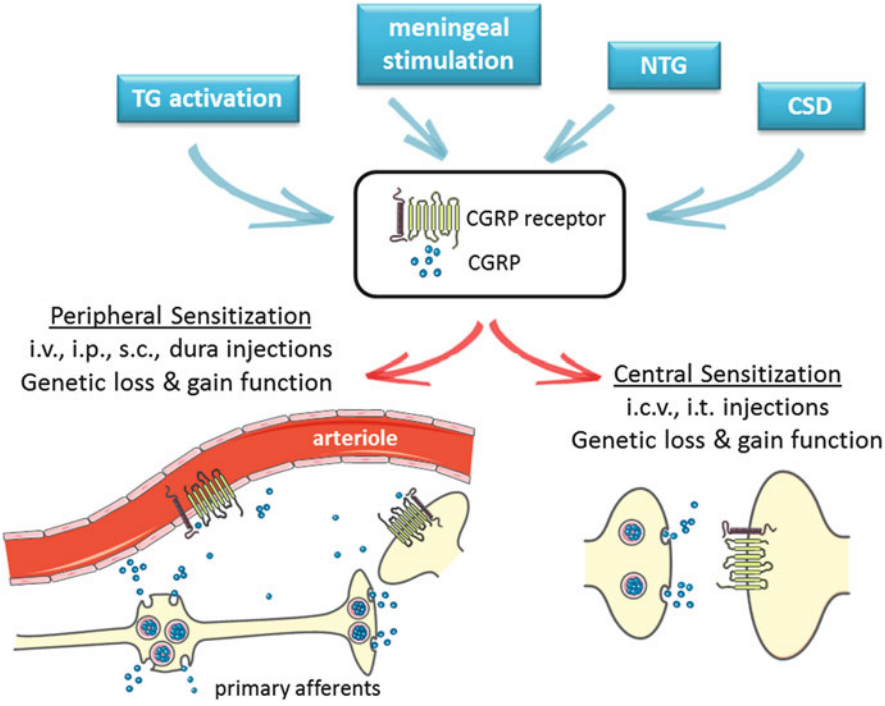

Fig. 1 CGRP in animal models of migraine. Animal models of migraine induced by activation of the trigeminal ganglia (TG), meningeal stimulation, infusion of nitroglycerin (NTG), or cortical spreading depression (CSD) have been shown to involve CGRP and its receptor. A schematic of the calcitonin-like receptor and RAMP1 complex is shown on vessels and neurons. Not shown are CGRP receptors on other cells, including mast cells and glia. While the exact sites and actions of CGRP that are important for migraine are not known, evidence from animal models suggests there are both peripheral and central sensitization mechanisms. Likewise, administration of CGRP by peripheral and central routes is believed to induce migraine-like phenotypes through these sensitization mechanisms. Peripheral delivery routes include intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.), and directly onto the dura. Central delivery includes intracerebroventricular (i.c.v.) and intrathecal (i.t.) routes. Genetic models involving loss or gain of CGRP and/or receptor subunits can also modulate peripheral and central CGRP actions

activation of craniovascular afferents causes release of vasodilatory peptides (Zagami et al. 1990). Using immunohistochemistry, Messlinger and colleagues later showed that the parietal dura mater of the rat was densely innervated by CGRP nerve fibers (Messlinger et al. 1995). Furthermore, it was shown that electrical stimulation of the dural surface caused a depletion of CGRP-immunopositive fibers, suggesting a release of CGRP, and an increase of the dural blood flow around branches of the medial meningeal artery (Messlinger et al. 1995). It was concluded that the stimulation of trigeminal afferents innervating the dura mater induced the release of CGRP from peptidergic afferent terminals, which in turn caused vasodilation and increased meningeal blood flow.

This increase in meningeal blood flow was inhibited in a dose-dependent manner by topical application of the CGRP antagonist $CGRP_{8–37}$ (Kurosawa et al. 1995). In separate studies, Williamson et al. showed that $CGRP_{8–37}$ and two different $5HT_{1B/1D}$ agonists (sumatriptan and rizatriptan) were able to reduce the dilation of dural vessels induced by electrical stimulation in rats and guinea pigs (Williamson et al. 1997, 2001), which mimicked for the first time clinical findings that triptans were able to normalize CGRP levels during a migraine attack (Goadsby et al. 1990). Intravenous injection of another CGRP antagonist, BIBN4096BS, was able to prevent the vasodilatory actions of endogenous CGRP released following transcranial electrical stimulation in rats (Petersen et al. 2004; Troltzsch et al. 2007), as well as inhibit trigeminocervical superior sagittal sinus-evoked activity in cats (Storer et al. 2004). Taken together, those studies show that across species, there are CGRP receptors in the trigeminocervical complex. These data supported the hypothesis that blocking CGRP would be an effective treatment of migraine.

Using isolated rat middle cerebral arteries, CGRP was shown to induce a concentration-dependent dilation with abluminal application, but not by luminal application (Edvinsson et al. 2007). This suggested that CGRP could act on smooth muscle cell CGRP receptors, but could not cross the endothelial barrier. CGRP blockers such as $CGRP_{8–37}$, BIBN4096BS, and CGRP antibody were able to inhibit CGRP-induced relaxation (Edvinsson et al. 2007).

Meningeal stimulation can also be achieved by application of substances directly on the dura. A recognized symptom of migraine is heightened sensitivity to stimuli. The perception of touch as a painful stimulus is reported by nearly half of migraineurs (LoPinto et al. 2006; Mathew et al. 2004). In animal models, this mechanical allodynia can be measured using von Frey filaments. Application of inflammatory mediators directly onto the dura elicits both facial and plantar allodynia that can be reversed by sumatriptan and $CGRP_{8–37}$ (Edelmayer et al. 2009), once again showing that targeting CGRP is a valid strategy to treat pain associated with migraine.

More recently, a study investigated sex differences in behavioral responses after application of inflammatory soup (IS) on the dura (Stucky et al. 2011). While both male and female rats showed behavioral responses (activity measures as well as withdrawal responses for periorbital and perimasseter mechanical testing) to IS application compared to saline, females showed effects at lower doses than males and for longer duration. However, males required fewer applications of IS to exhibit responses (Stucky et al. 2011). In the same study, levels of transcripts for CGRP and the different subunits of its principal receptor (RAMP1, receptor activity-modifying protein 1; CLR, calcitonin-like receptor; and RCP, receptor component unit) were assessed in different areas of the CNS, at baseline or after application of IS to the dura. At baseline, females had lower levels of the receptor components in the trigeminal ganglion and in the medulla, while their CGRP mRNA levels were higher in the medulla than males. After IS and saline application to the dura, CGRP transcript levels were upregulated in all groups (Stucky et al. 2011). This suggests that the CGRP pathway responds to changes in intracranial pressure or meningeal stretch, while migraine-like behaviors occur after meningeal inflammation.

2.3 Nitroglycerin-Induced Model

Nitric oxide (NO) is a regulator of cerebral blood flow and vessel diameter. Nitroglycerin (NTG), a NO donor, can be used to trigger migraine in migraineurs (Christiansen et al. 1999; Thomsen et al. 1994), and response to nitroglycerin is a diagnostic test for migraine (Ferrari et al. 2015). This has led to the use of NTG administration as a trigger for sensory hypersensitivity associated with migraine in laboratory animals. Considering that NO is an important signaling molecule involved in the synthesis and release of CGRP from trigeminal ganglion neurons (Bowen et al. 2006), many of the drugs designed to block CGRP to treat migraine symptoms are also effective in NTG-induced migraine models.

NTG administration induces thermal and mechanical allodynia as well as thermal hyperalgesia in rodents (Bates et al. 2010; Tassorelli et al. 2003). Sumatriptan, the gold-standard anti-migraine drug is a 5-HT_{1B} and 5-HT_{1D} agonist that can prevent the release of CGRP in plasma (Goadsby and Edvinsson 1994). Administered centrally (i.t.) and peripherally (i.p.), sumatriptan was able to reduce NTG-induced thermal hypersensitivity; only the central injection of sumatriptan was able to reduce mechanical hypersensitivity (Bates et al. 2010). Similarly, NO-induced increase in spinal trigeminal activity can be reduced by the CGRP receptor antagonists BIBN4096BS (later called olcegepant) and MK-8825 (Feistel et al. 2013; Koulchitsky et al. 2009). Subsequently, it was found that nitroglycerin (i.p.) administration to rats increased CGRP levels in the brainstem and trigeminal ganglia (Capuano et al. 2014). Additionally, those authors showed that an injection of CGRP in the whisker pads of rats only increased the time the rats spent in face rubbing when they were pre-treated with NTG (Capuano et al. 2014). This suggests that NTG can sensitize the trigeminal system for CGRP to induce a painful behavior in rats (Capuano et al. 2014). In order to study the progression from acute to chronic migraine, Pradhan and colleagues used chronic injection of NTG every other day for 9 days, which induced progressive and sustained hyperalgesia (Pradhan et al. 2014). This time however, systemic or central sumatriptan did not ameliorate NTG-induced chronic hyperalgesia (Pradhan et al. 2014).

2.4 Cortical Spreading Depression Model

Cortical spreading depression (CSD) is hypothesized to cause migraine auras (Cutrer and Huerter 2007). There is some evidence pointing toward an interaction between CSD events and CGRP actions (Close et al. 2018). A recent study showed that BIBN4096BS could decrease the amplitude and propagation rate of repeated retinal spreading depression episodes induced by potassium in chick retinal preparation (Wang et al. 2016). Blocking CGRP receptors with $CGRP_{8–37}$ attenuated CSD-associated hyperperfusion in the rat (Reuter et al. 1998) and reduced CSD-induced pial dilatation (Colonna et al. 1994; Wahl et al. 1994), suggesting that the release of CGRP by trigeminal sensory neurons is responsible, at least in part, for some of the vascular changes associated with CSD. In a recent study,

MK-8825 (CGRP receptor antagonist) did not alter CSD waves or CSD-induced change in regional cerebral blood flow (Filiz et al. 2017). It did however attenuate CSD-induced trigeminal nerve-mediated freezing and spontaneous responses (both body and head grooming, wet dog shakes, and head shakes). Other behaviors such as eating/drinking, rearing, and turning that are impaired after induction of CSD were not changed after administration of MK-8825 (Filiz et al. 2017). Finally, CSD-induced periorbital allodynia is reversed by administration of MK-8825 (Filiz et al. 2017). Taken together, those studies show that the blockade of CGRP can decrease the impact of CSD and seem to indicate that CGRP may be involved in the propagation of CSD (but not its initiation). Interestingly, a recent study showed that induction of CSD by KCl in rats resulted in increased CGRP protein expression in the trigeminal ganglia, although there was no change in CGRP transcript levels (Yisarakun et al. 2015), which points to a possible positive feedback loop between CSD and CGRP (Close et al. 2018).

3 Animal Models of Migraine Induced by Injection of CGRP

After it was reported that (1) CGRP levels are elevated during spontaneous migraine and in between attacks in patients with chronic migraine (Bellamy et al. 2006; Goadsby et al. 1990; van Dongen et al. 2017), and (2) an intravenous infusion of CGRP could induce a delayed migraine-like headache in migraineurs (Asghar et al. 2011; Hansen et al. 2010; Lassen et al. 2002), animal models of migraine induced by injection of CGRP were developed. Administration of CGRP by peripheral and central routes is believed to induce migraine-like phenotypes through peripheral and central sensitization mechanisms, respectively (Fig. 1). However, it must be emphasized that the mechanisms are not mutually exclusive. For example, peripheral sensitization can lead to central sensitization. In this section, we describe animal models based on the peripheral and central delivery routes, which are outlined in Fig. 1 and summarized in Table 1.

3.1 Intravenous CGRP Delivery

Intravenous infusion of CGRP at a dose able to induce vasodilation is sufficient to induce a migraine-like headache in 66% of migraineurs (Asghar et al. 2011; Guo et al. 2016; Hansen et al. 2010; Lassen et al. 2002). In contrast, it only provokes a mild headache in non-migraineurs (Petersen et al. 2005a), suggesting that migraineurs are more sensitive to CGRP (Russo et al. 2009). Based on these clinical observations, the effects of i.v. CGRP have been studied in animals (Table 1).

Up until the discovery of one unique wild-type rat displaying spontaneous episodic trigeminal allodynia (Munro et al. 2018; Oshinsky et al. 2012), scientists had not been able to witness any occurrence of spontaneous migraine symptoms in laboratory animals. It was therefore not possible to discriminate a migraineur vs. non-migraineur population in animals without evoking symptoms. Nevertheless,

Table 1 Potential migraine-related effects of CGRP administered by different routes in rodents

CGRP delivery	Phenotype	Species and reference
Intravenous	• ↓ Blood pressure • No c-Fos activation in in trigeminal nucleus caudalis • ↑ c-Fos protein in the brainstem • ↑ p-ERK in dura mater	Rat Bhatt et al. (2015)
	• ↓ Mean arterial pressure • tachycardia • ↑ Cardiac output • No change in stroke volume • ↓ Total peripheral resistance	Rat Lappe et al. (1987)
	• ↓ Mean arterial pressure • ↑ Heart rate and cardiac output • ↓ Total peripheral resistance • ↑ Mesenteric and hindquarter blood flow • Dose-dependent changes in renal blood flow • ↓ Resistance in all vascular beds	Rat Siren and Feuerstein (1988)
	• Dilation of middle meningeal artery • Facilitated vibrissal responses	Rat Cumberbatch et al. (1999)
	• Hypotension • Dilation of middle meningeal artery • ↑ Pial artery/arteriole diameter	Rat Petersen et al. (2004)
	• ↑ Dilation of cerebral arteries when applied abluminally but not luminally • ↑ Dilation of cerebral cortical pial arteries/arterioles • ↓ Blood pressure	Rat Petersen et al. (2005a, b)
	• ↑ Dural blood flow • No activation or sensitization of meningeal nociceptors	Rat Levy et al. (2005)
Intracerebroventricular	• No c-Fos activation in trigeminal nucleus caudalis	Rat Bhatt et al. (2014)
	• ↑ Light aversion • ↓ Locomotion in the dark	Mouse Kaiser et al. (2012)
	• ↑ Light aversion • ↑ Resting in dark	Mouse Mason et al. (2017)
	• ↑ Hindpaw withdrawal latency to thermal and mechanical stimulation (antinociception)	Rat Huang et al. (2000)
	• ↑ Tail-flick latencies to thermal stimulation (antinociception) • ↑ Response latencies on the hot plate (antinociception) • ↓ Evoked thalamic neuronal firing	Rat Pecile et al. (1987)

(continued)

Table 1 (continued)

CGRP delivery	Phenotype	Species and reference
	• ↑ Paw-withdrawal latencies in C57BL/6 mice • ↓ Depression-like behavior in forced swim test in both C57BL/6 and AKR mice	Mouse Schorscher-Petcu et al. (2009)
Intrathecal	• ↑ Mechanical and thermal hyperalgesia in AKR but not C57BL/6 mice	Mouse Mogil et al. (2005)
	• ↑ Hyperalgesia to mechanical noxious stimuli (pinching the hind paw)	Rat Oku et al. (1987)
	• ↑ Mechanical allodynia at high dose	Mouse Marquez de Prado et al. (2009)
Intraperitoneal	• ↑ Light aversion in CD1 and C57BL/6J • ↑ Resting in dark in both strains	Mouse Mason et al. (2017)
	• ↑ Facial signs of discomfort in CD1 and C57BL/6J • ↑ Squint in both strains	Mouse Rea et al. (2018)
	• ↑ Diarrhea	Mouse Kaiser et al. (2017)
Dural and epidural	• ↑ Dural blood flow • No activation or sensitization of meningeal nociceptors	Rat Levy et al. (2005)
	• ↓ Climbing hutch and face grooming • ↑ Immobile behavior	Rat Yao et al. (2017)
	• ↑ Periorbital hypersensitivity in female mice	Mouse Burgos Vega et al. (2017)
Subcutaneous and intradermal	• ↑ Blood flow • No change in thermal hyperalgesia	Rat Chu et al. (2000)
	• No change in mechanical hyperalgesia	Rat Nakamura-Craig and Gill (1991)
	• ↓ Paw withdrawal threshold to noxious heat (hyperalgesia) • Strain-dependent hyperalgesia with the hot plate assay • ↑ Sensitivity to mechanical stimulation with von Frey filaments	Mouse Mogil et al. (2005)
	• No tactile allodynia	Mouse Marquez de Prado et al. (2009)

scientists have studied the effect of i.v. CGRP in preclinical settings. Considering that migraine has historically been considered a vascular disorder, vascular actions of CGRP are important to take into account. In animals, an infusion of CGRP induced a dose-dependent decrease in blood pressure and increase in heart rate (Bhatt et al. 2015; Lappe et al. 1987; Siren and Feuerstein 1988). IV CGRP administration in rats caused a dilation of the cortical pial arteries and arterioles and of the middle meningeal artery and increased local cortical cerebral blood flow, all of which could be inhibited by the CGRP receptor antagonist BIBN4096BS (Cumberbatch et al. 1999; Petersen et al. 2004, 2005b). Surprisingly, and to our knowledge, there is very little in the literature about nociceptive actions of i.v. CGRP. IV CGRP facilitated vibrissal responses, which seemed to indicate that CGRP-induced vasodilation was activating primary afferent meningeal nociceptors (Cumberbatch et al. 1999). However, electrophysiological studies later showed that it was not the case (Levy et al. 2005). Additionally, a recent study showed that CGRP infusion in awake rats failed to increase c-Fos and Zif268 (neuronal pain markers) expression in the trigeminal nucleus caudalis (Bhatt et al. 2015).

Although i.v. CGRP is the most translational approach for CGRP administration, the inherent difficulty and stress from performing an i.v. injection in rodents, especially in mice, led to the use of other routes of injections in preclinical studies.

3.2 Intraperitoneal CGRP Delivery

A relatively easy method to deliver CGRP to the peripheral tissues of rodents to allow assessment of migraine-like symptoms is by intraperitoneal (i.p.) injections (Table 1). Notably, i.p. CGRP induced light aversion both in CD1 and C57BL/6J mice, which was attenuated by both sumatriptan and an anti-CGRP antibody (Mason et al. 2017). These results, coupled with results obtained centrally, suggest that CGRP actions to induce migraine-like behavior are mediated by both peripheral and central mechanisms (Mason et al. 2017).

Recently, we also described i.p. CGRP-induced spontaneous pain in mice. The mice showed increased facial signs of discomfort (grimace and squint) (Rea et al. 2018). Those phenotypes were also reversed by anti-CGRP antibody. Interestingly, sumatriptan partially inhibited CGRP-induced spontaneous pain in males but not females (Rea et al. 2018). Of importance, the dose of 0.1 mg/kg i.p. used in all of our studies is able to induce vasodilation visible as redness of the ears (Rea et al. 2018).

Additionally, migraine symptomatology includes gastrointestinal problems, which occur in 22% of migraineurs (Kelman 2004). Our team reported that i.p. CGRP administration induced diarrhea in C57BL/6J mice and that olcegepant (previously called BIBN4096BS), a CGRP receptor antagonist, was able to attenuate this symptom (Kaiser et al. 2017).

The use of triptans in the previously mentioned studies validates the symptoms as being migraine-related but also provides some clues about triptan mechanisms of action. It is known that triptans are vasoconstrictors that can also inhibit endogenous neuropeptide release via 5-HT_{1D} receptors (Durham and Russo 2002; Loder 2010).

Importantly, clinical studies demonstrated that triptans can reverse CGRP-induced vasodilation and headache in normal subjects and migraine patients (Asghar et al. 2010, 2011). Thus, in both mice and humans, triptans are able to override exogenous CGRP, suggesting that their mechanism of action must be more than just inhibition of CGRP release. Moreover, colocalization of 5-HT_{1D} and CGRP in the spinal trigeminal nucleus and other areas in the brainstem such as the parabrachial nucleus (Noseda et al. 2008) and the fact that triptans can downregulate nociceptive signal transmission in the spinal trigeminal nucleus (Levy et al. 2005; Mitsikostas et al. 1999) support the hypothesis that triptans can mask the effect of a bolus injection of CGRP injected either centrally or peripherally. It is very likely that triptan mechanism of action to relieve migraine-like symptoms involves actions at multiple sites (Ahn and Basbaum 2005; Kaiser et al. 2012).

3.3 Subcutaneous and Intradermal CGRP Delivery

In accordance to the results obtained with other routes of administration, intradermal CGRP (in rats and in rabbits) induced an increase in blood flow (Brain et al. 1985; Chu et al. 2000) (Table 1). However, intradermal CGRP did not induce any thermal hyperalgesia in rats (Chu et al. 2000). Early studies also showed a lack of effect of subplantar CGRP compared to that of substance P and neurokinin A in exacerbating the response to paw pressure (mechanical hyperalgesia) in Wistar rats (Nakamura-Craig and Gill 1991). This is consistent with early results in the human skin where CGRP was proposed to have a role in blood flow regulation and in mediating flare response but most likely had no direct role in nociception since the concentrations at which it induced histamine release exceeded normal physiologic concentrations (Brain et al. 1986; Weidner et al. 2000). In a later study, Mogil and colleagues reported a strain difference in the development of thermal hyperalgesia after subcutaneous (s.c.) CGRP injection into the plantar hindpaw. AKR mice but not in C57BL/6J mice seemed to become hypersensitive (Mogil et al. 2005). In our hands, C57BL/6J mice did not develop tactile allodynia assessed by von Frey filaments after intraplantar injection of CGRP (Marquez de Prado et al. 2009). Since we have shown that CD1 mice are more sensitive to CGRP-induced light aversion (Mason et al. 2017), this lack of effect may be strain specific.

3.4 Dural and Epidural CGRP Delivery

Similar to previously described models of meningeal stimulation, and because the activation of the trigeminal nerve leads to release of CGRP from perivascular nerve endings at meningeal blood vessels, dural/epidural delivery of CGRP was used as a model for migraine pathophysiology (Table 1). Dural delivery of CGRP induced a significant increase in dural blood flow, although it reportedly did not activate or sensitize meningeal nociceptors (Levy et al. 2005). Recently however, Yao and colleagues described a reduction in climbing and face-grooming behaviors

accompanied by increased immobile behavior after epidural CGRP administration in rats (Yao et al. 2017). Very interestingly, Dussor and colleagues have recently reported that CGRP can directly stimulate the dura of female but not male rodents to induce periorbital hypersensitivity (Burgos Vega et al. 2017). Additionally, CGRP was able to prime female rodents to a usually innocuous dural application of a pH 7.0 solution.

3.5 Intracerebroventricular CGRP Delivery

Since studies pointed towards a central mechanism of CGRP in migraine pathophysiology, intracerebroventricular (i.c.v.) injections of CGRP were studied (Table 1). Although i.c.v. injection in awake rats did not increase c-Fos expression in the trigeminal nucleus caudalis (Bhatt et al. 2014), a similar injection showed migraine-like behavioral effects. An important trigger and/or symptom experienced by migraineurs is photophobia or photosensitivity, which is an altered perception of light that elicits discomfort (Boulloche et al. 2010; Kelman 2007; Martin and Behbehani 2001; Mulleners et al. 2001; Rasmussen 1993; Spierings et al. 2001). In rodents, light aversion represents a surrogate for photophobia and can be measured using a conflict assay between a light and a dark chamber. Using this test, our lab has shown that i.c.v. injection of CGRP in mice induced light-aversive behavior when exposed to bright light (27,000 lux) but not to dim light (55 lux), which was attenuated by rizatriptan, a 5-$HT_{1B/D}$ agonist anti-migraine drug (Kaiser et al. 2012; Mason et al. 2017). Those animals also showed an increased time spent resting in the dark compartment, which is a behavior similar to migraineurs who tend to seek a dark place to rest during attacks (Kaiser et al. 2012; Mason et al. 2017). These findings indicate that CGRP can act in the CNS to cause light aversion.

Other studies assessed the role of i.c.v. CGRP on nociception. Antinociceptive effects of CGRP administered intracerebroventricularly into the nucleus raphe magnus, amygdala, nucleus accumbens, or the periaqueductal gray were reported in rats submitted to thermal and mechanical stimulations (Huang et al. 2000; Li et al. 2001; Pecile et al. 1987; Xu et al. 2003; Yu et al. 2003; Zhou et al. 2003). Additionally, it was reported that i.c.v. CGRP increased paw withdrawal latencies to thermal stimuli in C57BL/6 mice but not in AKR mice while decreasing depression-like behaviors in both strains in the forced swim test (Schorscher-Petcu et al. 2009). In the same study, i.c.v. CGRP and CGRP receptor antagonists failed to modulate activity in the elevated plus maze, a model of anxiety (Schorscher-Petcu et al. 2009).

3.6 Intrathecal CGRP Delivery

Studies showed that CGRP is located in small diameter dorsal root ganglion neurons (Hokfelt et al. 1992), dorsal horns (Hokfelt et al. 1992; Ishida-Yamamoto and Tohyama 1989), and intermediolateral and ventral horns of the spinal cord (Bennett

et al. 2000; Marti et al. 1987; Senba and Tohyama 1988). Spinal cord central sensitization following intradermal capsaicin injection has been shown to be mediated by CGRP and its receptors (Carlton et al. 1990; Sun et al. 2004). Thus, researchers explored the effects of intrathecal (i.t.) CGRP administration on pain responses (Table 1). Administration of i.t. CGRP induced mechanical and thermal hyperalgesia in rats (Mogil et al. 2005; Oku et al. 1987). Our team showed that a low dose of CGRP injected into the lumbar spinal region did not exacerbate the response to an innocuous mechanical stimulus (von Frey filaments), while a higher dose evoked mechanical allodynia (Marquez de Prado et al. 2009). These data indicate that CGRP can act centrally to sensitize mice to thermal and mechanical stimuli.

4 Genetic Manipulation of CGRP in Migraine Models

Genetic manipulations have been used to directly investigate CGRP signaling. Transgenic and knockout mice have been generated that have either a loss or gain of function of the CGRP ligand or receptor subunits. These genetic models allow modulation of peripheral and central CGRP actions (Fig. 1). A thorough review of all CGRP and receptor subunit mutant mice and their phenotypes can be found elsewhere (Sowers et al. 2017). We will focus on the few cases where migraine-like phenotypes were assessed.

4.1 Overexpression of Human RAMP1

With the goal to study migraine, our laboratory developed a subset of genetic constructs revolving around the overexpression of the human receptor activity-modifying protein 1 (RAMP1) subunit of the CGRP receptor. Using RAMP1, which has been shown to be functionally rate-limiting (Zhang et al. 2006, 2007), allowed the development of CGRP-sensitized mice. Overexpressing the human version of the gene provided the advantage that it can be targeted by drugs designed for clinical application and therefore allow more translatable models (Russo 2015b). To generate those models, the approach was to use double-transgenic mice that express hRAMP1 in a tissue-specific Cre-dependent manner or in all tissues.

Global overexpression of hRAMP1 in all tissues was achieved using mice expressing Cre recombinase under the control of the ubiquitous adenovirus EIIa promoter (Bohn et al. 2017). Cultures obtained from vascular smooth muscle and trigeminal ganglia from those global mice showed an increased CGRP receptor activity that could be blocked by drugs such as CGRP receptor antagonists telcagepant and $CGRP_{8–37}$ (Bohn et al. 2017). Mice with global hRAMP1 overexpression display increased vasodilation of the carotid and basilar arteries, and cerebral arterioles after CGRP application (Chrissobolis et al. 2010), and decreased angiotensin II-induced hypertension (Sabharwal et al. 2010).

In order to study more specifically the role of CGRP and its receptor in the nervous system, double-transgenic mice were developed using nestin-Cre to drive

expression in neurons and some glia cells (Zhang et al. 2007). The injection of CGRP in the whisker pads of those animals increased neurogenic inflammation by doubling plasma extravasation (Zhang et al. 2007). Those animals also display behaviors consistent with migraine such as mechanical allodynia and photosensitivity. While the *nestin/hRAMP1* mice have similar hindpaw withdrawal thresholds to von Frey filament stimulation than control littermates, their response frequency drastically increased after intrathecal CGRP injection, while the same dose of CGRP did not elicit a response in control animals (Marquez de Prado et al. 2009). *Nestin/hRAMP1* mice also show an increased sensitivity to tactile stimulation after capsaicin injection which extended to the contralateral hindpaw, suggesting central sensitization (Marquez de Prado et al. 2009). The transgenic *nestin/hRAMP1* mice display light-aversive behavior when confronted to bright light (Recober et al. 2009). This light aversion is enhanced after i.c.v. injection of CGRP even when exposed to very dim light (55 lux) (Recober et al. 2010). In the same conditions, those mice also display a decrease in motility behaviors once in the dark, such as rearing, distance travelled, time spent moving, and ambulatory velocity (Recober et al. 2010), which resembles the behavior of migraineurs who will seek out a dark room to rest during an attack.

As mentioned earlier, i.p. injection of CGRP in wild-type mice induced light aversion when exposed to very bright light (Mason et al. 2017). Interestingly, and contrasting to the results obtained with i.c.v. CGRP, *nestin/hRAMP1* transgenic mice were not sensitized to i.p. CGRP when exposed to dim lights (Mason et al. 2017). In conclusion, the hRAMP1 double transgenic mice enabled the understanding that CGRP is a key player in migraine both centrally through action on neurons and peripherally on receptors that are not located in the nervous system. Experiments are currently underway to assess the role of CGRP receptors on smooth muscle and the endothelium in the periphery.

4.2 Other Transgenic Models

A few other transgenic models affecting CGRP signaling assessed nociceptive and vascular changes that can have implications for migraine pathophysiology.

In terms of nociception, different lines of CGRP knockout mice have been developed that show maladaptation to pain. In contrast to wild-type mice, Zhang and colleagues reported a CT/αCGRP knockout mouse that showed no sign of secondary hyperalgesia after development of carrageenan-induced inflammation in the knee joint (Zhang et al. 2001). Another strain of αCGRP knockout showed an attenuated licking response to capsaicin and formalin injections as well as a reduction of the edema produced by carrageenan injection in the hindpaw (Salmon et al. 2001). This transgenic mouse also displayed no sign of thermal hyperalgesia after ATP-induced TRPV1 potentiation (Devesa et al. 2014) and reduced morphine analgesia (Salmon et al. 1999). CGRP knockout mice also present a reduced vestibule-ocular reflex (Luebke et al. 2014) and abnormal cochlear response (Maison et al. 2003) which can be of importance in the pathophysiology of migraine. Keeping

in mind that migraine has a vascular component, the effect of CGRP gene deletion on the cardiovascular system was assessed but remains controversial, with reports of a lack of effect (Lu et al. 1999) and reports of increased blood pressure (Gangula et al. 2000; Oh-hashi et al. 2001). In one study, RAMP1 knockout mice also had elevated blood pressure (Tsujikawa et al. 2007).

5 CGRP Antibodies: New Era in Migraine Treatment

Monoclonal antibodies that target either CGRP or its receptor have now been approved by the Federal Drug Administration for the preventive treatment of migraine. Erenumab (Amgen/Novartis) blocks CGRP receptors. Fremanezumab (Teva Pharmaceuticals) and galcanezumab (Eli Lilly) bind to CGRP and block its binding to the receptors. A fourth antibody, eptinezumab (Alder Biopharmaceuticals), also blocks CGRP and is on track for approval.

In the 1980s and 1990s, it was found that intrathecal injection of CGRP antisera could block the pain induced by thermal (Kawamura et al. 1989) and mechanical (Kawamura et al. 1989; Kuraishi et al. 1988) noxious stimuli in rats receiving injections of adjuvant arthritis or carrageenin in the paw. In addition, CGRP antiserum partially rescued the reduced nociceptive threshold evoked by repeated cold stress (Satoh et al. 1992). However, antibody studies that are more relevant to migraine have only been pursued in the past few years.

Several studies have examined the effect of CGRP-blocking antibodies on migraine-like symptoms in mice. Mason et al. studied the effect of one monoclonal anti-CGRP antibody (ALD405) in light aversion in mice (Mason et al. 2017). These mice were first treated with CGRP (i.p.) to establish the degree of their responsiveness to CGRP. The mice were then given anti-CGRP antibody (i.p.) 24 h before given CGRP (i.p.) a second time. The amount of anti-CGRP antibody injected was ~eightfold excess over exogenous CGRP. The results showed that CGRP antibody attenuated CGRP (i.p.)-induced light-aversive behavior (details about CGRP-induced light aversion in Sect. 3.2). The results suggest a peripheral action of CGRP in the induction of light aversion. Likewise, Rea et al. showed that ALD405 administration (i.p.) prevented CGRP (i.p.)-induced spontaneous grimace (indicator of facial discomfort) in CD1 mice both in males and females. ALD405 administration also prevented the grimace in restrained C57BL/6J mice independent of the light. Another measurement of facial discomfort is squint, which was the principle component of the grimace, accounting for 77% of the total variation of grimace scale. CGRP-induced squint in restrained CD1 mice and C57BL/6J mice was prevented by ALD405 administration (i.p.). This suggests that CGRP can act in the periphery to induce a pain response. Gastrointestinal issues are one of the most common symptoms of migraine including diarrhea (Kelman 2004) (see Sect. 3.4). CGRP injection induced diarrhea in C57BL/6J mice, while anti-CGRP antibodies blocked CGRP-induced diarrhea (Kaiser et al. 2017). Moreover, it has been reported that a different CGRP antibody suppressed CSD as indicated by increased latency of CSD, and this effect was blocked by exogenous CGRP (Jiang et al. 2018).

In a series of experiments in rats, i.v. administration of the CGRP-blocking antibody fremanezumab was shown to inhibit the activation of high-threshold trigeminovascular neurons that were responsive to mechanical stimulation of the dura, but not to either innocuous or noxious stimulation of the skin or cornea. Fremanezumab also prevented the activation of trigeminovascular high-threshold neurons by CSD induced mechanically by inserting a glass micropipette into the visual cortex (Melo-Carrillo et al. 2017a). Moreover, fremanezumab pretreatment inhibited the response of Aδ, but not C-fiber, neurons in response to CSD (Melo-Carrillo et al. 2017b). These results provide a mechanism by which fremanezumab could reduce the intracranial pain of migraine. In addition, it was demonstrated that fremanezumab can treat medication overuse headache symptoms in rats. For these experiments, rats were primed with repeated sumatriptan or morphine treatments. Fremanezumab significantly inhibited bright-light stress or NO donor-induced cutaneous allodynia (Kopruszinski et al. 2017). The data suggest that medication overuse headache may be CGRP-dependent and that the anti-CGRP antibody may be a potential therapeutic.

6 Conclusion

In conclusion, many animal models of migraine involve CGRP to some degree. The importance of CGRP in those models is confirmed by the ability of direct injection of CGRP to induce several migraine-like symptoms in rodents. Further, these preclinical observations are in full alignment with the recent success of CGRP-based migraine therapeutics in patients. Thus, CGRP in animal models of migraine is an excellent example of successful translation of science from the lab to the patient.

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CGRP in Human Models of Migraine

Håkan Ashina, Henrik Winther Schytz, and Messoud Ashina

Contents

Abstract

Over the past three decades, calcitonin gene-related peptide (CGRP) has emerged as a key molecule. Provocation experiments have demonstrated that intravenous CGRP infusion induces migraine-like attacks in migraine with and without aura patients. In addition, these studies have revealed a heterogeneous CGRP response, i.e., some migraine patients develop migraine-like attacks after CGRP infusion, while others do not. The role of CGRP in human migraine models has pointed to three potential sites of CGRP-induced migraine: (1) *vasodilation via cyclic adenosine monophosphate (cAMP) and possibly cyclic guanosine monophosphate (cGMP)*; (2) *activation of trigeminal sensory afferents,* and (3) *modulation of deep brain structures*. In the future, refined human experimental studies will continue to unveil the role of CGRP in migraine pathogenesis.

Keywords

CGRP · Human provocation models · Migraine

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1 Introduction

CGRP is a key signaling molecule in migraine pathophysiology and anti-CGRP drugs constitute a new promising target in migraine treatment (Khan et al. 2017a). CGRP is widely distributed in the central and peripheral nervous system (Amara et al. 1982; Rosenfeld et al. 1983; van Rossum et al. 1997; Hostetler et al. 2013), including in the perivascular trigeminal sensory afferents (Uddman et al. 1985), trigeminal ganglion (Uddman et al. 1985), and trigeminal nucleus caudalis (TNC) (Uddman et al. 2002). Aside from its function as a potent dilator of human cerebral arteries (McCulloch et al. 1986; Edvinsson et al. 1987), CGRP is released upon activation of the trigeminal ganglion (Goadsby et al. 1988) and induces migraine-like attack in migraine patients (Lassen et al. 2002). Thus, it seems that CGRP plays an important role in the development of migraine attacks. This chapter reviews studies on CGRP-induced migraine and discusses possible mechanisms underlying migraine induction following CGRP infusion.

2 Methodology in Human Migraine Models

Human headache models have greatly improved our understanding of migraine pathophysiology (Ashina et al. 2013; Schytz et al. 2017). The first human headache model was developed through systematic research using intravenous infusion of the nitric oxide donor glyceryl trinitrate (GTN) (Iversen et al. 1989). It has been demonstrated that GTN provokes migraine attacks without aura in migraine patients with and without aura (Thomsen et al. 1994; Christiansen et al. 1999). To date, other human models have been developed including migraine provocation models using intravenous infusion of CGRP (Lassen et al. 2002) and PACAP38 (Schytz et al. 2009).

We will briefly discuss the main aspects to consider when applying the human migraine model. For a more detailed description on this, readers are referred to recently published work (Fig. 1) (Ashina et al. 2017). In general, human migraine models use a double-blinded, crossover design (Olesen et al. 2009), where patients are randomly allocated to receive intravenous infusion of pharmacological migraine "triggers" or placebo (isotonic saline). An 11-point numeric scale (NRS 11) from 0 to 10 (0 no headache, 10 worst imaginable headache) is used to record headache characteristics up to 24 h after the start of infusion and to evaluate whether the patients develop headache with typical migraine features. It is important to notice that only pharmacological signaling molecules that induce sufficient headache in healthy volunteers should be tested on migraine sufferers. Furthermore, healthy volunteers are used to determine an optimal dose causing vascular responses (e.g., dilation of cranial arteries) and headache. Provocation experiments in migraine patients require that patients are headache-free for at least 5 days before the experimental days. Therefore, patients with a moderate frequency of migraine attacks are preferable for recruitment. In addition, patients should not take preventive medication because it might influence the outcomes. Healthy volunteers with susceptibility

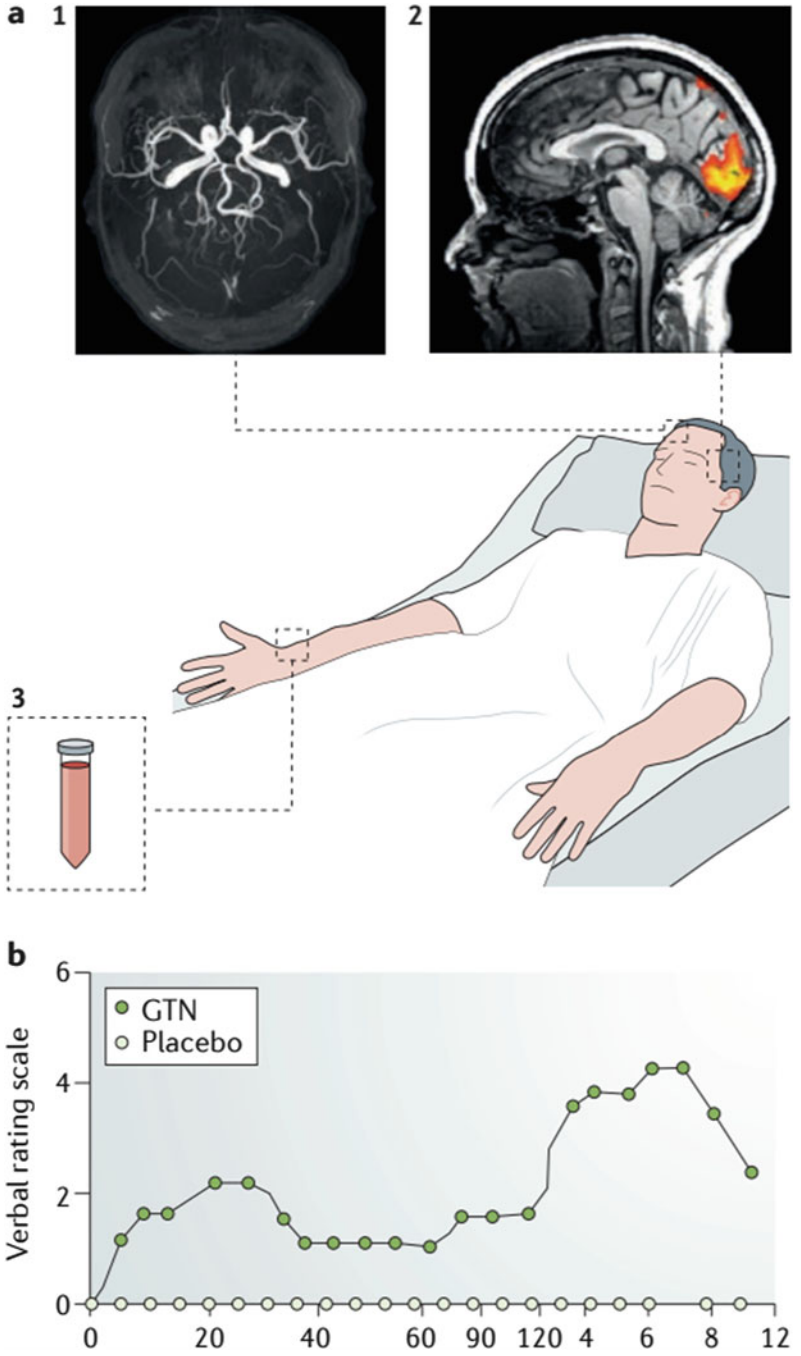

Fig. 1 The setup of an experimental human migraine study. (**a**) At baseline and at fixed and predefined intervals, the hemodynamic effects of the infusion are recorded, and might include

to migraine (first-degree relatives suffering from migraine) should be excluded to avoid triggering migraine.

Human provocation studies have also been combined with different recording techniques to investigate vascular biomarkers by transcranial Doppler ultrasonography (TCD) (Petersen et al. 2005; Lassen et al. 2008) and magnetic resonance imaging (MRI) angiography (Asghar et al. 2010, 2011) and CNS biomarkers (activation of deep brain structure) by functional MRI (fMRI) (Asghar et al. 2012, 2016) (Fig. 2).

3 CGRP-Induced Migraine

The first demonstration of CGRP-induced migraine-like attacks was documented in a double-blind crossover study (Lassen et al. 2002). Twelve patients suffering from migraine without aura (MO) were randomly allocated to receive CGRP (2 μg/min) or placebo infusion in the cubital vein over 20 min (Lassen et al. 2002). The final analyses excluded three of the patients, two of whom experienced hypotension associated with pallor and palpitations. The authors stated that the induced hypotension rendered the used CGRP dose to be the maximally tolerated dose. The remaining nine patients reported headache after CGRP infusion. All patients experienced flushing of the face, neck, and upper chest approximately 10 min after the start of CGRP infusion. Three patients reported migraine-like attacks according to the International Headache Society classification criteria for MO (Headache Classification Committee of the International Headache Society 1988). However, experimentally induced migraine attacks are not spontaneous and, therefore, we proposed different criteria for experimentally induced migraine (Hansen et al. 2010). By applying these criteria (Hansen et al. 2010) in the first CGRP provocation study (Lassen et al. 2002), we calculated that six out of nine patients (67%) experienced migraine-like attacks after CGRP infusion to only one after placebo. Later, two experimental studies also confirmed that CGRP induced migraine-like attacks in 67% of MO patients (Asghar et al. 2011; Guo et al. 2016). In addition, one of the studies reported that 40% of MO patients developed migraine-like attacks in the immediate phase (0–90 min post-infusion) (Guo et al. 2016) and the headache pain was predominantly frontally and temporally localized (Fig. 3) (Guo et al. 2016).

Fig. 1 (continued) recordings of the intracranial and extracranial arteries (1 – magnetic resonance angiography) or brain activity [2 – blood oxygen level-dependent functional MRI (fMRI)]. Vital signs, such as heart rate and blood pressure, are measured continuously throughout the study. Studies can be tailored to assess certain aspects – if the focus is to address imaging or plasma levels of a given substance, scans and blood sampling (3) are conducted at baseline, when effects are expected, and after treatment of the attack. (**b**) Headache intensity is recorded on a verbal rating scale from 0 to 10 (0, no headache; 5, moderate headache; 10, worst imaginable headache). Note the biphasic response, comprising an immediate headache followed hours later by a migraine-like headache (Ashina et al. 2017)

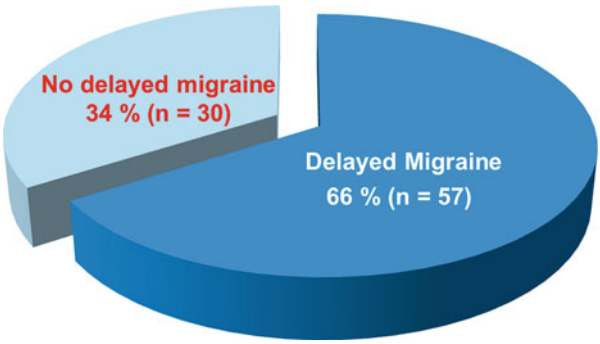

Fig. 2 Pie chart: percentage (numbers) of patients who developed delayed migraine-like attacks and patients who did not develop delayed migraine-like attacks after CGRP infusion (Lassen et al. 2002; Asghar et al. 2011; Guo et al. 2016; Hansen et al. 2010)

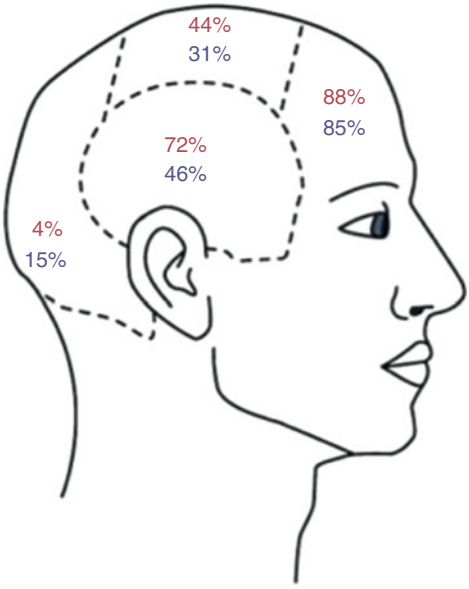

Fig. 3 Usual (●/red %) and CGRP-induced (●/blue %) localization of migraine attacks (Guo et al. 2016)

The role of CGRP in migraine with aura (MA) is not fully clarified (Hansen and Ashina 2014). Cortical spreading depression (CSD), a slowly propagated wave of depolarization followed by suppression of brain activity, is likely the underlying mechanism of the migraine aura (Charles and Baca 2013). In rats, endogenous CGRP was released from cortical slices and CGRP receptor antagonists had a dose-dependent inhibitory effect on CSD (Tozzi et al. 2012). In cats, CSD had no effect on the concentration of CGRP in the external jugular vein (Piper et al. 1993). One study investigated plasma CGRP collected from the carotid artery and the internal jugular vein in conjunction with cerebral angiography in MA patients (Friberg et al. 1994). Repeated measurements of plasma CGRP levels during MA attacks revealed no changes over time in arterial-venous plasma concentrations or in the release rates of CGRP (Friberg et al. 1994). Moreover, one provocation study

investigated whether CGRP would induce aura attacks in patients with MA who had *never* previously experienced attacks without aura (Hansen et al. 2010). CGRP infusion caused delayed migraine-like attacks *without* aura in 8 out of 14 patients (57%) and aura attacks in 4 out of 14 patients. To investigate whether the aura symptoms were due to CGRP infusion or experimental stress, it would require rechallenging these patients with CGRP. Interestingly, GTN infusion induces migraine-like attacks *without* aura in 50% of MA patients who have never previously experienced attacks *without* aura (Christiansen et al. 1999). In relation to familial hemiplegic migraine (FHM), an autosomal dominant subtype of MA, two provocation studies investigated the response to CGRP infusion in FHM patients. Both studies reported that CGRP infusion did not induce migraine-like attacks in FHM patients with (Hansen et al. 2008) and without known mutations (Hansen et al. 2011). These findings suggested that neurobiological pathways responsible for migraine headache in MO and MA patients may be distinct from pathways responsible for migraine headache in FHM patients (Hansen et al. 2008).

Taken together, studies in patients with common types of migraine demonstrated that CGRP infusion caused delayed migraine-like attacks in 66% of migraine patients with and without aura (Fig. 2) (Lassen et al. 2002; Asghar et al. 2011; Guo et al. 2016; Hansen et al. 2010).

4 Possible Mechanisms of CGRP-Induced Migraine

Potential sites of CGRP-induced migraine include *vasodilation* via *cyclic adenosine monophosphate (cAMP) and possibly cyclic guanosine monophosphate (cGMP)*, *peripheral activation of trigeminal sensory afferents*, and *central neuromodulation of higher brain centers*. Of these possible mechanisms of action, the peripheral vascular effects of CGRP have been the most extensively studied aspect. CGRP binds to its receptor on smooth muscle cells and acts as a potent dilator of human cerebral arteries (Edvinsson et al. 1987). In both healthy volunteers (Asghar et al. 2010) and MO patients (Asghar et al. 2011), MRA studies have demonstrated CGRP-induced dilation in the extracranial part of the middle meningeal artery (MMA), which was reversed following sumatriptan administration. The question then arises as to what extent CGRP-induced arterial dilation contributes to provoked migraine attacks. Studies have hypothesized that cAMP and possibly cGMP might be upregulated intracellularly following CGRP's extracellular binding to its receptor on vascular smooth muscle cells (Edvinsson et al. 1985; Uddman et al. 1985). Cilostazol, which induces cAMP elevation in vascular smooth muscle cells via inhibition of phosphodiesterase 3 dependent degradation, provokes migraine attacks in 86% of patients (Guo et al. 2014; Khan et al. 2017b). Nitric oxide production regulates cGMP formation and the nitric oxide donor GTN and sildenafil (a highly selective inhibitor of phosphodiesterase 5 that breaks down cGMP, and its inhibition leads to accumulation of cGMP) are powerful migraine triggers (Tvedskov et al. 2010; Kruuse et al. 2003).

To explore the possible relationship between nitric oxide and CGRP signaling pathways, one study investigated the effect of a nitric oxide-synthase inhibitor N(G)-monomethyl-L-arginine (L-NMMA) on CGRP-induced vasodilation (de Hoon et al. 2003). In a forearm skin model, L-NMMA infusion reduced CGRP-induced vasodilation in 40 healthy volunteers. Interestingly, L-NMMA did not have an inhibitory effect on CGRP-induced vasodilation when the highest CGRP dose was used. One provocation study investigated the effect of the CGRP receptor antagonist olcegepant in prevention of GTN-induced migraine (Tvedskov et al. 2010). In crossover fashion, all participants were pre-treated with olcegepant or placebo followed by infusion of GTN. This study showed no effect of olcegepant in prevention of GTN-induced migraine (Tvedskov et al. 2010). Collectively, these data suggest that activation of CGRP signaling pathway through intracellular cGMP increase is unlikely to be involved in mechanisms underlying CGRP-induced migraine.

One of the important questions that should be addressed is if CGRP may directly act on the trigeminal sensory afferents. An in vitro study reported CGRP release from capsaicin sensitive nerve fibers and dilation of human cerebral arteries (Jansen-Olesen et al. 1996). In rats, CGRP-induced meningeal vasodilation did not activate or sensitize meningeal nociceptors (Levy et al. 2005). In humans, CGRP injection into the forearm skin did not elicit a pain sensation (Pedersen-Bjergaard et al. 1991). CGRP receptors are expressed in three levels of the trigeminovascular system (Lennerz et al. 2008): peripheral nerve fibers associated with the cranial dura mater, the trigeminal ganglion, and the spinal trigeminal nucleus. Theoretically, exogenously administered CGRP could act on these receptors and induce migraine. In support, CGRP activates transcription of pro-nociceptive receptors on cultured trigeminal neurons through protein kinase A-dependent mechanisms (Giniatullin et al. 2008). In humans, noxious heat stimuli applied to the V1 area of the trigeminal nerve after CGRP infusion caused blood oxygen level-dependent (BOLD), a surrogate marker of neuronal activity, changes in the insula, brain stem, caudate nuclei, thalamus, and cingulate cortex that were reversed by administration of sumatriptan (Asghar et al. 2016). Given that neither CGRP nor sumatriptan are likely to cross the blood–brain barrier (BBB), these data indicated that CGRP might modulate nociceptive trigeminal transmission without having a direct effect in the CNS (Asghar et al. 2016). Furthermore, an fMRI study showed that visual sensory input by checkerboard stimulation did not cause any BOLD signal changes in the visual cortex after CGRP infusion (Asghar et al. 2012). In addition, none of the CGRP provocation studies reported any CNS side effects after CGRP infusion (Lassen et al. 2002; Asghar et al. 2011; Guo et al. 2016; Hansen et al. 2010). Collectively, these data support the notion of a predominantly peripheral site of action of CGRP in migraine. A preclinical study on anesthetized rats provided further support for peripheral mechanism by demonstrating that a monoclonal anti-CGRP antibody, fremanezumab, inhibited naive high-threshold neurons, but not wide-dynamic range trigeminovascular neurons (Melo-Carrillo et al. 2017a). Moreover, the inhibitory effects on the neurons were limited to their activation from the intracranial dura but *not* facial skin or cornea (Melo-Carrillo et al. 2017a). Interestingly, fremanezumab

inhibited the meningeal nociceptors of Aδ-fiber neurons but not C-fiber neurons (Melo-Carrillo et al. 2017b). The importance of these studies (Melo-Carrillo et al. 2017a, b) is twofold. First, fremanezumab selectively inhibited meningeal nociceptors of Aδ-fiber neurons peripherally and naive high-threshold trigeminovascular neurons centrally. Therefore, it has been suggested (Melo-Carrillo et al. 2017a, b) that the headache generating phase of migraine relies on activation of meningeal nociceptors because the site of action for monoclonal anti-CGPR antibodies is situated outside the BBB. Secondly, it has been proposed (Melo-Carrillo et al. 2017a, b) a possible explanation to why monoclonal anti-CGRP antibodies are not effective in all migraine patients in that fremanezumab only exhibited selective inhibition of meningeal nociceptors. Thus, it could be speculated that inhibition of meningeal nociceptors of Aδ-fiber neurons and naive high-threshold trigeminovascular neurons is not sufficient to block the generation of a migraine attack in all patients.

The question is whether CGRP could induce migraine via centrally mediated mechanisms? In this context, we should point to arguments favoring a central site of action for CGRP in migraine pathogenesis: (1) CGRP is widely distributed in central migraine-relevant structures such as the TNC and the spinal cord at the C1-level (Eftekhari and Edvinsson 2011); (2) Gene expression of the CGRP receptor has been documented within the spinal trigeminal nucleus, pons, and spinal cord (Eftekhari et al. 2016); (3) CGRP could also act on second-order neurons in the trigeminocervical complex (TCC) leading to nociceptive transmission to the thalamus and higher brain centers (Akerman et al. 2011). CGRP has also been implicated in modulation of central nociceptive transmission in the dorsal horn of the spinal cord (Yu et al. 2002). In addition, one study reported that olcegepant, CGRP receptor antagonist, inhibited trigeminovascular nociceptive transmission when injected into the periaqueductal gray of the midbrain (Pozo-Rosich et al. 2015).

To study the possible CNS-mediated migraine inducing effects, one study investigated premonitory symptoms as a surrogate for CNS involvement before onset of migraine headache (Guo et al. 2016). This study found that MO attacks were not associated with premonitory symptoms – suggesting a peripheral origin of CGRP-induced migraine. In support, one in vivo positron emission tomography (PET) study demonstrated that telcagepant, a CGRP receptor antagonist, at an efficacious dose only achieved low human CGRP receptor occupancy (Hostetler et al. 2013) – indicating that inhibition of central CGRP receptors is not needed for migraine amelioration. Furthermore, the PET tracer concentration was highest in the cerebellum in both anesthetized rhesus monkeys and healthy male volunteers – consistent with the known CGRP receptor distribution. Taken together, these data suggest a peripheral mechanism of action in CGRP-induced migraine.

5 Why Are Human Migraine Models Important?

Migraine is characterized by multiphasic events and its key feature is that it can be provoked by various triggers. Provocation models allow us to study migraine during different attack phases, i.e., from the beginning to the end. The clinical phenotype of

pharmacologically induced migraine-like attacks mimics those of spontaneous migraine attacks. Thus, the unique value of human migraine models has been twofold.

First, it has led to the discovery of novel signaling pathways implicated in cascade of events that leads to a migraine attack. Studies have documented the importance of signaling peptides such as CGRP, GTN, and PACAP in inducing migraine-like attacks. In this context, the potential role of the cAMP and cGMP signaling is supported by provocation studies demonstrating that phosphodiesterase inhibitors induce migraine-like attacks with an aptitude superior to CGRP, GTN, and PACAP (Guo et al. 2014; Kruuse et al. 2003; Thomsen et al. 1994; Lassen et al. 2002; Schytz et al. 2009). The disparity suggests that phosphodiesterase inhibitors likely act downstream from the abovementioned signaling peptides in the migraine-generating cascade.

Secondly, studies on migraine provocation by CGRP provided strong support for development of anti-CGRP drugs for the treatment of migraine. Currently, several randomized clinical trials have demonstrated efficacy of anti-CGRP monoclonal antibodies in migraine prevention (Khan et al. 2017a). The CGRP provocation studies have also suggested the heterogenic CGRP response of migraine patients (Lassen et al. 2002; Asghar et al. 2011; Guo et al. 2016; Hansen et al. 2010), i.e., some patients developed attacks while others did not. Thus, the human migraine model could theoretically be used for stratifying migraine patients in CGRP responders and nonresponders in order to predict the response to anti-CGRP drugs. However, it should be noted that the lack of CGRP response has not been reproduced in the same group of nonresponders. Reproducibility is important to minimize the risk of fluctuating susceptibility to migraine, which could theoretically affect the CGRP response. Moreover, CGRP retest reproducibility would cement the value of CGRP as a migraine-specific biomarker.

6 Future Perspectives and Concluding Remarks

Future studies should seek to optimize study designs and explore unanswered questions concerning CGRP-induced migraine. One pivotal aspect would be to investigate possible innate fluctuations in migraine attack susceptibility. This could be addressed by examining the migraine provoking effect of CGRP in patients whose frequency of attacks has been documented for an extended period before and after infusion day. For this purpose, it would be interesting to investigate the CGRP response in migraine sufferers who only experience attacks very few times on a yearly basis. This would allow us to evaluate whether headache frequency impacts the likelihood of developing migraine-like attacks after CGRP infusion. Another approach would be to rechallenge a population of CGRP nonresponders and examine whether the lack of response is reproducible. If so, this would delineate CGRP as potent and reliable inducer of migraine attacks. In this context, future studies should also examine whether the efficacy of anti-CGRP antibody treatment can be predicted in migraine sufferers based on hypersensitivity to CGRP.

Human migraine models provide a unique opportunity to study migraine-specific mechanisms after CGRP infusion. The knowledge acquired from these studies has validated CGRP as potent inducer of migraine, thereby, paving the way for development of drugs targeting the CGRP molecule or its receptor. In the future, refined human experimental studies will continue to unveil the role of CGRP in migraine pathogenesis.

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Role of CGRP in Migraine

Lars Edvinsson

Contents

Abstract

Migraine is a common neurological disorder that afflicts up to 15% of the adult population in most countries, with predominance in females. It is characterized by episodic, often disabling headache, photophobia and phonophobia, autonomic symptoms (nausea and vomiting), and in a subgroup an aura in the beginning of the attack. Although still debated, many researchers consider migraine to be a disorder in which CNS dysfunction plays a pivotal role while various parts of the trigeminal system are necessary for the expression of associated symptoms.

Treatment of migraine has in recent years seen the development of drugs that target the trigeminal sensory neuropeptide calcitonin gene-related peptide (CGRP) or its receptor. Several of these drugs are now approved for use in frequent episodic and in chronic migraine. CGRP-related therapies offer considerable improvements over existing drugs, as they are the first to be designed

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specifically to act on the trigeminal pain system: they are more specific and have little or no adverse effects. Small molecule CGRP receptor antagonists, gepants, are effective for acute relief of migraine headache, whereas monoclonal antibodies against CGRP (Eptinezumab, Fremanezumab, and Galcanezumab) or the CGRP receptor (Erenumab) effectively prevent migraine attacks. The neurobiology of CGRP signaling is briefly summarized together with key clinical evidence for the role of CGRP in migraine headache, including the efficacy of CGRP-targeted treatments.

Keywords
CGRP · CGRP receptor · Gepants · Migraine · Monoclonal antibodies

1 Background

Migraine is a neurological disorder that afflicts up to 15% of the adult population, with predominance in females. It is a chronic, complex neurological disorder that manifests as recurrent attacks of moderate to severe headache pain lasting 4–72 h. It is characterized by episodic headache, photophobia and phonophobia, autonomic symptoms (nausea and vomiting), and in a subgroup an aura in the beginning of the attack (IHS 2018 Classification). Although still debated, nowadays migraine is considered a neurological disorder in which CNS dysfunction plays a pivotal role while various parts of the trigeminal system are necessary for the expression of peripheral associated symptoms.

In an early study, Weiller et al. (1995) suggested a "migraine generator region" in the brainstem. This has recently been revisited and a series of imaging studies have suggested that already on the day before the start of a migraine attack signs of activation in the hypothalamus, possibly involving thalamus and the limbic system, are present (May and Schulte 2016). Further connectivity imaging studies suggested that once in the attack brainstem regions and the trigeminovascular pathway are recruited eliciting many of the components classically linked to the symptomatology of the migraine attack (May 2017). The progression of the disease into different phases has nicely been elucidated recently (Charles 2018; Dodick 2018).

The trigeminal system is by and large involved in the pain part of the attack exemplified by the release of calcitonin gene-related peptide (CGRP) in the headache phase of the migraine attack and its termination by triptan administration (Goadsby and Edvinsson 1993; Goadsby et al. 1990). Interestingly, about half of all neurons in the trigeminal ganglion express CGRP, as visualized using immunohistochemical staining with CGRP antibodies (Edvinsson et al. 2018). Neurobiology studies have so far only shown CGRP to be directly linked to the attack, but reasonably other neuronal messengers could, in addition, be involved. Recent findings provide new insight into the intricate role of CNS and the trigeminal system in the pathophysiology of migraine. Current specific migraine therapies have been found to interact with the trigeminal system to limit symptoms of the migraine attack (Edvinsson et al. 2018).

2 CGRP in the Cranial System

The expression of mRNA from the calcitonin gene is tissue specific in which CGRP mRNA is predominantly expressed in nerves and calcitonin mRNA in the thyroid (Russell et al. 2014). The 37 amino acid peptide CGRP and calcitonin belong to a family of related molecules that include the peptides adrenomedullin, primarily produced by non-neuronal tissues, especially vascular tissues, and amylin that is mainly produced in the pancreas. They share some structural homology (approximately 25–40%) and there are also some similarities in biological activities (Hay et al. 2018). CGRP is abundant in the body and has a wide distribution throughout the central and peripheral nervous systems (Goadsby et al. 2017). CGRP is an extremely potent and long-lasting vasodilator that is active at all levels of the cardiovascular system (Uddman et al. 1985, 1986a). It is now realized that CGRP is a widely expressed neuropeptide that has a major role in sensory neurotransmission.

Soon after CGRP was discovered in 1982 (Amara et al. 1982), it was linked to the trigeminovascular system with implications for migraine. Our interest in this peptide began due to a general interest in neuronal messengers in autonomic and sensory innervation of the cranial circulation. We had already made progress upon challenging the classical dogma that "one nerve only has one neuronal messenger" (law of Canon) (Edvinsson and Uddman 2005) and could verify that neurons in the trigeminal ganglion co-localized CGRP, substance P, neurokinin A, neuronal nitric oxide synthase, pituitary adenylate cyclase activating peptide (PACAP), inter alia (Edvinsson and Uddman 2005). However, their individual and collective functional roles were still unknown.

The first data on CGRP in the trigeminovascular system were presented in 1984 at meeting on Regulatory Peptides (Edvinsson 1985) and later in a series of publications. In the animal and human trigeminal ganglion, over half of the neurons contain CGRP (Uddman et al. 1986b; Tajti et al. 1999). This was confirmed in subsequent work where two main populations of neurons in the trigeminal ganglion were quantified in several species including man (Eftekhari et al. 2010): half of them contain CGRP (C-fibers) and half the CGRP receptor elements (Aδ-fibers). In addition, >90% of the cell count consisted of small satellite glial cells organized around the neurons. The origin of the CGRP containing nerve fibers was demonstrated by immunohistochemistry and quantitation by radioimmunoassay, following trigeminal nerve denervation (Uddman et al. 1985). Subsequent tracing studies from intra- and extracranial arteries revealed that the sensory CGRP-positive fibers originate in the trigeminal neurons and co-localize with substance P (Edvinsson et al. 1989; Hara et al. 1989; Uddman and Edvinsson 1989).

There exist two forms of CGRP in the body; the α-CGRP is encoded by the CT/CGRP gene that is relevant for the cerebral circulation and migraine. The β-CGRP (with a structural homology of 90%) is primarily found in the gut and formed by a different gene. The α-CGRP is synthesized in neurons by tissue-specific splicing of mRNA transcribed from the calcitonin/CGRP gene located on chromosome 11. CGRP is generated by cleavage of a pro-peptide precursor, and then

processed through the Golgi apparatus, packaged into dense core vesicles for transport to axon terminals for storage and release.

Functional studies showed that CGRP is a very potent vasodilator of cerebral arteries and arterioles, activating adenylyl cyclase in the smooth muscle cells and is unrelated to functional endothelium (Edvinsson 1985). In vivo, CGRP potently relaxed cortical arterioles but not venules (McCulloch et al. 1986). Lesioning of the perivascular sensory CGRP nerves, however, did not modify resting cerebral blood flow, flow-metabolism coupling, chemical regulation, or autoregulation of brain circulation (Edvinsson et al. 1986). Instead CGRP was demonstrated to play a key role in a protective trigeminovascular reflex (McCulloch et al. 1986), whereby CGRP is released by trigeminal nerves in response to local cerebral vasoconstriction to cause dilation and maintain cerebral blood flow. These findings suggested that CGRP was involved in migraine pathophysiology (Edvinsson 1985). The decisive results for demonstration of its involvement in primary headaches came a few years later: CGRP is selectively released from the trigeminal system during acute migraine headache attacks, and this release is prevented by anti-migraine drugs such as triptans (Edvinsson et al. 2018).

3 Components of CGRP Transmission Relevant to Migraine Therapies

In CGRP nerves, the presynaptic terminals take the form of focal swellings, called axonal varicosities that occur at regular intervals along the axon like a strand of pearls. Upon nerve stimulation or depolarization, CGRP is released from its storage vesicles via calcium-dependent exocytosis. Capsaicin, a component of chili peppers, also will cause release of α-CGRP, and this compound has been useful as an experimental tool to release and ultimately deplete the peptide from CGRP nerves. Presynaptic receptors located on trigeminal neurons regulate CGRP release. Presynaptic serotonin 5-HT_{1B} and 5-HT_{1D} receptors (Hou et al. 2001) may inhibit CGRP release, and they are of particular relevance to migraine treatment. These receptors are the target for the therapeutic effects of the triptan drugs, e.g., sumatriptan, in relief of migraine headache, while we consider the vasomotor response to triptans mainly as an unnecessary bi-product (Edvinsson et al. 2018).

In bipolar trigeminal sensory neurons located in the trigeminal ganglion, CGRP is released at both peripheral and central nerve terminals. In the trigeminovascular system, the peripheral branch of CGRP axons form perivascular nerves that run along the adventitial-medial border in the wall of intracranial cerebral and dural blood vessels (Eftekhari et al. 2013). Axon varicosities are separated from the adjacent smooth muscle cell by a relatively wide cleft (100–500 nm). CGRP fibers also terminate in nonvascular regions of the dura mater, where they play a role in meningeal nociception.

The central branch of trigeminovascular CGRP axons project to the spinal trigeminal nucleus and C1–2-levels of the spinal cord, notably laminae I and II of the dorsal horn (Edvinsson 2011; Eftekhari and Edvinsson 2011). Central CGRP

release sites, as demonstrated by immunohistochemical co-localization with the synaptic vesicle protein synaptophysin (Eftekhari and Edvinsson 2011), also exhibit a beaded appearance consistent with axonal varicosities. In addition to the peripheral and central terminations, GGRP is released within the trigeminal ganglion itself, where it likely regulates sensory processing at an early stage in the pain pathway. Within the ganglion, CGRP is expressed in thin, beaded fibers that are likely release sites on local branches of CGRP axons (Edvinsson et al. 2018).

Following release, CGRP is broken down by metalloproteases (Kim et al. 2013). Amidation at the C-terminus helps protect the peptide and increases its half-life. Human plasma levels are usually within the low picomolar range and generally attributed to spillover from sites of neuronal release. The plasma half-life of CGRP in humans was estimated following CGRP infusion to be 7 min for a fast decay phase and 26 min for a slower phase of decay (Kraenzlin et al. 1985). Release of CGRP after stimulation of the trigeminal ganglion in humans has been measured in blood collected from the external jugular vein draining the extracerebral tissues (Goadsby et al. 1988). Because of the instability of the peptide, the blood samples were collected close to the release site using a protocol to limit proteolysis.

4 CGRP Receptor Family

In line with other G protein-coupled receptors the CGRP receptor can trigger diverse signaling pathways and undergo regulatory control (Hay et al. 2018). The CGRP receptor consists of a seven transmembrane G protein-coupled calcitonin receptor-like receptor (CLR) with a single membrane-spanning receptor activity modifying protein (RAMP1) (Hay et al. 2018). CLR is a member of the secretin receptor family (Class B GPCR), and it is a required element in receptors for CGRP and adrenomedullin (AM_1 and AM_2). In order to create a functional membrane receptor with specific affinity for CGRP, CLR must form a heterodimer with the RAMP1 (McLatchie et al. 1998). RAMP proteins alter the pharmacology, functionality, and cell trafficking of specific GPCRs. The ligand-binding domain of the CGRP receptor is located at the interface between the RAMP1 and CLR proteins (Hay et al. 2018; Mallee et al. 2002). Thus, co-expression of both CLR and RAMP1 is necessary for a cell to respond to CGRP. Recently Liang and colleagues reported the detailed structure of the human CGRP receptor (Liang et al. 2018). The work illustrated the site of binding of CGRP and gepants at the interface of CLR and RAMP1. If CLR is coupled with RAMP2 or RAMP3, the two forms of adrenomedullin receptors, AM_1 and AM_2, are formed, respectively.

The other part of this group of receptors is that based on calcitonin. CT per se can act as agonist at the CT receptor (CTR). The CTR does not require combination with a RAMP molecule. When the CTR is combined with either of the RAMPs the amylin family of receptors is formed: AMY_1 (RAMP1), AMY_2 (RAMP2), and AMY_3 (RAMP3) (Hay et al. 2018). The role of the different receptors and ligands of the CGRP family is less well understood.

The CGRP receptor complex includes two cytoplasmic proteins that associate with the CLR-RAMP1 heterodimer to mediate signal transduction. CLR is coupled to a G-protein containing the Gs α subunit (Gαs) that activates adenylyl cyclase and cAMP-dependent signaling pathways. In addition, the CGRP receptor associates with a receptor coupling protein (RCP) that amplifies G-protein activation and is important for optimal signal transduction (Egea and Dickerson 2012). Receptor-mediated increases in intracellular cAMP activate protein kinase A (PKA), resulting in the phosphorylation of multiple downstream targets, including potassium-sensitive ATP channels (K_{ATP} channels), extracellular signal-related kinases (ERKs), or transcription factors such as cAMP response element-binding protein (CREB). In cerebrovascular smooth muscle, elevation of cAMP by CGRP results in vasorelaxation.

An important feature of CGRP signaling is the regulation and desensitization of the receptor following agonist activation. After CGRP has bound to its receptor, the CLR component is rapidly phosphorylated; and the receptor is subsequently internalized via recruitment of β-arrestin. Classically, the receptor binding is thought to signal only at the cell surface but is now recognized that some G protein-coupled receptors including the CGRP receptor undergo sustained signaling from endosomes, once internalizes in response to the ligand to signal pain (Yarwood et al. 2017). Studies using labeled receptor components were found to co-localize together suggesting that both receptor elements co-internalize (Kuwasako et al. 2000; Padilla et al. 2007). Thus, transient stimulation by CGRP induces internalization of the receptor to endosomes that allows rapid recycling back to the plasma membrane. Chronic exposure to CGRP, however, initiates an internalization process that traffics the receptor to lysosomes for degradation. Thus, the CGRP receptor can signal not only via the cell membrane but also from endosomes within cells. The regulation of CGRP receptors in disease is another challenge for researchers.

5 CGRP Receptor Antagonists

Migraine is a prevalent, disabling neurological disorder involving the trigeminovascular system. Previous and current treatments were either originally intended for other conditions and/or associated with intolerable adverse effects. As stated above CGRP is the most prevalent neuropeptide in the trigeminal system and plays an important role in the pathophysiology of migraine (Edvinsson et al. 2018). The gepants and the monoclonal antibodies are the first treatments created specifically for migraine, modulating pain signaling pathways.

The first specific antagonist for CGRP receptors was olcegepant which was soon found to be very potent and specific, but was a di-peptide and hence only works when given parenterally (Doods et al. 2000). A number of other small molecule non-peptides antagonists have since been identified that potently and selectively block CGRP responses in both experimental and clinical studies (Holland and Goadsby 2018). Although chemically unrelated, this class of antagonists is collectively called “gepants” due to their common mode of action. Significantly, all

gepants tested to date are efficacious in migraine patients. Interestingly, a common feature of the clinically relevant gepants is their high affinity for CGRP receptors of humans and nonhuman primates relative to other species. This is due to a species-specific residue located at the interface between the RAMP1 and CLR proteins, indicating this region as the site of antagonist binding (Mallee et al. 2002). The recent characterization of the CGRP receptor structure has revealed the exact location of the binding of CGRP and gepants to the receptor complex (Liang et al. 2018).

Gepants potently block the binding of CGRP to its receptor in cells and in tissue preparations and produced a rightward shift of CGRP concentration-response curves in signaling (cAMP production) and functionality (vasodilation). The newer gepants that has been developed still exhibited high potency and good oral bioavailability, e.g., telcagepant (Paone et al. 2007), but were discontinued due to liver enzyme issues (Edvinsson and Linde 2010). Currently, three of the newer gepants, ubrogepant, aterogepant, and rimegepant, have passed phase III testing and are now in the finale for registration. It will be interesting to learn how they will be positioned in the therapy of migraine.

6 CGRP and CGRP Receptor Antibodies for Prophylaxis

An alternative strategy for blocking CGRP transmission in migraine patients is to use selective monoclonal antibodies that bind either CGRP or the CGRP receptor. This approach has been remarkably successful for decreasing the frequency of migraine attacks. Four such antibodies have now completed Phase III clinical trials and are in the process of approval worldwide.

The therapeutic goal for migraine prophylaxis is to reduce the number of migraine days experienced by patients who suffer frequent attacks. Antibodies to either CGRP or the CGRP receptor have demonstrated efficacy in reducing migraine days in patients with episodic (<15 days/month) or chronic (>15 days/month) migraine. The anti-receptor antibody Erenumab (Aimovig) is a human IgG2 monoclonal antibody targeted to the CGRP binding site on the CGRP receptor. It is administered once a month by subcutaneous injection to effectively reduce migraine frequency. Erenumab is approved in the USA by FDA (May 2018) and by EMA for Europe, and is on the market. Currently, there are three different monoclonal antibodies targeted to sites on the CGRP peptide itself, which have all concluded Phase III testing for migraine prevention. Overall, these anti-CGRP antibodies appear to show similar efficacy and safety profiles. Eptinezumab is a genetically engineered humanized IgG1 monoclonal antibody that is formulated for intravenous administration and intended for once per quarter dosing. The other two antibodies to CGRP have now been approved by the FDA (September 2018): Fremanezumab (Ajovy) and Galcanezumab (Emgality). Fremanezumab is a fully humanized IgG2a monoclonal antibody that is given once per month by subcutaneous injection. Galcanezumab is a fully humanized IgG4 monoclonal antibody that also is administered subcutaneously on a monthly basis to reduce the number of migraine days.

The antibodies are particularly suited for use as a prophylactic treatment for migraine with the advantages of patient adherence and tolerability. They have a prolonged serum half-life (20–30 days) that enables patients to take their medication less frequently for prevention of migraine attacks. Antibodies bind their target site with high affinity and selectivity, thus reducing the potential for unwanted, off-target effects. In contrast to small exogenous molecules such as the gepants, antibodies are not processed by the liver, thus avoiding the potential for liver toxicities and hepatic drug interactions. No adverse cardiovascular or cerebrovascular effects have been reported for these antibodies. A primary disadvantage of the antibodies, however, is they are not orally active and must be administered by injection. Injection-site reactions, including pain, are the most commonly reported adverse events. These reactions are usually mild and transient, and are less likely with intravenous as compared to subcutaneous administration. Thus, overall, the anti-migraine antibodies have been shown in clinical trials to be well tolerated and safe in patients. Because no head-to-head comparisons have been done, it is difficult to discuss if one or the other is to prefer; however, published data show more or less similar results.

7 Conclusion

The development of the understanding of the CGRP family of peptides and receptors has shed new light on migraine pathophysiology and provided several options to treat this large group of patients with migraine. The promising new gepants and antibody medications are on their way to the patients and it will be rewarding to observe their clinical effects in the general population.

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Understanding CGRP and Cardiovascular Risk

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Contents

Abstract

Increasing knowledge about the role of calcitonin gene-related peptide (CGRP) in migraine pathophysiology has led to the development of antibodies against this peptide or its receptor. However, CGRP is widely expressed throughout the body, participating not only in pathophysiological conditions but also in several physiological processes and homeostatic responses during pathophysiological events. Therefore, in this chapter, the risks of long-term blockade of the CGRP pathway will be discussed, with focus on the cardiovascular system, as this peptide has been described to have a protective role during ischemic events, and migraine patients present a higher risk of stroke and myocardial infarction.

Keywords

Cardiovascular safety · CGRP · CGRP (receptor) antibodies · Migraine · Myocardial infarction · Stroke

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1 Introduction

Migraine is a highly disabling neurovascular disorder (Stovner et al. 2018). As mentioned in the previous chapters, calcitonin gene-related peptide (CGRP) has been described to play an important role in migraine pathophysiology (Edvinsson 2017; Goadsby et al. 2002). As a result, the CGRP pathway has become a promising target.

Initially, CGRP receptor antagonists (gepants) were developed for the acute treatment of migraine and proved to be effective (Doods et al. 2000; Edvinsson and Linde 2010). Unfortunately, pharmacokinetic limitations and hepatotoxicity cases did not allow the initial gepants to reach the market (Negro et al. 2012). New gepants are currently in Phase II trials for the acute and prophylactic treatment of migraine, with no hepatotoxicity reported (Holland and Goadsby 2018; Tepper 2018); nevertheless, the concerns about the hepatotoxicity reports led to the development of CGRP (receptor) antibodies for the prophylactic treatment of migraine (Deen et al. 2017; Schuster et al. 2015; Wrobel Goldberg and Silberstein 2015). Preliminary results of the clinical trials are promising and have not reported serious side effects (Mitsikostas and Reuter 2017); however, it is important to consider the physiological role of this peptide and the possible side effects after long-term blockade of the CGRP pathway.

In this chapter, the role of CGRP in physiological processes will be described, with focus on the cardiovascular system, as migraine patients present a higher risk of stroke and myocardial infarction (Etminan et al. 2005; Kurth et al. 2009; Sacco et al. 2013; Scher et al. 2005).

2 CGRP and the Cardiovascular System

CGRP and its fibers are widely distributed in peripheral and central structures. In the cardiovascular system, sensory CGRPergic fibers have been described to innervate the blood vessels and the heart (Opgaard et al. 1995; Uddman et al. 1986; Wimalawansa and MacIntyre 1988). Several studies have shown that CGRP plays an important role in the regulation of blood pressure and in the homeostatic responses during ischemic events and hypertension (HT) (Edvinsson et al. 1998; Keith et al. 2000; Lindstedt et al. 2006; MaassenVanDenBrink et al. 2016; McCulloch et al. 1986; Russell et al. 2014).

2.1 CGRP and Hypertension

As mentioned above, CGRP has been demonstrated to be involved in the regulation of blood pressure. Although its role under physiological conditions may be limited (Smillie and Brain 2011), it seems to act as a protective/compensatory mechanism during HT (Smillie et al. 2014). In accordance with this hypothesis, in the

deoxycorticosterone-salt HT model, CGRP knockout mice had a significant increase in 24-h mean arterial pressure (MAP) and renal damage when compared to wild types (Jianping et al. 2013), while in non-treated animals, only the 7-day average of the daytime MAP was significantly increased (Mai et al. 2014). Moreover, in a model of angiotensin II-induced HT, CGRP knockout mice exhibited an enhanced increase in MAP and aortic hypertrophy. This was accompanied by an upregulation of the CGRP receptor components expression, reinforcing the role of CGRP release as a safeguard mechanism against the onset and maintenance of HT (Smillie et al. 2014). This increase in blood pressure has been associated to an elevated sympathetic activation, as CGRP knockout mice show an increase in urine and plasma markers of catecholamine release (Mai et al. 2014). Indeed, bolus injections of the CGRP antagonist olcegepant enhance the vasopressor sympathetic outflow in pithed rats (Avilés-Rosas et al. 2017). Moreover, CGRP is not only involved in peripheral mechanisms, but it also participates in the maintenance of cerebrovascular reactivity during chronic HT (Wang et al. 2015).

The abovementioned studies support the role of CGRP in blood pressure regulation during HT. As a result, a novel CGRP analogue was recently developed to improve and reverse cardiovascular disease. Results from in vivo preclinical models of hypertension and cardiac failure showed positive antihypertensive effects, an attenuation of cardiac remodeling, and an increase in angiogenesis and cell survival after administration of the CGRP analogue (Aubdool et al. 2017).

2.2 CGRP and Ischemia

During severe HT and focal cerebral ischemia, CGRP has been demonstrated to act as a neuroprotector, by increasing cerebral blood flow (Moskowitz et al. 1989; Sakas et al. 1989; Zhang et al. 2011). In rats, if CGRP is administrated at the beginning of reperfusion after experimental cerebral artery occlusion, a reduction in brain edema is observed, probably due to a decrease in the blood-brain barrier disruption (Liu et al. 2011). In patients with subarachnoid hemorrhage (SAH), higher levels of plasma CGRP have been associated with delayed vasospasm (Juul et al. 1990) and infusion of CGRP further reduced vasospasm (Juul et al. 1994). Similarly, in another cohort of patients with SAH, CGRP levels in cerebrospinal fluid of patients without vasospasm were significantly higher than the levels of patients with vasospasm, with the former group not developing cerebral ischemia (Schebesch et al. 2013). In an experimental rat model of SAH, CGRP expression was decreased; however, an enhanced CGRP-dependent vasodilation was observed (Edvinsson et al. 1990). Finally, vasospasm after induction of SAH by placing a clot around the internal carotid artery bifurcation was significantly ameliorated in monkeys that were treated with slow-release CGRP tablets, consisting of compressed microspheres containing CGRP, and that were placed in the cerebrospinal fluid (Inoue et al. 1996). Due to their composition, these compressed microsphere tablets released CGRP for a period of several weeks, providing proof-of-concept data suggesting CGRP agonism as a possible therapeutic target for SAH patients.

In myocardial ischemia, CGRP is also considered to be released as a protective mechanism. Preclinical studies in rats and mice show protective hemodynamic and metabolic changes mediated by CGRP in response to ischemic events (Chai et al. 2006; Gao et al. 2015; Homma et al. 2014; Lei et al. 2016). Moreover, in clinical studies, intravenous administration of CGRP resulted in a decrease of both systolic and diastolic arterial pressure and an increase of heart rate (Gennari and Fischer 1985). Furthermore, when infused in patients with congestive heart failure, myocardial contractility is improved (Gennari et al. 1990). Interestingly, lower plasma levels of CGRP have been reported in patients with diabetes mellitus and coronary artery disease, when compared to controls, suggesting an alteration in the CGRP (cardioprotective) pathway (Wang et al. 2012). Obviously, these observations need to be confirmed in future, and it should be elucidated whether potential changes in patients with cardiovascular disease reflect a cause or consequence of this disease.

2.3 CGRP and Preeclampsia

CGRP also seems to be involved in the vascular adaptations during pregnancy, as plasma levels increase through the gestation period, reaching their maximum during the last trimester and normalizing after delivery. However, in preeclampsia, a pregnancy disorder characterized by high blood pressure and proteinuria, CGRP levels are lower (Yadav et al. 2014). The mechanisms behind this are not yet known but indicate an alteration in the CGRP signaling, similar as observed in patients with cardiovascular disease.

3 Cardiovascular Risk and Migraine

Numerous studies have shown that migraine patients present an increased risk of hemorrhagic and ischemic stroke, with the risk being higher for women (Chang et al. 1999; Etminan et al. 2005; Sacco et al. 2013; Schurks et al. 2009; Spector et al. 2010; Tzourio et al. 1995). Moreover, a higher risk of myocardial infarction, coronary artery disease, and altered arterial function has also been described (Scher et al. 2005; Vanmolkot et al. 2007). Unfortunately, the mechanisms behind these increases are not clear, but it is thought to involve genetic aspects and vascular dysfunction, among other factors. This poses a concern, as currently the main novel therapeutic target for migraine treatment is blocking CGRP or its receptor, which could increase cardiovascular risk (Deen et al. 2017; MaassenVanDenBrink et al. 2016).

3.1 Cardiovascular Risk, Migraine, and Women

Migraine is almost three times more prevalent in women than in men (Buse et al. 2013). Frequency, intensity of headaches, disability, and chronification have also

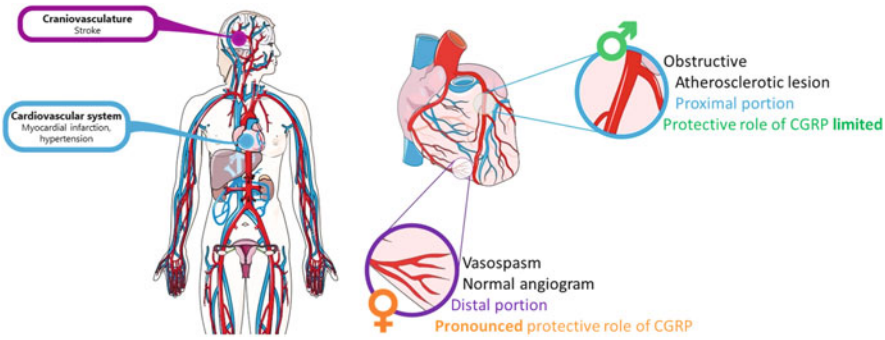

Fig. 1 Theoretical concerns after long-term blockade of CGRP (receptor). Migraine patients present an increased risk of cardiovascular disease, and CGRP participates as a safeguard during ischemic events which suggest that after CGRP blockade, the (cardio)vascular risk could increase further. In myocardial ischemia, CGRP seems to have a more prominent role in the distal portion than in the proximal portion of the coronary arteries, which may represent a downside for women, as ischemic events in the distal portion are more common in female patients, while proximal obstructions are more prevalent in male patients

been reported to be higher in female patients (Buse et al. 2013; Labastida-Ramirez et al. 2017). In addition, women with migraine present a higher risk of stroke when compared to men with migraine, and, as before menopause the prevalence of cardiovascular events is rather low, after menopause the occurrence rises sharply (Bushnell et al. 2014; Mieres et al. 2014).

In myocardial infarction, sex-related differences have also been observed. Women usually present angina-like chest pain and a positive response to stress testing but no visible obstructions during angiography as it is caused by vasospasms of the small intramyocardial portions of the coronary arteries (Humphries et al. 2008; Kaski et al. 1995). On the contrary, men usually present with occlusions of the proximal conducting portion, which are evident during an angiography (Fig. 1). This disparity may represent a downside for female migraine patients undergoing treatment with CGRP (receptor) blockade, as CGRP-dependent vasodilation (and cardioprotection) in coronary arteries is more pronounced in the distal portions than in the proximal portions (Chan et al. 2010; Gulbenkian et al. 1993; MaassenVanDenBrink et al. 2016). Moreover, CGRP signaling seems to be modulated by ovarian steroid hormones, as women have higher plasma levels than men, and the levels increase when patients are under contraceptives (Valdemarsson et al. 1990). Furthermore, the decrease in blood pressure and the positive inotropic effect induced by CGRP administration have been described to be enhanced when 17β-estradiol or progesterone is co-administered (Al-Rubaiee et al. 2013; Gangula et al. 2002). This evidence, taken together, strongly suggests a (protective) synergistic interaction between ovarian steroid hormones and CGRP and reiterates the concerns about CGRP (receptor) blockade in women, as this could increase their risk of suffering an ischemic event even more, especially after menopause.

4 Safety Assessment of CGRP Blockade

Considering the increased cardiovascular risk of migraine patients discussed in the previous section, it is important to perform studies that *correctly* assess the safety of CGRP (receptor) blockade. For such a purpose, cardiovascularly compromised subjects should be included that properly represent the population of migraine patients potentially using these drugs.

Unfortunately, even though the grand majority of the CGRP (receptor) antibodies have been approved, currently only one group has evaluated their cardiovascular safety profile in cardiovascularly compromised patients (Depre et al. 2018). In this study, a randomized, double-blind, placebo-controlled trial was performed to evaluate the effect of erenumab, a fully human monoclonal antibody directed against the CGRP receptor, on exercise time during a treadmill test in patients with stable angina pectoris. The authors reported no alterations in performance between patients receiving erenumab and placebo. Apart from serious pharmacological concerns about the validity of this specific study, because no evidence was presented on whether effective CGRP receptor blockade was achieved at the time of the treadmill test (Maassen van den Brink et al. 2018), the study population needs further attention.

In the study from Depre et al., the patients included suffered from stable angina pectoris, most likely due to stenosis of the epicardial conducting portions of the coronary artery. As discussed previously, the role of CGRP is limited in the proximal coronary artery (Chan et al. 2010). Whereas most patients using the antibodies will be female, this study included 78% males, as stable angina related to epicardial stenosis is mainly present in male patients. Thus, women, who pose a major concern and may suffer from microvascular disease, where CGRP may be a relevant mediator, were underrepresented in this study.

While in some cases performing appropriate studies in relevant patient groups may be ethically and practically challenging, preclinical studies are excellent to shed more light on the role of CGRP in cardiovascular regulation. In this light, it is important also to take into account potential differences between short-term and long-term blockade of CGRP or its receptor in models of cardiovascular disease in both male and female animals.

5 Conclusion

CGRP plays an important role in (cardio)vascular protection. However, it is also involved in migraine pathophysiology, and the current novel treatments involve CGRP (receptor) blockade. As migraine patients present higher cardiovascular risk, with women at higher risk, chronic blockade of the CGRP pathway poses a concern. While the initial clinical trials don't indicate frequent adverse events, it is of crucial importance to correctly evaluate the safety profile of these novel drugs, in order to prevent serious adverse effects when these drugs will be used on a large scale.

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CGRP and Painful Pathologies Other than Headache

David A. Walsh and Daniel F. McWilliams

Contents

Abstract

CGRP has long been suspected as a mediator of arthritis pain, although evidence that CGRP directly mediates human musculoskeletal pain remains circumstantial. This chapter describes in depth the evidence surrounding CGRP's association with pain in musculoskeletal disorders and also summarises evidence for CGRP being a direct cause of pain in other conditions. CGRP-immunoreactive nerves are present in musculoskeletal tissues, and CGRP expression is altered in musculoskeletal pain. CGRP modulates musculoskeletal pain through actions both in the periphery and central nervous system. Human observational studies, research

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on animal arthritis models and the few reported randomised controlled trials in humans of treatments that target CGRP provide the context of CGRP as a possible pain biomarker or mediator in conditions other than migraine.

Keywords
Back pain · Central sensitisation · Gastrointestinal · Neck pain · Neuropathic pain · Osteoarthritis · Pain · Pancreatitis · Peripheral sensitisation · Rheumatoid arthritis

Abbreviations

ATF3	Activating transcription factor-3
BDNF	Brain-derived neurotrophic factor
CCL	Chemokine ligand
CGRP	Calcitonin gene-related polypeptide
CNS	Central nervous system
COX2	Cyclooxygenase-2
CRPS	Complex regional pain syndrome
CSF	Cerebrospinal fluid
DRG	Dorsal root ganglion
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
MIA	Monoiodoacetate
NGF	Nerve growth factor
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PGP9.5	Protein gene product 9.5
PKA	Protein kinase-A
RA	Rheumatoid arthritis
RAMP	Receptor activity-modifying protein
TNF	Tumour necrosis factor-alpha
TrkA	Receptor for nerve growth factor
TRPV	Transient receptor potential cation channel subfamily V member
VAS	Visual analogue scale
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

1 Introduction

CGRP has long been explored as a neuromodulating peptide in diverse painful conditions, due to its localisation in fine unmyelinated first-order sensory nerves that are activated by nociceptive and painful chemical stimuli. Clinical benefit in migraine may reflect a particular contribution of perivascular CGRP-containing

nerves in that condition, either or both due to sensory and efferent vasomotor activities of CGRP. Substantial evidence has been accumulated that CGRP might also be important for musculoskeletal pain and possibly also neuropathic and abdominal pains. To date, however, the translational relevance of these findings remains unproven for treating human pain states other than migraine. More sophisticated models of chronic pain now recognise the importance of peripheral and central sensitisation and contributions from inflammation and the vasculature. Pain is not a single entity, and different treatments might specifically reduce its different components, for example, nociceptive or neuropathic and constant or intermittent. Patient-reported outcomes designed to assess benefits from one class of analgesics might not detect important effects of an analgesic agent acting through different mechanisms. Understanding potential mechanisms by which CGRP might contribute to chronic pain is important in the design of clinical trials to test analgesic efficacy in humans.

This chapter updates previous reviews on CGRP's role in pain (Schou et al. 2017; Walsh et al. 2015). A recent systematic review of the reported associations between CGRP and pain found that outside of migraine and headache translationally robust findings were limited (Schou et al. 2017). Only one randomised controlled trial of CGRP inhibition outside of headache/migraine was identified, and that failed to demonstrate benefit for knee osteoarthritis (OA) pain. However, these findings underestimate CGRP as a target of interest and might underestimate the potential for blocking CGRP to relieve chronic pain.

2 CGRP in Musculoskeletal Pain

Arthritis is the commonest cause of chronic pain, and OA and rheumatoid arthritis (RA) each remains a major burden on both individuals and society. In the UK alone, 7.5 million working days are lost per year due to musculoskeletal conditions. Current treatments often provide incomplete relief of arthritis pain or are associated with important risk of adverse events. Even total joint replacement surgery, one of the most effective treatments to improve quality of life across any condition, leaves a substantial minority of patients with chronic pain and is only appropriate for people with end-stage disease who have often suffered for many years from their arthritis.

CGRP has long been suspected as a mediator of arthritis pain, although evidence that CGRP directly mediates human musculoskeletal pain remains circumstantial. Human joints, bone and muscle are richly innervated with CGRP-immunoreactive sensory nerves, and arthritis has been associated with increased CGRP-like immunoreactivity in joint fluids suggesting increased peripheral CGRP release. Nerve growth factor (NGF) expression is increased in arthritic joints, where it both sensitises peripheral nerves and increases their expression of CGRP. Antibodies to NGF can provide clinically important analgesia in patients with OA. Research in rodents and larger mammals shows that CGRP sensory pathways are remarkably conserved across species, and demonstrate upregulation of CGRP by sensory nerves during arthritis. In animal models, inhibiting CGRP in the CNS or in the joint can

reduce arthritis pain behaviour. A more recent clinical trial of antibody blockade of CGRP receptors however did not demonstrate benefit for OA pain in humans (Jin et al. 2018). Does this represent another example of preclinical models not predicting translation to human disease or a failure of clinical trial design to reveal clinically important benefit for human pain? In this chapter, we summarise contributions of CGRP to musculoskeletal pain (Fig. 1) and attempt to interpret apparent inconsistencies in the available evidence.

2.1 Musculoskeletal Innervation by CGRP-Immunoreactive Nerves

CGRP is expressed by fine, unmyelinated sensory nerves supplying articular tissues (Walsh et al. 2015; Dirmeier et al. 2008). In skeletal muscle, CGRP is also present in motoneurons where peripheral release might be myotrophic. Within human synovial joints, CGRP-immunoreactive sensory nerves have been localised to synovium and tendons, ligaments, menisci in the knee and temporomandibular joint as well as periosteum and subchondral bone (Walsh et al. 2015; Dirmeier et al. 2008). Synovial joints studied include knees (Pereira da Silva and Carmo-Fonseca 1990), temporomandibular joints and spinal facet joints (Inami et al. 2001; Kallakuri et al. 2004). CGRP-immunoreactive nerves have also been localised to non-synovial components of human sacroiliac joints (Szadek et al. 2008, 2010), muscle (Ohtori et al. 2012), tendon insertions (Spang and Alfredson 2017), intervertebral discs (Ashton et al. 1994; Gruber et al. 2012), vertebral end plates (Brown et al. 1997) and peridural membrane (Bosscher et al. 2016) and in uncovertebral joints of the cervical spine (Brismee et al. 2009). Musculoskeletal innervation by CGRP-immunoreactive nerves has been demonstrated similarly across species in rats (Iwasaki et al. 1995; Ahmed et al. 1993; Hukkanen et al. 1991, 1992a, b; Shinoda et al. 2003), rabbits (Kallakuri et al. 1998) and sheep (Tahmasebi-Sarvestani et al. 1996).

Retrograde neuronal tracing in rodents has demonstrated specific routes through which CGRP-immunoreactive musculoskeletal nerves course before reaching their dorsal root ganglia (DRG). Articular nerves conduct CGRP-immunoreactive fibres from peripheral joints, whereas sympathetic trunks might make important contributions from intervertebral discs (Suseki et al. 1998) or facet joints (Aoki et al. 2004). This is essential information if developing targeted nerve ablation procedures. CGRP-immunoreactive nerves in peripheral joints such as knees or wrists (Kuniyoshi et al. 2007) have their cell bodies in restricted ipsilateral lumbosacral or cervical DRGs. Temporomandibular joints are innervated by CGRP-immunoreactive nerves originating in the ipsilateral trigeminal ganglion. CGRP-immunoreactive nerves from vertebral bodies (Ohtori et al. 2007), facet joints (Ohtori et al. 2000, 2002; Kras et al. 2013; Ishikawa et al. 2005), intervertebral discs or spinal ligaments and sacroiliac joints (Murata et al. 2007) may have cell bodies along a broader range of DRGs, on either side of the body. Dual retrograde labelling experiments suggest that CGRP-immunoreactive axons from knee and lumbar vertebrae might originate from a single DRG cell, perhaps providing one anatomical basis for referred musculoskeletal pain (Ohtori et al. 2003).

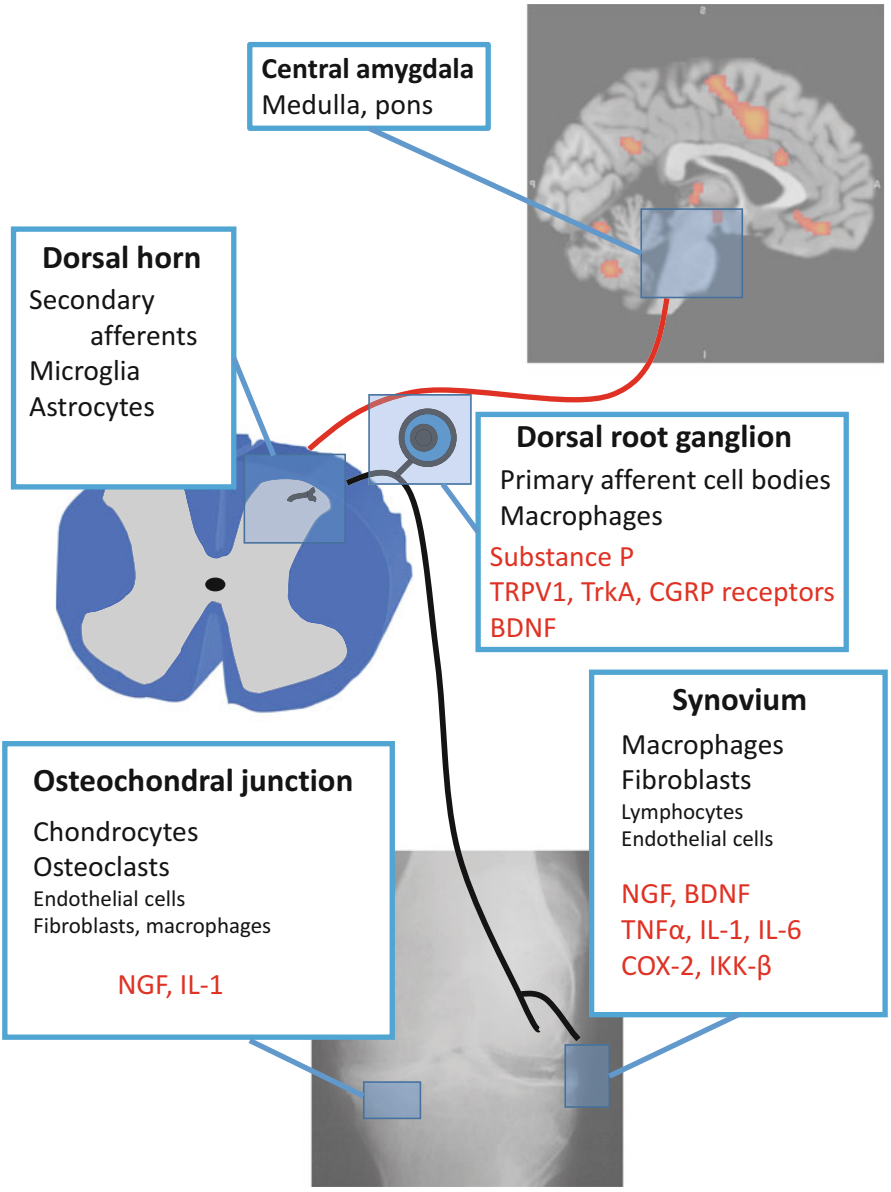

Fig. 1 Arthritis pain mechanisms associated with CGRP. Schematic diagram displaying joint and afferent nervous system structures involved in arthritis pain mechanisms that are associated with CGRP. Nociceptive signals from subchondral bone and synovium are processed within the spinal cord and brain stem. Cytokines and growth factors released within the joint as well as CGRP released from peripheral terminals of articular sensory nerves can sensitise peripheral nociceptive nerves. Altered gene expression in the dorsal root ganglion and increased CGRP release within the dorsal horn modulate activity in secondary afferents. CGRP is widely expressed in the central nervous system, acting in the amygdala, pons and other brainstem regions to augment nociceptive

Arthritis can modify joint innervation, CGRP expression and release (Walsh et al. 2015). In osteoarthritic human joints, CGRP-immunoreactive nerves have been demonstrated in vascular channels within articular cartilage and deep to the outer third of the knee meniscus, structures which are normally avascular and aneural (Ashraf et al. 2011). Osteophytes, regions of new bone formation, also contain CGRP-immunoreactive nerves. Higher densities of CGRP-immunoreactive nerves in the synovium have been associated with human OA (Saxler et al. 2007). Increased CGRP innervation has also been reported at vertebral end plates from people with low back pain (Brown et al. 1997) and in the annulus fibrosus of spondylotic intervertebral discs (Gruber et al. 2012). OA (Ichiseki et al. 2018; Miyamoto et al. 2017), inflammatory arthritis (Shinoda et al. 2003; Weihe et al. 1988; Wu et al. 2002; Kar et al. 1991; Imai et al. 1997a; Ghilardi et al. 2012), myositis (Reinert et al. 1998), inflammation of thoracolumbar fascia (Mense and Hoheisel 2016) and laminectomy (Saxler et al. 2008) in rodents have also been associated with proliferation of CGRP-immunoreactive nerve terminals. This neoinnervation is perhaps in response to local production of neurotrophic factors such as NGF. NGF expression is increased in human synovium (Stoppiello et al. 2014), subchondral bone (Walsh et al. 2010), articular chondrocytes (Iannone et al. 2002) and intervertebral discs (Krock et al. 2014) in painful arthritic conditions. NGF inhibition can reduce CGRP-immunoreactive articular innervation (Ghilardi et al. 2012). Spondylotic human intervertebral discs produce factors which stimulate sensory neurite outgrowth in vitro, an effect which was blocked by antibodies to NGF (Krock et al. 2014).

Higher densities of CGRP-immunoreactive nerves in the synovium have been associated with worse pain in people with OA undergoing arthroplasty (Takano et al. 2017). Similar associations between CGRP innervation density and pain behaviour have been noted in rodent models of OA (Miyamoto et al. 2017) or RA (Ghilardi et al. 2012), thoracolumbar fascial inflammation (Miyagi et al. 2011a) and intervertebral disc injury (Miyagi et al. 2011b, 2013). NGF blockade abrogated the increased CGRP innervation in synovium and reduced pain behaviour in CFA-induced arthritic rats (Ghilardi et al. 2012). Changes in neuroanatomy therefore might contribute to arthritis pain.

Human (Dirmeier et al. 2008; Pereira da Silva and Carmo-Fonseca 1990; Gronblad et al. 1988; Mapp et al. 1990), rat (Murakami et al. 2015; Mapp et al. 1993; Konttinen et al. 1990, 1992; Buma et al. 2000), mouse (Buma et al. 1992) and ovine (Tahmasebi-Sarvestani et al. 2001) arthritis have been associated in some studies with reduced, rather than increased CGRP-immunoreactive nerve densities in synovium. Such apparent denervation might result from synovial hyperplasia

Fig. 1 (continued) signalling. CGRP receptor antagonists with restricted access across the blood-brain barrier have helped elucidate contributions of both peripheral and central CGRP systems to musculoskeletal pain. NGF, nerve growth factor; COX2, cyclooxygenase-2; TNF, tumour necrosis factor-alpha; TRPV, transient receptor potential cation channel subfamily V member; BDNF, brain-derived neurotrophic factor; IKK-β, inhibitor of nuclear factor kappa-B kinase subunit beta; TrkA, receptor for nerve growth factor

outstripping the capacity for nerves to grow into new tissues (Buma et al. 2000). Increased peptide release from nerve terminals might render them invisible to immunohistochemistry for CGRP, despite increased axonal transport of CGRP from the DRG. However, similar reductions in nerve terminal densities detected by immunohistochemistry for constitutive proteins such as protein gene product 9.5 (PGP9.5) suggest that terminal rarefaction is not entirely attributable to peptide release. Nerve terminals might retract from chemically hostile environments, for example, due to the induction of proteases or free radicals during inflammation. CGRP-immunoreactive nerve densities might also be reduced in OA synovium, possibly also associated with synovitis. In animal models, chemical induction of OA by monoiodoacetate (MIA) or surgical induction might directly damage sensory nerves, and increased activating transcription factor-3 (ATF3) in peptidergic DRG cells might indicate nerve injury in rat models of inflammatory arthritis (Nascimento et al. 2011). Early nerve damage might be followed by reinnervation during the repair phase of inflammatory arthritis models (Imai et al. 1997b). Peripheral nerve damage can lead to neuropathic pain, and the sharp and burning pain characteristics described by some people with arthritis (Hochman et al. 2013) might indicate neuropathic pain mechanisms.

2.2 Altered CGRP Expression in Musculoskeletal Pain

Musculoskeletal disease or injury are associated with increased expression of CGRP by sensory ganglia innervating the affected joint (Miyamoto et al. 2017; Kar et al. 1991, 1994; Kuraishi et al. 1989; Nohr et al. 1999; Weihe et al. 1995; Ikeuchi et al. 2009; Hutchins et al. 2000; Hanesch et al. 1993, 1997; Bulling et al. 2001; Carleson et al. 1997; Chen et al. 2008; Ahmed et al. 1995; Damico et al. 2012; Taniguchi et al. 2015; Walker et al. 2000; Donaldson et al. 1992; Smith et al. 1992; Staton et al. 2007; Nieto et al. 2015), intervertebral disc (Lee et al. 2009; Koshi et al. 2010; Kobori et al. 2014) or muscle (Ambalavanar et al. 2006a). Arthritis can induce CGRP expression, although an initial decrease in CGRP immunoreactivity might reflect release of preformed peptide. Arthritis might increase CGRP expression through the production of NGF in the joint. CGRP expression in DRGs is increased by intra-articular injection of NGF (Omae et al. 2015), and NGF inhibition can reduce CGRP expression in DRGs (Iwakura et al. 2010).

Increased CGRP expression by DRGs is maintained for many weeks in rodent models of chronic OA (Ichiseki et al. 2018; Ferland et al. 2011; Ferreira-Gomes et al. 2010; Kawarai et al. 2018), RA (Kuraishi et al. 1989; Staton et al. 2007) or myositis (Ambalavanar et al. 2006b), and this has been associated with increased pain behaviour (Staton et al. 2007; Lee et al. 2009; Ferreira-Gomes et al. 2010; Fernihough et al. 2005). However, increased CGRP expression by DRGs might persist long after remission of Freund's Complete Adjuvant-induced arthritis in rats and after resolution of overt pain behaviour, indicating that increased CGRP expression alone is not a valid biomarker of musculoskeletal pain (Calza et al. 2000). Inhibitory mechanisms, perhaps involving opioids (Calza et al. 2000), might balance

CGRP-induced pain augmentation in this model. Deficiencies in endogenous pain inhibition might explain persistent pain in people with RA whose inflammatory disease is in remission (Walsh and McWilliams 2014). Conversely, genetic depletion of tumour necrosis factor-alpha (TNF) prevented the increase in CGRP in DRGs from mice with MIA-induced OA, but did not affect pain behaviour, suggesting that changes in CGRP expression might not be a major drive to OA pain in this model (Taniguchi et al. 2015).

Inflammation is a major driver of increased CGRP expression by musculoskeletal nerves. Anti-inflammatory treatments such as diclofenac (Kuraishi et al. 1989), the cyclooxygenase-2 (COX2) inhibitor rofecoxib (Staton et al. 2007), corticosteroids (Nohr et al. 1999; Weihe et al. 1995), the TNF blocker etanercept (Horii et al. 2011), inhibition of the inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) (Kobori et al. 2014) or the proteasome inhibitor MG132 (Ahmed et al. 2012) each blunt CGRP upregulation in rodent models of arthritis. Sustained nociceptor activation might also contribute to CGRP upregulation in DRGs. Local anaesthetic blunts increased neuropeptide expression in arthritis (Donaldson et al. 1994), as do other strategies that might reduce nociceptive transmission. Increased CGRP expression in spinal tissues of rats with shoulder OA is reduced by mesenchymal stem cell therapy aiming to reduce joint pathology (Ichiseki et al. 2018). Successful intervertebral fusion also abrogates the increased CGRP expression in disc innervation in a rat intervertebral disc injury model (Koshi et al. 2010). Further research is required to determine whether CGRP upregulation is driven by direct effect of inflammatory mediators on CGRP expression or by the increased nociceptive input resulting from peripheral inflammation. Increased expression of CGRP was noted in periodontal inflammation that is not typically painful, suggesting a possible direct stimulus from inflammation (Abd El-Aleem et al. 2004).

Bisphosphonates might also reduce CGRP upregulation and pain in rats with monoiodoacetate (MIA)-induced OA (Yu et al. 2013). Bisphosphonates reduce osteoclast activity but also might have anti-inflammatory actions, so mechanisms by which they reduce pain or CGRP expression remain incompletely understood. The bisphosphonate, alendronic acid, reduced DRG expression of CGRP and pain behaviour in ovariectomised rats without arthritis, suggesting a direct link between bone mineral density and mechanical hyperalgesia (Naito et al. 2017).

2.3 CGRP and Mechanisms of Musculoskeletal Pain

CGRP is released from the central terminals of joint afferents during nociceptive transmission. CGRP immunoreactivity in the dorsal horn (Mapp et al. 1993; Marlier et al. 1991; Sluka and Westlund 1993) and spinal CGRP release (Ichiseki et al. 2018; Nieto et al. 2015; Schaible et al. 1994; Collin et al. 1993; Ogbonna et al. 2013; Puttfarcken et al. 2010; Nanayama et al. 1989) might be increased in animal models of arthritis, although this has not been demonstrated in all models (Malcangio and Bowery 1996). Release exceeding replenishment might sometimes reduce CGRP-immunoreactive nerve densities in the superficial dorsal horn (Kar et al. 1991), in

much the same way as described above in peripheral nerve terminals in musculoskeletal tissues. Reduction in spinal CGRP immunoreactivity might be dependent on joint movement (Nakabayashi et al. 2016), consistent with CGRP release during mechanical activation of nociceptors. Reductions in CGRP agonist binding sites in dorsal horn of rats with adjuvant arthritis (Kar et al. 1994) might further represent receptor activation by locally released CGRP. In animal models, spinal release of CGRP might increase soon after the onset of arthritis (Schaible et al. 1994) and persist for many weeks (Collin et al. 1993; Ballet et al. 1998), although release can be tonically suppressed by endogenous opioids, which act on mu and, in arthritis, on delta opioid receptors (Collin et al. 1993; Ballet et al. 1998). CGRP-like immunoreactivity has been detected in cerebrospinal fluid from patients (Lindh et al. 1999) and rats (Carleson et al. 1996) with musculoskeletal pain, although human knee pain was associated with reduced CGRP levels in the cerebrospinal fluid (CSF).

CGRP released into the spinal dorsal horn can modulate second-order neurones and increase nociceptive transmission (Bird et al. 2006). These effects were inhibited by the CGRP receptor antagonist CGRP(8-37), as was spinal gliosis, in collagen-induced arthritis (Nieto et al. 2015). Increased CGRP in the spinal cord induces nocifensive behaviours in response to mechanical stimulation of the joint, an effect which is inhibited by CGRP(8-37), and might be mediated by spinal protein kinase-A (PKA) and by glial fibrillary acidic protein (GFAP) expressing astrocytes (Ogbonna et al. 2013; Cornelison et al. 2016). Intrathecal administration of a CGRP-blocking antibody also reduced hyperalgesia in rats with adjuvant-induced arthritis (Kuraishi et al. 1988). Central sensitisation is a key pain mechanism in complex regional pain syndrome (CRPS). In a mouse model of CRPS following tibial fracture, genetic deficiency of receptor activity-modifying protein-1 (RAMP1), an essential component of the CGRP receptor complex, reduced sensitisation (Li et al. 2018; Shi et al. 2015; Guo et al. 2012). Genetic deletion of CGRP also reduced central sensitisation in a rat knee arthritis model (Zheng et al. 2010).

CGRP might also contribute to pain processing in the brain. Local administration of CGRP receptor antagonists (CGRP(8-37) or BIBN4096BS (olcegepant)) reversed the sensitisation of nociceptive neurons in the central nucleus of the amygdala in anaesthetised arthritic rats (Han et al. 2005). This local CGRP receptor blockade also inhibited hindlimb withdrawal reflexes and ultrasonic vocalisations in awake arthritic rats, the latter possibly indicative of roles of CGRP in affective as well as purely sensory aspects of pain.

Peripheral pain mechanisms involving CGRP might also contribute to musculoskeletal pain. CGRP-like immunoreactivity has been detected in synovial fluids from a range of human joints, including temporomandibular joints (Appelgren et al. 1991, 1995; Holmlund et al. 1991), hips (Wang et al. 2015) and knees (Appelgren et al. 1993; Larsson et al. 1989). Increased peripheral release of CGRP during arthritis might be indicated by local concentrations in synovial fluid which exceed those in plasma (Appelgren et al. 1993). CGRP might also be expressed by non-neuronal tissues including macrophages and fibroblasts within the joint during synovitis (Walsh et al. 2015) and from cells within the intervertebral discs (Ahmed et al. 2019).

CGRP induces sensitisation of joint nociceptors, reducing their thresholds to mechanical stimuli (Bullock et al. 2014). Increased CGRP in the joint can stimulate neuronal and glial expression of proteins implicated in the development of peripheral and central sensitisation including P_2X_3, GFAP and OX-42 (Cady et al. 2011). Osteoarthritic knees appear particularly prone to the sensitising actions of CGRP, and CGRP receptor antagonists administered locally to the joint innervation can reduce this sensitisation and suppress the mechanical hyperalgesia in a rat OA (Bullock et al. 2014). Administration of the peripherally restricted, non-peptide CGRP antagonist, olcegepant (Hirsch et al. 2013), or an antibody directed against CGRP, galcanezumab (LY2951742) (Puttfarcken et al. 2010; Benschop et al. 2014), each reduced weight-bearing asymmetry in rats with OA.

CGRP-immunoreactive nerves in joint tissues are frequently in close association with blood vessels, and CGRP induces synovial vasodilatation. Vascular contributions to musculoskeletal pain have been suggested, but remain unproven (Mapp and Walsh 2012). Reducing blood flow might be expected to increase ischaemic pain in arthritis, although reduced blood flow or angiogenesis by CGRP receptor inhibition might suppress inflammation and thereby reduce inflammatory joint pain (Walsh et al. 2015).

Plasma concentrations of CGRP-like immunoreactivity might represent activity of peripheral sensory nerves. Plasma CGRP levels decreased after treatment of rheumatoid arthritis with etanercept, and this might reflect effective reduction either of pain or of inflammation (Origuchi et al. 2011). Higher concentrations of CGRP in synovial fluids (Appelgren et al. 1995) or blood (Dong et al. 2015a) have been associated with human arthritis pain, but further validation would be required to characterise circulating CGRP as a biomarker of musculoskeletal pain (Schou et al. 2017) or, indeed, of synovitis. Synovial fluid levels of CGRP differed in one study between osteoarthritis and the inflammatory arthritides gout and rheumatoid arthritis, whereas plasma levels showed no differences between groups (Hernanz et al. 1993).

2.4 Disrupting CGRP Pathways to Reduce Musculoskeletal Pain

Several therapeutic strategies might target CGRP and its receptors with the aim of reducing musculoskeletal pain. Sensitisation of CGRP-containing nerves can be reduced by inhibiting arthritis pathology or inhibiting specific sensitising agents. CGRP innervation into musculoskeletal tissues might be interrupted by surgical, physical or chemical means. Pharmacological approaches include small molecule receptor inhibitors, or blocking antibodies targeting CGRP or its receptors. Other strategies might reduce the upregulation of CGRP that is associated with musculoskeletal pathology. Genetic deletion of CGRP or its receptors in animal models provides supportive evidence for the potential of anti-CGRP approaches (Zhang et al. 2001).

As described above, anti-inflammatory treatments including non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids or cytokine inhibitors can each

reduce neuronal CGRP expression, CGRP peptide levels and nociception in rodent models of arthritis. Inflammation causes joint pain by the sensitisation of articular nerves, in which NGF plays a key role. Inhibiting NGF in the periphery reduces pain in people with knee OA or with low back pain, as demonstrated consistently by randomised controlled trials (Lane et al. 2010; Sanga et al. 2013; Tiseo et al. 2014). NGF displays specificity for peptidergic sensory nerves, strongly implicating contributions to arthritis pain from one or more of their colocalised neurotransmitters or neuromodulators. Clinical trials of neurokinin-1 receptor antagonists in arthritis have not demonstrated useful analgesia (Goldstein et al. 2000). Preclinical evidence that CGRP contributes to peripheral sensitisation in arthritis is consistent with a role for this peptide in the clinical benefit observed with NGF blockade. NGF blocking antibody administration has been associated with rare but important adverse events, including rapidly progressive OA (Hochberg 2015). Alternatives are desirable that might replicate the clinical benefits of NGF blockade while avoiding adverse events.

Local denervation of osteoarthritic joints can also be achieved by cryotherapy or radiofrequency nerve ablation (Oladeji and Cook 2019), and this can be associated with important pain relief. Surgical denervation might also contribute to the analgesic benefit of joint arthroplasty or osteotomy, where subchondral nerves are inevitably sectioned during surgery. Joints receive CGRP fibres through multiple nerve trunks and along blood vessels, such that complete denervation using local procedures might not be possible. Reinnervation can follow denervation procedures, just as regrowth of CGRP-immunoreactive nerves into joint structures can occur during the healing phases of animal arthritis models. Iatrogenic nerve damage has the potential to cause neuropathic pain, which might contribute to persistent pain that sometimes follows joint surgery (Vergne-Salle 2016).

Targeted disruption of CGRP-containing sensory nerves has been achieved using the transient receptor potential cation channel subfamily V member-1 (TRPV1) agonist, capsaicin. Repeated or high-dose capsaicin application can reduce CGRP innervation in joints. Neonatal capsaicin in rodents can induce sustained reductions in articular CGRP immunoreactivity, whereas low doses in adults deplete CGRP from peripheral nerve terminals. Depletion of capsaicin-sensitive nerves in rodents reduced OA-induced pain behaviour (Kalff et al. 2010). Capsaicin injection in osteoarthritic knees has reduced knee pain in a randomised clinical trial (Stevens et al. 2019). Capsaicin injection in rat knees, however, can also induce synovitis, in part due to the release of proinflammatory neuropeptides including CGRP (Mapp et al. 1996). Synovitis has not been reported as an important adverse event with intra-articular capsaicinoids used in clinical trials, possibly due to the careful formulation of this relatively insoluble chemical.

Topical application of capsaicin can locally deplete neuropeptides including CGRP from cutaneous tissues, although penetration into deep joint tissues might be limited. Topical capsaicin was licenced for clinical use following positive randomised controlled trials in knee or hand OA and might also have some benefit for RA pain (Derry et al. 2017). As with other analgesic treatments, approximately half the clinical benefit from topical capsaicin might be attributed to contextual (or placebo) effects (Persson et al. 2018). Full blinding is difficult to achieve in

clinical trials due to the temporary burning sensation experienced after capsaicin application.

Pharmacological approaches have now been developed that inhibit activation of CGRP receptors. These include competitive inhibition by small molecules and prevention of receptor engagement using antibodies that bind either CGRP itself or the CGRP receptor. CGRP receptor antagonists can reduce arthritis pain behaviour in rodent models of arthritis (Ogbonna et al. 2013; Cornelison et al. 2016; Kuraishi et al. 1988; Han et al. 2005) or muscle inflammation (Romero-Reyes et al. 2015). Neutralisation of CGRP by the monoclonal antibody galcanezumab dose-dependently reduced pain behaviour as measured by weight-bearing differential in the rat MIA and meniscal tear models of OA pain (Benschop et al. 2014).

Analgesia from CGRP blockade might in part be attributed to reductions in peripheral inflammation resulting from inhibition of CGRP-induced vasodilatation in the joint (Walsh et al. 2015). A CGRP receptor antagonist has also been purported to reduce cartilage degeneration and subchondral bone sclerosis in osteoarthritic mice (Nakasa et al. 2016). Disease modification by CGRP receptor blockade has not, however, been to date demonstrated in human arthritis. Peripheral (intra-articular or closed arterial) administration of CGRP antagonists also reduce nociceptive signalling in joint afferents, suggesting inhibition of CGRP-induced peripheral sensitisation in these animal models (Bullock et al. 2014). Antibodies have very limited penetration across the normal blood-brain (or blood-spinal cord) barrier, and analgesia induced by antibody blockade of CGRP or its receptors in animal models of arthritis might be assumed to be due to effects on the joint rather than directly within the CNS. However, additional contributions of CGRP released in the spinal cord are suggested by reduced hyperalgesic behaviour following intrathecal administration of CGRP receptor antagonist (CGRP(8-37)) in a rat model of inflammatory arthritis (Nieto et al. 2015). Spinal administration of CGRP-blocking antibodies also reduced nociceptive signalling from arthritic joints (Kuraishi et al. 1988). The relative contributions of peripheral and central actions of CGRP to human arthritis pain remain incompletely defined.

A double-blind placebo-controlled trial of 8 weeks of treatment of people with knee OA with the monoclonal antibody to CGRP, galcanezumab, was terminated after interim analysis indicated lack of efficacy in its primary pain outcome (Jin et al. 2018). A celecoxib arm within the same trial showed good improvement (1.2 on a 10 cm visual analogue pain scale), but no dose of galcanezumab was associated with clinically important analgesia above placebo (mean specific analgesic effects were all $\leq$0.5 cm). Clinical development of CGRP-blocking agents has subsequently focussed on migraine rather than arthritis pain (Paemeleire and MaassenVanDenBrink 2018). This single trial however cannot exclude potential benefit from CGRP blockade for people with arthritis. The trial evaluated pain outcomes using visual analogue scale (VAS) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), well-validated patient-reported outcome measures for OA pain. However, given the heterogeneous mechanisms and diverse impacts of arthritis pain, it remains possible that CGRP plays a more

important contribution for aspects of pain that are not captured by WOMAC items. In rat OA models, analgesia from CGRP blockade was independent of NSAID-responsive pain and additional to the analgesic benefits of NSAIDs, suggesting that CGRP inhibition and anti-inflammatory treatments might act on different aspects of OA pain (Benschop et al. 2014).

CGRP might particularly contribute to OA pain when there is overactivity of the peripheral peptidergic sensory system. However, post hoc analysis of clinical trial data did not reveal clear prediction of analgesic response by circulating CGRP levels (McNearney et al. 2017), a putative biomarker of high peptidergic nerve activity. However, the study was powered for its primary comparison with placebo rather than for predictive or subgroup analyses. Baseline CGRP levels in OA patients were not associated with WOMAC or VAS pain scores, although they were modestly associated with radiographic OA severity (McNearney et al. 2017).

Lessons might be learned from benefits observed in migraine, where CGRP blockade might be most useful in reducing the frequency and severity of painful episodes, rather than inhibiting ongoing pain. Arthritis pain is similarly episodic, with both intermittent and constant components (Hawker et al. 2008). Patient-reported outcome measures such as ICOAP which capture the important impact of intermittent arthritis pain might be more sensitive to analgesic benefits from CGRP blockade than might be questionnaires that are influenced by constant OA pain, such as WOMAC. Antibodies to CGRP and its receptors have to date had favourable safety profiles, and further exploration of potential benefit for musculoskeletal pain might be justified.

3 CGRP and Pain in Non-musculoskeletal Conditions

CGRP might also be involved in chronic pain states other than migraine and musculoskeletal pain, although human evidence is only observational (Schou et al. 2017). Neuropathic and abdominal pain are major clinical problems, often resistant to existing treatments.

CGRP might contribute to the development and severity of central and peripheral neuropathic pain (Iyengar et al. 2017). Damage to central nociceptive pathways might be traumatic or ischaemic. In an animal model of central neuropathic pain following spinal cord injury, mechanical and thermal allodynia were reduced by intrathecal administration of CGRP(8-37) (Bennett et al. 2000).

Damage to peripheral nerves might be metabolic, as in diabetic neuropathy, traumatic, as in radiculopathy, or iatrogenic as after chemotherapy or surgery. Peripheral neuropathy might be associated both with central and with peripheral sensitisation, mediated by microglial cells, immune cells and immune regulators (Kwiatkowski and Mika 2018). Animals genetically susceptible to neuropathic pain have elevated DRG expression of CGRP (Nitzan-Luques et al. 2013).

Randomised controlled trials of topical capsaicin at sufficient doses to deplete cutaneous nerves of CGRP demonstrate analgesic efficacy above placebo for cutaneous neuropathic pain (Derry et al. 2009). In L5 and L6 lumbar nerve injuries in

rats, CGRP was involved in the establishment of hyperalgesia and progression of pain. Intrathecal injections of antagonists (L703,606 and CGRP(8-37)) delayed the induction of mechanical hyperalgesia after nerve ligation (Lee and Kim 2007; Malon et al. 2011). Spinal chemokine ligand (CCL) 5 and p38 might contribute to CGRP-mediated peripheral neuropathy, suggesting inflammatory mechanisms (Malon and Cao 2016). Despite evidence of contributions from CGRP to peripheral neuropathic pain, effects of CGRP receptor antagonists on nerve injury models have shown inconsistencies. Systemic administration of the CGRP receptor antagonist, olcegepant, revealed strong reductions in mechanical allodynia in a model using infraorbital nerve ligation, but no differences were detected using sciatic nerve ligation (Michot et al. 2012). The authors also reported evidence of synergism between CGRP inhibition and naratriptan, a selective 5-HT_1 receptor agonist used in migraine, in reducing allodynia following infraorbital nerve ligation, but no synergism was observed after the sciatic nerve ligation (Michot et al. 2012).

CGRP-containing nerves might contribute to gastrointestinal (GI) pain by directly functioning as nociceptive neurones and also by regulating gastrointestinal motility (Evangelista 2014) and inflammation. Inflammatory bowel disorders are often associated with pain, even during remission (Norton et al. 2017; Zielinska et al. 2019). Small and large bowels are densely innervated by CGRP-immunoreactive nerves. Indeed, the major source of CGRP in the gastrointestinal tract is neurons, although neuroendocrine cells also synthesise neuropeptides. CGRP is also found in the diseased gastrointestinal tract (Mozsik et al. 2007) as are CGRP receptors (Cottrell et al. 2012). In people with faecal urgency and incontinence with rectal hypersensitivity, a large increase in CGRP-containing nerve fibre densities was observed, along with other neuropeptides (Chan et al. 2003).

Animal models of GI pain can mimic aspects of inflammation, distension or irritable bowel syndrome and suggest possible roles of CGRP. In models, CGRP nerve density correlates with painful (Qiao and Grider 2009) or hyposensitive (Dong et al. 2015b) phenotypes. Systemic and intrathecal administration of CGRP receptor antagonists each demonstrate analgesic efficacy in GI models (Plourde et al. 1997). Intraperitoneal injection of acetic acid induces immobility and writhing pain behaviours in mice. Targeted genetic deletion of CGRPα from TRPV1-expressing neurons in mice, using CRE-LOX recombination, reduced acetic acid-induced immobility (Spencer et al. 2018). Hypersensitivity to rectal distension in rodents can also be induced by administering acetic acid; and this was completely reversed by CGRP(8-37) (Plourde et al. 1997). Muscle contractions taken as indicative of visceral pain have likewise been inhibited by CGRP(8-37) (Julia and Bueno 1997; Bueno et al. 1997).

People with pancreatitis report that pain is their most common symptom (often referred to the back), pain which is often resistant to treatment (Kuhlmann et al. 2019). Pain mechanisms in pancreatitis might be nociceptive, inflammatory or neuropathic (Kuhlmann et al. 2019), and nociceptive transmission is through the spinal dorsal column (Vera-Portocarrero and Westlund 2005). Experimental pancreatitis can be induced by infusion of trinitobenzene sulfonic acid into the pancreatic duct of rats. In this model, CGRP is upregulated in the DRGs (Winston et al. 2005),

and pain sensitivity to electrical stimulation of the pancreas and mechanical stimulation of the abdomen were both reduced by intrathecal administration of CGRP(8-37) (Liu et al. 2011). CGRP upregulation was mediated by NGF (Liu et al. 2011).

In some circumstances, CGRP might display protective actions, and this might be an obstacle to the therapeutic inhibition of CGRP receptors for abdominal pain. CGRP release through activation of TRPV1 appears to be gastroprotective in some models of gastric ulcer (summarised in Evangelista (2014), and the severity of caerulein-induced pancreatitis in rats can be reduced by administration of CGRP during initiation (Warzecha et al. 1997). However, protective effect of CGRP might not be apparent at later stages of pancreatitis when CGRP might exacerbate the condition (Warzecha et al. 2001).

4 Conclusions

Overwhelming evidence implicates CGRP in chronic pain in addition to migraine, even though, to date, this has not translated into clinical benefit from specific CGRP blockade. Musculoskeletal pain has been of major interest, due to its frequency, personal and economic burden and the sparsity of safe and effective treatments. However, chronic neuropathic and gastrointestinal pain might also involve CGRP. The balance between CGRP's involvement in peripheral and central pain mechanisms might be critical to its targeting for non-migraine pain. Peripherally restricted pharmacological agents have advantages of lack of potential for adverse effects within the central nervous system, but their benefit depends on a predominant role of peripheral CGRP receptors rather than those in the dorsal horn of the spinal cord or in the brain. CGRP contributes to sensitisation of peripheral nerves, which we now know makes an important contribution to arthritis pain. Blocking pain caused by sensitisation is attractive in leaving normal protective nociceptive reflexes intact while suppressing pathological pain. Vascular pain might also contribute to musculoskeletal symptoms, although evidence for this remains somewhat circumstantial, and traditional outcome measures might not detect its impact on the patient. Non-selective or selective (e.g. with capsaicin) ablation of musculoskeletal CGRP-containing nerves has entered clinical practice to help relieve arthritis pain. The potential of CGRP-blocking antibodies or receptor antagonists to do likewise deserves further research.

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Calcitonin Gene-Related Peptide Antagonists and Therapeutic Antibodies

Roxana-Maria Rujan and Christopher A. Reynolds

Contents

Abstract

The calcitonin gene-related peptide (CGRP) receptor is composed of the calcitonin receptor-like receptor (CLR, a class B GPCR) and a single-pass membrane protein known as receptor activity modifying protein type 1 (RAMP1). The levels of the CGRP peptide increase during a migraine attack and infusion of CGRP can provoke a migraine attack. Consequently, there is much interest in inhibiting the

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actions of CGRP as a way to control migraine. Here we describe the development of small molecule antagonists designed to bind to the CGRP receptor to block its action by preventing binding of the CGRP peptide. We also describe the development of antibody drugs, designed to bind either to the CGRP receptor to block its action, or to bind directly to the CGRP peptide. The field has been very active, with one antibody drug approved and three antibody drugs in phase III clinical trial. Initial programs on the development CGRP antagonists were frustrated by liver toxicity but the current outlook is very promising with five small molecule antagonists in various stages of clinical trial.

Keywords
Antibody drugs · Calcitonin-receptor-like receptor · CGRP antagonist · CGRP peptide · Migraine

1 Background

The calcitonin gene-related peptide (CGRP) receptor is composed of the calcitonin receptor-like receptor (CLR), a member of the class B GPCR family, and a single-pass membrane protein known as receptor activity-modifying protein type 1 (RAMP1), involved in modulation of hormone selectivity (Poyner et al. 2002; Pal et al. 2012). Detailed studies have also shown that a new intracellular peripheral membrane protein known as CGRP-receptor component protein (RCP) is required to enable signal transduction (Dickerson 2013).

The cognate ligand of the CGRP receptor, CGRP, consists of 37 amino acids that present a disulfide-bonded ring at positions 2 and 7 of its N-terminus which plays an important part in receptor activation (Barwell et al. 2013; Liang et al. 2018). In addition, C-terminal amidation of the peptide plays a key part in ligand-receptor interaction (O'Connell et al. 1993; Hay and Walker 2017). It has been shown that there are actually two types of CGRP, αCGRP and βCGRP, which in humans differ only by three amino acids but share similar activities (Hay and Walker 2017). The predominant form, αCGRP, is a result of the alternative splicing of the calcitonin (CT) gene *CALCA*, while βCGRP is a transcription product of its own gene, *CALCB*, which shares a high homology to the CT gene (Poyner et al. 2002; Hay and Walker 2017). However, in this review the term CGRP will be used without differentiating the two forms of the peptide, except if it is important. CGRP is widely expressed in the central and peripheral nervous system, including the trigeminovascular pathways, and is consistent with modulation of vasodilatation and transmission of nociceptive information (Deen et al. 2017). CGRP and its receptor are also present in the cardiovascular system where they play a protective role (Deen et al. 2017). During migraine, CGRP is released from the trigeminovascular system. At peripheral synapses, CGRP release is associated with vasodilatation on the smooth muscle cells of meningeal and cerebral blood vessels (Deen et al. 2017). At central synapses, it has been assumed that CGRP release is associated with pain transmission via the brain stem and midbrain to the thalamus and higher cortical pain regions (Eftekhari

and Edvinsson 2011). Several studies also revealed that the level of CGRP increases during a migraine attack and infusion of CGRP can provoke this kind of attack in predisposed individuals (Durham and Vause 2010).

To this end, different approaches are being investigated to target CGRP or its receptor to diminish their activity and hence to prevent or treat a migraine attack. Interestingly, a functional antagonist of the CGRP receptor ($CGRP_{8–37}$) can be obtained by deletion of the first seven residues of the CGRP, which are important for receptor activation (Bell 2014). It has been shown that $CGRP_{8–37}$ inhibits vasodilatation and neurogenic inflammation in animal models, but was not useful in clinical studies because its short half-life adds to the lack of potency in vivo (Durham and Vause 2010). However, information gained from these studies supported the development of non-peptide molecules that can block the activity of the peptide at its receptor (Durham and Vause 2010). Therefore, this review aims to focus on the mechanism of action of existing CGRP antagonists and antibodies.

2 CGRP Antagonists

2.1 Olcegepant (BIBN4096BS)

Olcegepant or BIBN4096BS was the first non-peptide antagonist that presented a very high affinity and specificity for the CGRP receptor and that inhibited the nociceptive and vasodilatory effects of the endogenous peptide, CGRP (Durham 2004; Olesen et al. 2004). This non-peptide antagonist was developed by Boehringer Ingelheim GmbH, Germany, following a high-throughput screening (HTS) campaign around dipeptide derivatives (Bell 2014). Subsequently, studies on structure-activity relationship of these compounds led to discovery of an intermediate compound which was further optimized at its benzoxazolone ring to yield olcegepant (CHEMBL AlogP = 2.78) (Fig. 1) (Bell 2014). However, due to the difficulties encountered in developing an oral formulation, high molecular weight (MW) = 869 Da and polar surface area (PSA) = 181 $Å^2$, olcegepant studies were discontinued (Bell 2014; Kuzawinska et al. 2016).

2.1.1 Drug-Receptor Interaction Studies

The affinity of olcegepant for the GCRP receptor was found to be >100-fold higher for primate over non-primate receptors (Poyner et al. 2002; ter Haar et al. 2010).

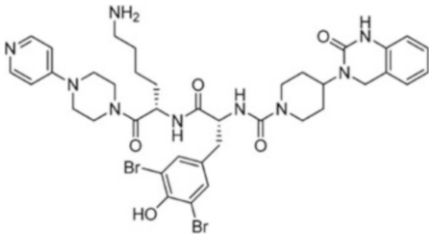

Fig. 1 Olcegepant. Source: Bell (2014)

Several studies revealed that olcegepant shows a specific affinity for the extracellular region of RAMP1, rather than the CLR receptor or the intracellular RCP subunit (Durham and Vause 2010). The crystallographic model of the extracellular domain of the CGRP receptor in complex with olcegepant (PDB 3N7S) showed that tryptophan at position 74 of the RAMP1 is the key residue for higher affinity of olcegepant for primate CGRP receptors (Fig. 2a) (Kandepedu et al. 2015; ter Haar et al. 2010); this important RAMP1 residue position (74) is highly variable outside of the primate family. The decrease in activity for rodent CGRP receptor is conferred by replacement of Trp 74 with Lys (Kandepedu et al. 2015). When olcegepant binds to the CGRP receptor, it stretches around 18 Å from a hydrogen bond donor site at Thr 122 of the CLR receptor into the deep hydrophobic pocket, consisting of the helix αC1 of the CLR receptor and helix αR2 of RAMP1 (ter Haar et al. 2010). Trp 74 (helix αR2) and Trp 84 (in the loop connecting helixes αR2 and αR3 of RAMP1) form the roof and the back surface of the binding pocket (ter Haar et al. 2010). Therefore, replacement of Trp 74 by lysine would reduce the ligand-protein hydrophobic area and sterically hinder the access to the hydrophobic pocket (ter Haar et al. 2010). Other RAMP1 amino acids that may play a small role in selectivity are represented by Ala 70, Asp 71, His 75, Phe 83, Trp 84, and Pro 85 (ter Haar et al. 2010).

One important movement encountered when the antagonist binds to the receptor is represented by the 70° rotation of the side chain of Trp 72 belonging to the CLR receptor. The rotation creates a "Trp shelf" where the piperidine ring of the antagonist settles (Fig. 2b) (ter Haar et al. 2010). The dibromotyrosyl group reaches deep into the pocket and binds by both hydrophobic and electrostatic interactions (ter Haar et al. 2010). Moreover, the carbonyl of the second amide bond forms a hydrogen bond with the NH of Trp 72 belonging to the CLR receptor (ter Haar

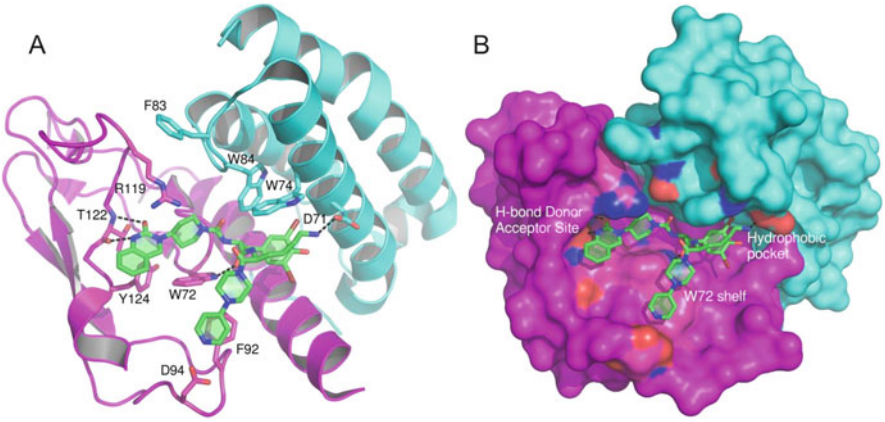

Fig. 2 Structures of olcegepant in complex with the CGRP receptor: (**a**) ribbon representation and (**b**) surface representation. The hydrogen bonds between olcegepant and the CLR receptor (pink) or the RAMP1 protein (cyan) are indicated with dashed lines. Interactions were taken from ter Haar et al. (2010)

et al. 2010). Olcegepant also forms a salt bridge between its Lys 6-amino terminus and the carboxyl of Asp 71, belonging to RAMP1 (ter Haar et al. 2010).

2.1.2 Antimigraine Effects of Olcegepant and Interaction with Other Receptors

Clinical studies revealed that olcegepant was effective for migraine only after intravenous administration and its effect was visible after 30 min and continued to improve over a few hours (Kuzawinska et al. 2016). The success of treating migraine with this antagonist achieved a rate of 60% using doses ranging between 0.25 and 10 mg (Kuzawinska et al. 2016). This success represented a major step in the development of CGRP antagonists for migraine. The most notable side effect encountered in patients during the phase I and II clinical trials was associated with paresthesias (Recober and Russo 2007; Kuzawinska et al. 2016). Furthermore, results from the trials demonstrated that no side effects regarding blood pressure or heart rate have been noticed after its administration (Durham and Vause 2010). Moreover, this drug had no constrictor effect on the superficial, radial, and cerebral temporary arteries and did not present cerebral blood flow changes (Benemei et al. 2017). The lack of the vasoconstrictor properties represents an advantage over the triptans, the most effective current abortive drugs in the treatment of migraine (Durham and Vause 2010). Interestingly, studies on anesthetized rat closed cranial models concluded that olcegepant appears to act outside of the blood-brain barrier (BBB), more specifically in the wall of meningeal arteries which do not have a BBB (Recober and Russo 2007; Benemei et al. 2017).

On the other hand, olcegepant is not efficient in treating migraine caused by factors other than CGRP. For example, Tvedskov et al. (2010) showed that this antagonist is not efficient in preventing migraine caused by the nitric oxide (NO) donor glyceryl trinitrate. The explanation for this is that NO does not cause headache attacks by releasing CGRP. These findings are in concordance with studies demonstrating that olcegepant exhibits an affinity for the binding site of the endogenous ligand at the CGRP receptor (Durham 2004). Instead, other studies revealed that olcegepant presents a relatively low antagonism for the amylin receptor 1 (AMY1), which is composed of the calcitonin (CT) receptor and RAMP1 (Hay and Walker 2017); this is important because CGRP can also bind to the amylin receptor (Hay et al. 2018). This is perhaps expected, since both CGRP and AMY1 receptors share RAMP1 as a common subunit. Moreover, Walker et al. (2017) examined the ability of olcegepant to block CGRP stimulation of intracellular signaling molecules relevant to pain (cAMP, p38, ERK 1/2, and CREB phosphorylation) in rat trigeminal ganglia neurons and transfected Cos7 cells. They showed that olcegepant antagonism of CGRP-stimulated cAMP accumulation in Cos7 cells transfected with CGRP and AMY1 receptors is approximately 130-fold more potent at the CGRP receptor than at the AMY receptor. Results also showed that for this pathway olcegepant is approximately 14-fold more potent in blocking CGRP rather than in blocking amylin at the AMY1 receptor in Cos7 cells. However, the selectivity of olcegepant for the AMY1 receptor depends on the pathway measured (Walker et al. 2017). Interestingly, a concentration of 1 μmol L^{-1} olcegepant is enough to

block both receptors, but in this way the selectivity of the antagonist is lost (Hay and Walker 2017). However, the antagonism at the AMY1 receptor may be underestimated, and further studies are required to clarify its potential side effect (Walker et al. 2017).

2.2 Telcagepant (MK-0974)

MK-0974 (or telcagepant) was developed at Merck Research Laboratories under a program that aimed to discover the first orally active CGRP receptor antagonist. The benzodiazepinone moiety was identified as a potential lead after a HTS campaign (Bell 2014). Subsequent research led to the discovery of several compounds that underwent further optimization to obtain telcagepant (CHEMBL AlogP = 3.35), which presented an attractive combination of potency, selectivity, and oral bioavailability (Fig. 3) (Bell 2014).

2.2.1 Drug-Receptor Interaction Studies

Surprisingly, it has been shown that telcagepant (K_D = 1.9 nM) is not as potent as olcegepant (K_D = 45 pM) (ter Haar et al. 2010). However, while having a lower molecular weight, it has fewer but more productive interactions (ter Haar et al. 2010). The key substituent that provides high affinity for CGRP receptors is the 2,3-difluorophenyl substituent (Bell 2014). However, just like olcegepant, telcagepant is also RAMP-1 dependent and shows less affinity for non-primate species. Surprisingly, telcagepant showed 1,500 lower affinity compared to 100 lower affinity of olcegepant for these species (Kandepedu et al. 2015). One possible explanation for the huge difference between the antagonists for non-primate species could be the fact that telcagepant is smaller and not sterically hindered (Kandepedu et al. 2015). Therefore, replacement of tryptophan 94 in the human RAMP1 with lysine in rodents shows a higher impact on affinity for olcegepant (Kandepedu et al. 2015).

Despite its lower molecular weight (MW = 566 Da), telcagepant binds in a similar way to olcegepant by acting as a lever between Thr 122 of the CLR receptor and the hydrophobic pocket of RAMP1 and breaking nearby interactions which are important for peptide binding (Fig. 4) (Miller et al. 2010; ter Haar et al. 2010). Besides its interactions with Trp 74 and Trp 84, olcegepant presents an additional hydrogen bond between its azabenzimidazolone ring and the carbonyl belonging to

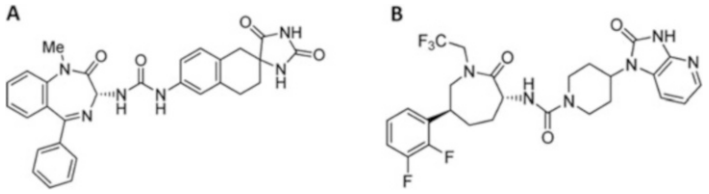

Fig. 3 Structures of the benzodiazepinone-based lead (**a**) and telcagepant (**b**). Source: Bell (2014)

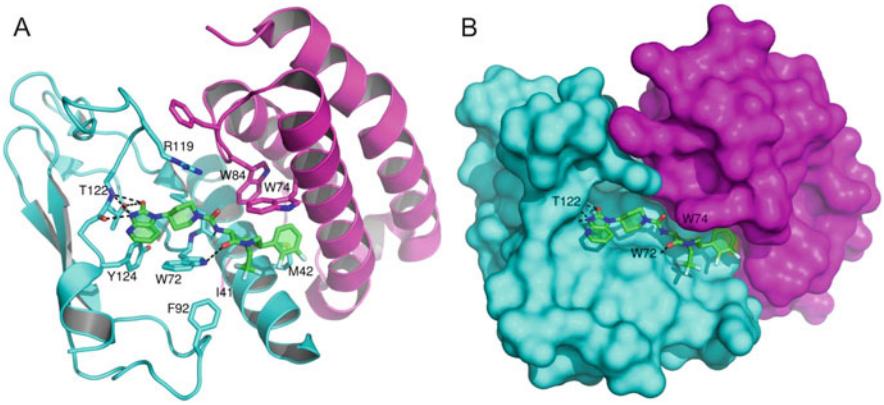

Fig. 4 Structures of telcagepant in complex with the CGRP receptor: (**a**) ribbon representation and (**b**) surface representation. The hydrogen bonds between telcagepant and the CLR receptor (pink) or the RAMP1 protein (cyan) are indicated with dashed lines. Interactions were taken from ter Haar et al. (2010)

Thr 122 of the CLR receptor (ter Haar et al. 2010). Moreover, the difluorophenyl group of telcagepant reaches deeper into the pocket than the dibromotyrosyl group of olcegepant, but it only relies on hydrophobic interactions with Met 42 of the CLR receptor (ter Haar et al. 2010). This residue, which is involved in binding of both antagonists, is more important for telcagepant affinity as shown by Miller et al. (2010). Another hydrophobic interaction of telcagepant arises between its trifluoromethyl group and Ile 41 of the CLR receptor.

2.2.2 Antimigraine Effects of Telcagepant and Interaction with Other Receptors

Like olcegepant, telcagepant is a highly selective antagonist for the GCRP receptor that can stop migraine pain and other migraine symptoms like nausea, photophobia, and phonophobia (Ho et al. 2008; Durham and Vause 2010). Telcagepant lacks vasoconstrictor properties which may allow a safe administration in patients suffering from migraine and cardiovascular disease, though further studies are required to assess the safety in this class of patients (Ho et al. 2008). Pharmacokinetic studies on telcagepant demonstrated that it has fairly good absorption, with plasma concentrations that decrease in a biphasic way and a half-life of about 6 h (Edvinsson and Linde 2010). Moreover, it showed relief from pain 30 min after administration, and a steady state was achieved in 3–4 days of multiple dosing (Edvinsson and Linde 2010). A phase II study compared the clinical effects of 25, 50, 100, 200, 300, 400, and 600 mg telcagepant with 10 mg of rizatriptan. Doses of 300, 400, and 600 mg were significant versus placebo, and 300 mg of telcagepant seemed to have outcomes as effective as rizatriptan (Edvinsson and Linde 2010). The study confirmed that telcagepant was effective in relieving pain at 2 h and provided sustained freedom from pain for up to 24 h (Edvinsson and Linde

2010). A phase III clinical study evaluated the efficacy and tolerability of telcagepant 150 and 300 mg in comparison with zolmitriptan 5 mg and showed that telcagepant (300 mg) was superior to telcagepant (150 mg) and placebo (Ho et al. 2008). Furthermore, telcagepant (300 mg) had a similar 2 h efficacy to zolmitriptan (5 mg) but showed fewer adverse effects than this compound (Ho et al. 2008). Another study also demonstrated that administration of 600 mg of telcagepant did not show any side effects on arterial blood pressure (Edvinsson and Linde 2010). However, data from a long-term safety trial reported that taking telcagepant (140 or 280 mg) twice daily for 12 weeks for migraine prevention led to raised concentration of liver transaminases (Edvinsson and Linde 2010). This suggests that the risk of liver toxicity may be dependent on dose and time (Kuzawinska et al. 2016). Consequently, concerns about hepatic toxicity terminated telcagepant development (Kuzawinska et al. 2016).

Regarding the interaction with other receptors, Walker et al. (2017) also revealed that telcagepant can act as an antagonist to the AMY_1 receptor in transfected Cos7 cells. Though, this antagonist was 35-fold more potent at the CGRP receptor compared to the AMY_1 receptor when CGRP-stimulated cAMP accumulation was measured (Walker et al. 2017). The antagonism of telcagepant when measuring CREB phosphorylation was not significantly different than that for cAMP accumulation. Telcagepant was approximately tenfold more potent at the GCRP receptor than at the AMY1 receptor. Surprisingly, compared to olcegepant, telcagepant selectivity seems to not depend on the pathway being measured (Walker et al. 2017).

2.3 BI 44370 TA

BI 44370 TA is another small-molecule antagonist at the GCRP receptor developed by Boehringer Ingelheim. It was discovered by focusing on reducing the molecular weight and polar surface area of olcegepant, as well as identifying an oral formulation (Fig. 5) (Bell 2014). In phase I trials, BI 44370 TA displayed good tolerability and minimal adverse effects (Diener et al. 2010). A phase II trial was conducted to assess the efficacy of three doses of BI 44370 TA, 50, 200, and 400 mg, for the treatment of acute migraine attacks (Diener et al. 2010). The three doses of BI 44370 TA were compared with eletriptan 40 mg and placebo. The study showed that the

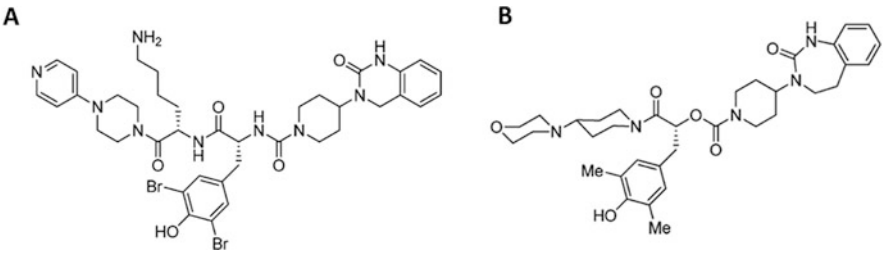

Fig. 5 Structures of olcegepant (**a**) and BI 44370 TA (**b**). Source: Bell (2014)

primary endpoint (pain-free at 2 h) was achieved by the subjects in the BI 44370 TA 400 mg and the eletriptan groups (Diener et al. 2010). The BI 44370 TA 400 mg group displayed similar endpoints as subjects treated with other CGRP antagonists (Diener et al. 2010). The BI 44370 TA 50 mg effect was similar to placebo, while the effect of the 200 mg BI 44370 TA was superior to placebo, but it failed to reach the primary endpoints (Diener et al. 2010). Moreover, the frequency of adverse effects was low in all the groups investigated, and no changes were found regarding ECG, pulse rate, and blood pressure (Diener et al. 2010). However, the development of the molecule was terminated for unknown reasons (Diener et al. 2015).

2.4 MK-2918

After the discovery of telcagepant, Merck Research Laboratories focused on developing another oral antagonist with a lower anticipated clinical dose than telcagepant (Paone et al. 2011). Therefore, they targeted improvements in potency and pharmacokinetic profile by increasing solubility and reducing plasma protein binding (Paone et al. 2011). An increased solubility, especially at acidic pH, was achieved by replacement of the caprolactam ring of telcagepant with imidazoazepane (Paone et al. 2011). In addition, the utilization of the azabenzoxazinone spiropiperidine structure decreased metabolism, and the tertiary methyl ether was found to be a good substituent for potency enhancement (Paone et al. 2011). Further optimization achieved the selection of MK-2918 (Fig. 6) (Paone et al. 2011). Studies showed that MK-2918 is more potent than telcagepant, but its bioavailability is only moderate in rats and low in dogs and rhesus monkeys (Paone et al. 2011; Bell 2014). However, after administration of MK-2918, substantial levels of an active metabolite (the alcohol derived from demethylation of the ether) were observed (Bell 2014). Therefore, it was expected that this metabolite contributes to the clinical efficacy, leading to a lower projected clinical dose (Paone et al. 2011; Bell 2014). Moreover, MK-2918 showed more than 6,000-fold selectivity in a panel of assays containing over 160 receptors, transporters, and enzymes and was selected as a preclinical candidate based on its profile although its current development status is still ambiguous (Paone et al. 2011; Bell 2014).

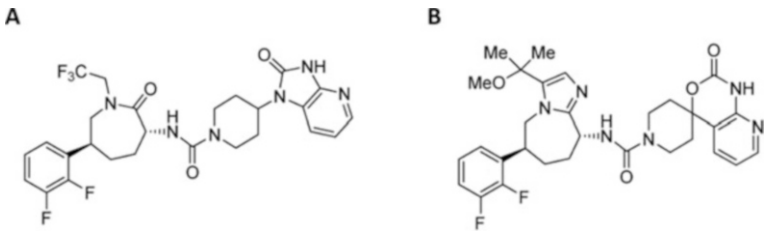

Fig. 6 Structures of telcagepant (**a**) and MK-2918 (**b**). Source: Bell (2014)

2.5 MK-3207

In parallel with the work that led to discovery of telcagepant and MK-2918 from the benzodiazepinone-based lead, the Merck group focused on developing another oral antagonist (Bell 2014). They followed an approach in which the spirohydantoin portion of the lead was retained and the benzodiazepinone group was replaced (Bell 2014). Further optimization of the spirohydantoin-based structure led to attractive intermediate lead structures which underwent several replacements to obtain MK-3207 (Fig. 7) (Bell 2014). The incorporation of a spirocyclopentyl-substituted piperazinone was the peak modification which allowed the optimal potency, selectivity, and pharmacokinetics of MK-3207 (CHEMBL MW = 557.6 Da, AlogP = 3.4) (Fig. 1) (Bell 2014). Detailed evaluation of MK-3207 revealed that it has a high affinity for the rhesus monkey GCRP receptor and a low affinity for the rat CGRP receptor (Bell 2014). Moreover, it was shown that MK-3207 is more potent than telcagepant both in vitro (>50-fold) and in vivo (>100-fold) (Bell 2014). Studies on binding using the tritiated analog [^{3}H]MK-3207 showed that the compound dissociated from the CGRP receptor more slowly ($t_{1/2}$ = 59 min) compared to telcagepant ($t_{1/2}$ = 1.3 min) (Bell 2014). Furthermore, the efficacy of MK-3207 in the treatment of migraine was evaluated in a randomized trial. Doses of 2.5, 5, 10, 20, and 50 mg of MK-3207 were chosen in the first part of the study. MK-3207 doses of 2.5 and 5 mg were shown to have insufficient efficacy, but only the 2.5 mg dose was discontinued from the study (Hewitt et al. 2011). In addition, due to the low efficiency of the other doses, a 200 mg dose was added in the second part of the trial (Hewitt et al. 2011). The study found that the pain-free rate after 2 h administration of 200 mg MK-3207 was superior to placebo and nominally significant for doses of 100 and 10 mg (Hewitt et al. 2011). Moreover, the authors concluded that the compound was well-tolerated and effective in acute treatment of migraine and the incidence of adverse effects (nausea, dizziness, sleepiness) did not appear to enhance with increasing dose (Hewitt et al. 2011). However, the studies on MK-3207 were soon terminated after it was found that MK-3207 caused elevated levels of transaminases (Bell 2014). This was a major discouraging effect in the global search for CGRP antagonists given that telcagepant had been discontinued due to the same adverse effect.

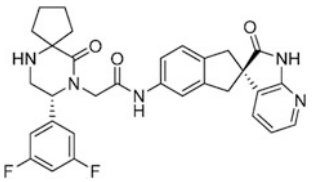

Fig. 7 Structure of MK-3207. Source: Bell (2014)

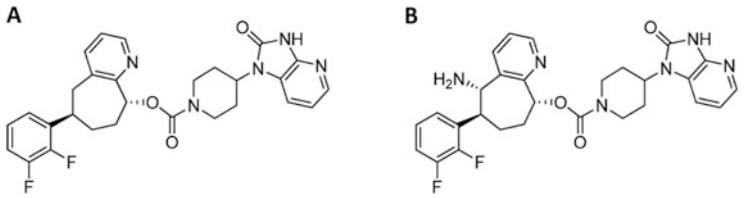

Fig. 8 Structures of BMS-846372 (**a**) and BMS-927711 (rimegepant) (**b**). Source: Bell (2014)

2.6 Rimegepant (BMS-927711/BHV3000)

Bristol-Myers Squibb also initiated a program that aimed to identify GCRP receptor antagonists for the treatment of acute migraine (Bell 2014). They identified a potent oral antagonist, BMS-846372, that contained a cyclohepta[b]pyridine core (Fig. 8a). The compound had a high resemblance to telcagepant and was an attractive clinical lead (Bell 2014). However, it proved to have a very low aqueous solubility (CHEMBL AlogP = 4.2) and created a significant challenge for further development (Luo et al. 2012). Several attempts like formation of salts of the core pyridine or phosphate-related prodrugs were made to address this problem, but they were unsuccessful (Luo et al. 2012). Therefore, they hypothesized that addition of an amino substituent to the cyclohepta[b]pyridine core will improve the solubility and will maintain the attractive properties of the lead compound (Luo et al. 2012; Bell 2014). This led directly to the discovery of BMS-927711 or rimegepant (CHEMBL MW = 534.6 Da, AlogP = 2.9) which presented substantial improvement in aqueous solubility and no serious challenges regarding development (Fig. 8b) (Bell 2014). Moreover, BMS-927711 was a more potent antagonist both in vitro and in vivo and displayed a good oral bioavailability in both rats and monkeys (Bell 2014). Currently, BMS-927711, rimegepant (recently named BHV-3000), is under development by Biohaven Pharmaceuticals. BMS-927711 efficacy was evaluated in a phase II trial at doses of 10, 25, 75, 150, 300, and 600 mg, with sumatriptan 100 mg and placebo as comparators (Marcus et al. 2014). The study showed that doses of 75, 150, and 300 mg were superior to placebo regarding being pain-free at 2 h after administration (Marcus et al. 2014). The 150 mg dose was the most effective in this case. However, for this endpoint, the 600 mg dose was not superior to placebo, and one reason for this may be because of the inherent variability of the patients in this group (Marcus et al. 2014). For the other endpoints, such as sustained pain-free to 24 h post-dose, doses ranging from 25 to 600 mg were all superior to placebo (Marcus et al. 2014). The incidence of adverse effects was low, and the most common effects were nausea, dizziness, and vomiting (Bell 2014; Marcus et al. 2014). To this end, the authors concluded that BMS-927711 is superior to placebo and has an excellent tolerability profile (Marcus et al. 2014). A phase III clinical trial to assess the efficacy of rimegepant versus placebo started in the middle of 2016 and is expected to come to completion at the end of March 2018 (ClinicalTrials.gov: NCT03237845). Another active phase III study with a focus on the tolerability and

safety of rimegepant is estimated to conclude in April 2019 (ClinicalTrials.gov: NCT03266588).

2.7 BHV-3500

Another compound acquired by Biohaven Pharmaceuticals from Bristol-Myers Squibb is BHV-3500 for the prevention of episodic and chronic migraine (www.biohavenpharma.com). According to Biohaven, BHV-3500 is a highly soluble, potent, and selective drug at the human CGRP receptor. Preliminary preclinical evaluations on the marmoset model following oral delivery showed no significant cardiovascular safety or systemic toxicity concerns (www.biohavenpharma.com). Biohaven also reported that they are confident in BHV-3500's chemical properties, which allow multiple routes of delivery such as oral, nasal, inhalation, or subcutaneous administration (www.biohavenpharma.com). At the moment, Biohaven is progressing to submit an investigational new drug application (IND) to the FDA in the first half of 2018. The company also plans to conduct a phase I trial in the second half of 2018 to evaluate the pharmacokinetics, safety, and tolerability of BHV-3500 in healthy volunteers (www.biohavenpharma.com).

2.8 Ubrogepant (MK-1602)

Ubrogepant or MK-1602 (CHEMBL MW = 549.5 Da, AlogP = 2.9) is a novel small molecule drug that has been identified to act as a receptor antagonist to CGRP for the treatment of migraine (Voss et al. 2016). Initially, ubrogepant was developed by Merck Research Laboratories. However, in July 2015, Merck signed a licensing agreement with Allergan to cede exclusive worldwide rights to the new CGRP Migraine Development Program (www.allergen.com). Ubrogepant could be obtained after an amide bond formation between two intermediates, an amino lactam and a spiro acid (Fig. 9) (Yasuda et al. 2017). The preparation of these two intermediates represented a real synthetic challenge, and the discovery of new routes to these compounds became essential for the program to go further into clinical trials (Yasuda et al. 2017). For example, Yasuda et al. (2017) reported the asymmetric synthesis of the lactam intermediate by an enzyme mediated dynamic kinetic

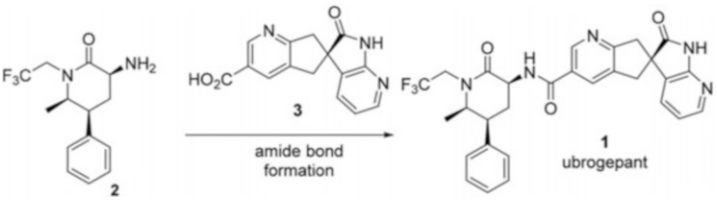

Fig. 9 The synthesis of ubrogepant. Note: (1) ubrogepant, (2) amino lactam intermediate, (3) spiro acid intermediate. Source: Yasuda et al. (2017)

transamination approach. They also described the asymmetric synthesis of the second intermediate using a novel doubly quaternized phase transfer catalyst spirocyclization (Yasuda et al. 2017).

Ubrogepant is a human p-glycoprotein substrate with moderate permeability that was shown to be absorbed quickly (T_{max} of 0.7–1.5 h) and to have a half-life of ~3 h for the α phase or ~5–7 h for the β phase (Voss et al. 2016). A phase IIb randomized, double-blind, placebo-controlled trial was conducted to assess the efficacy and tolerability of ubrogepant for the acute treatment of migraine (Voss et al. 2016). The doses of ubrogepant tested were 1, 10, 25, 50, and 100 mg. Ubrogepant 25, 50, and 100 mg were shown to be superior to placebo for the primary endpoint of 2 h pain-free, with the dose of 100 mg to be the most effective (Voss et al. 2016). However, for the 2 h headache response, only ubrogepant 100 mg showed a high response, but it was not significantly superior compared to placebo (Voss et al. 2016). Ubrogepant was well tolerated, and the side effects were similar to the ones encountered in the placebo group (Voss et al. 2016). The only two exceptions were for nausea and dizziness which were normally mild and self-limited. No elevated levels of liver enzymes were found in this present study (Voss et al. 2016).

ACHIEVE 1, a phase III study, that evaluated two doses of ubrogepant (50 and 100 mg) was completed at the end of 2017 (ClinicalTrials.gov: NCT02828020). Top-line results from Allergan showed that 19.2% of the patients in the 50 mg dose group and 21.2% of the patients in the 100 mg dose group experienced pain freedom at 2 h after the initial dose, compared to 11.8% patients in the placebo group. Moreover, the absence of the most bothersome migraine-associated symptoms including nausea, photophobia, and phonophobia were absent 2 h after the initial dose in 38.6% patients in the lower-dose group and 37.7% patients in the higher-dose group, versus 27.8% in the placebo set (www.allergan.com).

Another two phase III studies of ubrogepant began in the second half of 2016. ACHIEVE 2 evaluated the efficacy, safety, and tolerability of ubrogepant 25 and 50 mg for a single migraine attack (ClinicalTrials.gov: NCT02867709). It was completed in February 2018, and the results are expected in the first half of 2018. An extension phase III study to evaluate the long-term safety and tolerability of ubrogepant 50 and 100 mg is anticipated to end in October 2018 (ClinicalTrials.gov: NCT02873221). Allergan is expected to file for the new drug application (NDA) to the FDA in 2019 (www.allergan.com).

2.9 Atogepant (MK-8031)

Another CGRP inhibitor acquired by Allergan from Merck Research Laboratories following the licensing agreement is atogepant or MK-8031 (Fig. 10). Atogepant is the first oral CGRP antagonist being developed for migraine prophylaxis (www.allergan.com). Currently, atogepant is under a phase II/III study that will evaluate the efficacy, safety, and tolerability of once-daily dosing (10, 30, 60 mg) and twice-daily dosing (30, 60 mg) for the prevention of episodic migraine (ClinicalTrials.gov: NCT02848326). The study is expected to be completed by April 2018.

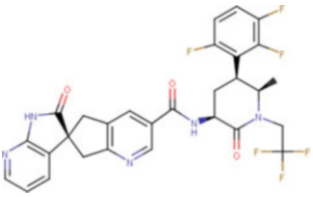

Fig. 10 Structure of atogepant or MK-8031. Source: PubChem

2.10 HTL0022562

HTL0022562 is a novel, potent, and highly selective CGRP antagonist designed by Heptares Therapeutics (owned by the Sosei Group), using its structure-based drug design platform. HTL0022562 was selected as the lead candidate following a laborious selection process based on positive preclinical data under an alliance with Teva Pharmaceutical Industries. A phase I trial in healthy volunteers is expected to begin later in 2018 (www.heptares.com).

3 Anti-CGRP Ligand and Receptor Monoclonal Antibodies

A distinctive approach to inhibit GCRP and its receptor is represented by the development of humanized monoclonal antibodies for the prophylactic treatment of migraine (Kuzawinska et al. 2016). In comparison with gepants, monoclonal antibodies (mAbs) present a slower onset of action, a prolonged half-life, an enhanced target specificity, no abnormal liver side effects, a low risk of drug-drug interactions, and the inability to cross the blood-brain barrier (Kuzawinska et al. 2016; Silberstein 2017). However, due to their large size, they can only be administered parenterally (Silberstein 2017). Currently, there are four mAbs being developed for migraine prevention.

3.1 Erenumab (AMG 334)

The only antibody that binds to the CGRP receptor is erenumab or AMG 334. Erenumab was developed by Amgen Inc. However, in August 2015, Amgen started a global collaboration with Novartis Pharmaceuticals which also includes the erenumab program. AMG 334 is a fully human mAb of the IgG2 subtype that has a half-life estimated at 21 days (Silberstein 2017).

The efficacy and safety of doses of AMG 334 of 7, 21, and 70 mg administered subcutaneously for a period of 12 weeks to patients with episodic migraine were evaluated in a phase II study (ClinicalTrials.gov: NCT01952574). The primary endpoint was the mean change in monthly migraine days (MDM) from baseline to week 12 of the study (Sun et al. 2016). A significant reduction of −3.4 days in the primary endpoint from baseline at week 12 was encountered in the AMG 334 70 mg group versus a reduction of −2.3 days in the placebo group ($p = 0.021$) (Sun et al.

2016). The lower doses did not significantly reduce migraine days. Adverse events such as fatigue, headache, and nasopharyngitis were mild to moderate and occurred in 50–54% of the patients in the AMG 334 groups compared with 54% in the placebo group (Sun et al. 2016). Nine patients also presented neutralizing antibodies (five in the AMG 334 7 mg group, three in the 21 mg group, and one in the 70 mg group) (Sun et al. 2016). This trial was followed by an open-label phase to assess the long-term safety and efficacy of AMG 334 for up to 5 years (Ashina et al. 2017). Results from the first completed year of this open-label follow-up evaluated changes in mean of migraine days (MMD) for patients who received AMG 334 70 mg every 4 weeks (Ashina et al. 2017). The researchers reported that an ongoing preventive effect exists, with a 5-day reduction in MMD in week 64 compared to the baseline of the initial study (Ashina et al. 2017). Moreover, at this week, 65, 42, and 26% of the patients achieved response rate of $\geq$50%, $\geq$75%, and 100%, respectively (Ashina et al. 2017).

Data from ARISE, a phase III study of erenumab 70 mg for episodic migraine, has recently become available (ClinicalTrials.gov: NCT02483585). The study showed that patients in the AMG 334 group experienced −2.9 days change in MMD versus −1.8 days for placebo group (Dodick et al. 2018). As well, a reduction in MMD of more than 50% was present in 29.5% in the placebo group and in 39.7% of the patients treated with AMG 334 (Dodick et al. 2018). Results from STRIVE (ClinicalTrials.gov: NCT02456740), a 6-month phase III trial, demonstrated a decrease in migraine days by 3.7 in the 140 mg dose of AMG 334, 3.2 in the 70 mg group, and 1.8 in the placebo group (Goadsby et al. 2017). Two other trials sponsored by Novartis Pharmaceutical are currently ongoing. Both studies are assessing AMG 334 for the treatment of episodic migraine. However, one evaluates AMG 334 in countries beyond the USA and the European Union by using a single-cohort, three-treatment arm (ClinicalTrials.gov: NCT03333109), while the other is evaluating AMG 334 in patients who have failed prophylactic migraine treatments (ClinicalTrials.gov Identifier: NCT03096834).

AMG 334 was also studied in a phase II trial for chronic migraine. The study demonstrated a reduction of −6.6 days in MMD for AMG 334 70 and 140 mg groups compared to −4.2 days for the placebo group (Tepper et al. 2017). The most frequent side effects were nausea, injection-site pain, and upper respiratory tract infection (Tepper et al. 2017).

All the findings from these phase II and phase III trials suggest that AMG 334 can be beneficial for the prevention of episodic migraine (Goadsby et al. 2017). While no cardiovascular safety concerns were identified in these trials, there is a theoretical cardiovascular risk associated with inhibition of the GCRP pathway (Ashina et al. 2017). Therefore, long-term studies are required to assess this matter and to determine the durability of AMG 334 effects (Ashina et al. 2017; Goadsby et al. 2017).

In July 2017, the AMG 334 license was accepted for review by the US Food and Drug Administration (FDA). AMG 334, trade name Aimovig, has recently been approved and is the first monoclonal antibody on the market that targets the CGRP receptor (www.amgen.com).

3.2 Fremanezumab (TEV-48125, LBR-101, PF-04427429, RN-307)

Fremanezumab, initially known as RN-307 and PF-04427429, is a fully humanized monoclonal antibody of the IgG2 isotype for the prevention of episodic and chronic migraine that was initially developed by Rinat Neuroscience, a company acquired by Pfizer in 2006 (Peroutka 2014; www.tevapharm.com). In 2012, after completing a phase I program for fremanezumab, Pfizer ceded the worldwide rights on the drug to Labrys Biologics. However, in June 2014, Labrys Biologics including the program that was in phase IIb clinical trials was acquired by Teva Pharmaceutical Industries Ltd. (www.tevapharm.com). In December 2017, Teva Pharmaceutical Industries announced that the FDA accepted a priority review for the license for TEV-48125 (www.tevapharm.com).

Unlike AMG 334 which binds to the CGRP receptor, TEV-48125 binds directly to CGRP and blocks its ability to interact with the receptor (Peroutka 2014). TEV-48125 presents a half-life of 39–48 days (Peroutka 2014). Results from five separate phase I trials showed that single intravenous infusions of TEV-48125 ranging between 0.2 and 2,000 mg and multiple infusions of doses up to 300 mg (once at 14 days) were well-tolerated (Peroutka 2014). However, a maximally tolerated dose was not identified (Peroutka 2014).

A phase IIb trial assessed the safety, efficacy, and tolerability of TEV-48125 225 mg and 675 mg administered subcutaneously in the preventive treatment of high-frequency episodic migraine (ClinicalTrials.gov Identifier: NCT02025556). The least square mean (LSM) change in the number of migraine days from baseline to weeks 9–12 decreased with −3.46 days in the placebo group compared to −6.27 days for the 225 mg dose group and −6.09 days for the 675 mg group (Bigal et al. 2015a, b). Adverse events took place in 46% of patients subjected to the lower-dose group, 59% patients in the higher-dose group, and 56% patients in the placebo group (Bigal et al. 2015a, b). The majority of reported adverse events were related to injection-site pain or erythema. No liver function abnormalities, cardiovascular changes, or immunological dysfunctions were found (Bigal et al. 2015a, b). However, two patients presented antibodies against TEV-48125 before and after the treatment. However, no increases in antibody titers were found, and no treatment-emergent antibody response was documented (Bigal et al. 2015a, b).

Other phase IIb trials assessed subcutaneous TEV-48125 675/225 mg and 900 mg for the preventive treatment of chronic migraine (ClinicalTrials.gov: NCT02021773). They randomly assigned patients to three 28-day treatment cycles of TEV-48125 (675 mg in the first cycle followed by 225 mg in the second and third cycle, 900 mg in all three cycles), or placebo (Bigal et al. 2015a, b). The mean decrease from baseline to week 9–12 in headache hours were significantly greater for the active groups (−60 h for the 675/225 mg group, −68 h in the 900 mg group) compared to placebo group (−37 h) (Bigal et al. 2015a, b). The LSM difference in the reduction of headache hours between placebo and active groups was also assessed. For the placebo and 675/225 mg groups was −23 h, while for placebo and 900 mg groups was −30 h. No serious treatment-related adverse effects were found (Bigal et al. 2015a, b).

Results from a 12-week phase III trial to assess the efficacy and safety of subcutaneously administered TEV-48125 225 and 675 mg regimens for the treatment of chronic migraine have recently become published (ClinicalTrials.gov: NCT02621931). Patients were randomly assigned to receive TEV-48125 quarterly (a single dose of 675 mg at baseline followed by placebo at weeks 4 and 8), monthly (675 mg dose at baseline, followed by 225 mg dose at weeks 4 and 8), or placebo (Silberstein et al. 2017). The mean number of headache days was reduced by 4.6 ± 0.3 days for the quarterly group, 4.6 ± 0.3 days for the monthly group, and 2.5 ± 0.3 days in the placebo group (Silberstein et al. 2017). The patients who received the monthly regimen had a 41% reduction in the average headache days per month, the quarterly regimen had a 38% reduction, and the placebo group had an 18% reduction (Silberstein et al. 2017). Other phase III studies of TEV-48125 for both episodic and chronic migraine preventive treatments are ongoing, as well as for cluster headache.

3.3 Galcanezumab (LY2951742)

Galcanezumab or LY2951742 is a humanized antibody of the IgG4 subtype with a half-life of 28 days that also targets CGRP for the prevention of migraine and cluster headache (Schuster and Rapoport 2016). It was initially developed by Eli Lilly and Co. In 2011, LY2951742 was licensed to Arteaus Therapeutics (Peroutka 2015). However, in 2014 Eli Lilly reacquired the rights to the experimental drug (Peroutka 2015).

In a phase II proof-of-concept study, LY2951742 150 mg given subcutaneously every 2 weeks for a duration of 12 weeks in patients suffering episodic migraine was assessed (ClinicalTrials.gov: NCT01625988). LY2951742 was found to be superior to placebo, with a mean change from baseline to week 12 in migraine days of −4.2 compared to −3.0 days (Dodick et al. 2014a, b). Moreover, the patients in the LY2951742 group also experienced high responder rates. The migraine headache was reduced by >50% in 70% of the patients and >75% in 49% of the patients, and there was complete elimination of the attacks in 32% of patients (Dodick et al. 2014a, b). Injection-site reactions and erythema were the most frequent adverse events that occurred in the active group. Viral infections and upper respiratory infections were the most common events in both groups (Dodick et al. 2014a, b).

Another phase II trial evaluated the effect of subcutaneous administration of galcanezumab 5, 50, 120, and 300 mg for the prevention of episodic migraine during a 3-month period (ClinicalTrials.gov: NCT02163993). The primary outcome for this study was represented by superiority, which was determined when the posterior probability of a greater boost for any active group compared with placebo measured by the mean change from baseline in the number of migraine headache days (MHD) in month 3 was ≥95% (Bayesian analysis) (Skljarevski et al. 2018). The 120 mg dose of LY2951742 met the primary outcome and significantly reduced the mean of migraine days (99.6% posterior probability −4.8 MHD; 90% Bayesian credible intervals, −5.4 to −4.2 MHD) compared to placebo (−3.7 MHD, 90% BCI, −4.1

to −3.2 MHD) (Skljarevski et al. 2018). The overall change from baseline in the number of MHD was also significant for the 120 mg group (−4.3 MHD; 95% CI, −4.9 to −3.7 MHD; $P = 0.02$) and 300 mg group (−4.3 MHD; 95% CI, −4.9 to −3.7 MHD; $P = 0.02$) compared with the placebo group (−3.4 MHD; 95% CI, −3.8 to −2.9 MHD) (Skljarevski et al. 2018). The adverse effects that occurred in this study were consistent with other findings and included nausea, injection-site pain, dysmenorrhea, and upper respiratory tract infections (Skljarevski et al. 2018).

The efficacy and safety of several doses of galcanezumab were also studied in healthy volunteers in a phase I trial (ClinicalTrials.gov: NCT01337596). It was found that subcutaneous injections of LY2951742 were well tolerated in all single doses (1–600 mg) and consecutive doses (150 mg). LY2951742 was found to induce a durable, robust, and dose-dependent inhibition of the capsaicin model, a target engagement biomarker that leads to a CGRP-mediated increase in dermal blood flow (Monteith et al. 2017). The adverse effects were generally similar between active groups and placebo and included headache, nasopharyngitis, and contact dermatitis (Monteith et al. 2017). An increased level of alanine and aspartate aminotransferase were found in five patients in the active group, but researchers concluded that these were unrelated to LY2951742 (Monteith et al. 2017).

Currently, LY2951742 is being studied in several phase III trials. In EVOLVE-1 (ClinicalTrials.gov: NCT02614183) and EVOLVE-2 (ClinicalTrials.gov: NCT02614196), galcanezumab is evaluated for prevention of episodic migraines. The completion dates are October 2018 for EVOLVE-1 and April 2019 for EVOLVE-2. Galcanezumab is also undergoing evaluation in REGAIN, a phase III trial conducted for the prevention of chronic migraine with an expected completion date in July 2019 (ClinicalTrials.gov: NCT02614261). Two other phase III trials to assess the efficacy and safety of LY2951742 in episodic (ClinicalTrials.gov: NCT02397473) and chronic (ClinicalTrials.gov: NCT02438826) cluster headache are ongoing. The estimated completion dates are June 2018 and July 2019, respectively. The last phase III trial that is expected to complete in December 2018 aims to evaluate the long-term safety of LY2951742 in patients with episodic and chronic migraine (ClinicalTrials.gov: NCT02614287).

3.4 Eptinezumab (ALD403)

Eptinezumab or ALD403 is another humanized monoclonal antibody that binds selectively and potently to inhibit CGRP (Silberstein 2017). The plasma half-life after infusion is 31 days (Silberstein 2017). ALD403 is being developed by Alder Biopharmaceuticals for the preventive treatment of episodic and chronic migraine. Interestingly, ALD403 which is of the IgG1 subtype is produced using yeast, not mammalian cells (Kuzawinska et al. 2016; Silberstein 2017). Moreover, ALD403 is the only anti-CGRP antibody designed to be administered by quarterly infusion (www.alderbio.com).

A phase II trial investigated the safety and efficacy of ALD403 1,000 mg administered as a one intravenous infusion in patients suffering frequent episodic

migraine (ClinicalTrials.gov: NCT01772524). The active group showed a reduction of 5.6 migraine headache days (MHD) in weeks 5–8 compared with baseline, while the placebo group presented a reduction of 4.6 days (Dodick et al. 2014a, b). Moreover, after the single infusion, the responder rate of 100% was found in 41% of the patients in the active group versus 17% in the placebo at weeks 9–12. The same responder rate was also found in 16% of the patients in the ALD403 group versus 0% in the placebo group at weeks 1–12 post-hoc analysis (Dodick et al. 2014a, b). Adverse events were experienced by 57% of the patients in ALD403 group and by 52% of the patients in the placebo group. Some of the most frequent events were upper respiratory tract infection, urinary tract infection, and back pain (Dodick et al. 2014a, b). Results from the anti-drug antibody assays suggested that 14% of the patients in the ALD403 group had the potential to form antibodies against ALD403 during the study. Reassuringly, the anti-drug titers were low, and no evident effects of immunogenicity on tested parameters were observed (Dodick et al. 2014a, b).

Results from a phase II trial assessing intravenously administered ALD403 in chronic migraine (ClinicalTrials.gov: NCT02275117) were presented at the fifth European Headache and Migraine Trust International Congress in September 2016 (Reichert 2017). The doses of eptinezumab evaluated were of 10, 30, 100, and 300 mg. The study showed that all doses were safe and well-tolerated (Silberstein 2017). The primary efficacy endpoint represented by the percentage of people that achieved a 75% reduction in migraine days (weeks 1–12) was met by 300 mg (33%) and 100 mg (31%) doses compared to placebo (21%) (Silberstein 2017). Moreover, ALD403 300, 100, and 30 mg showed a significant difference versus placebo for the mean change from baseline to weeks 1–12 in migraine days per month (Reichert 2017).

At the moment, there are three ongoing phase III trials that assess the efficacy and safety of ALD403 in migraine prevention. According to ClinicalTrials.gov, the PROMISE 1 study (ClinicalTrials.gov: NCT02559895) had an estimated completion date of December 2017. Top-line results were published on Alder Biopharmaceutical's webpage. PROMISE 1 evaluates ALD403 30, 100, and 300 mg administered intravenously once every 12 weeks in patients with frequent episodic migraine (www.alderbio.com). The results showed that patients in the 300 mg dose group had a reduction of 4.3 migraine days from baseline over weeks 1–12, while the patients in the 100 mg group experienced a reduction of 3.9 fewer migraine days per month compared to placebo (−3.2 days) (www.alderbio.com). Furthermore, in this period a responder rate of 75% reduction was achieved by 29.7% patients in the 300 mg group and 22.2% patients in the 100 mg group versus 16.2% patients in placebo set (www.alderbio.com). Over months 1–6, an average of one in five patients (20.6%) receiving ALD403 300 mg had a 100% responder rate with no migraine days. The safety profile was similar to placebo and consistent with other ALD403 studies (www.alderbio.com).

PROMISE 2, a second phase III trial that has an estimated primary completion date of June 2018, is studying ALD403 300 mg and 100 mg for the prevention of chronic migraine (ClinicalTrials.gov: NCT02974153). Top-line results were

presented by the company in January 2018. The doses were administered as a single infusion once every 12 weeks. Patients in the study experienced an average of 16.1 migraine days per month at baseline (www.alderbio.com). Both doses met the primary endpoint. A reduction of 8.2 monthly migraine days from baseline over the 12 weeks versus 5.6 days for placebo was found. A responder rate of $\geq$75% reduction from baseline was achieved by 33% of the patients versus 15% in the placebo group (www.alderbio.com).

An open-label study to assess the safety and tolerability of ALD403 in chronic migraine is also underway (ClinicalTrials.gov: NCT02985398). The study has an estimated primary end date of June 2018.

The results from these phase III trials will support the eptinezumab license submission to the FDA, which Alder Biopharmaceutical plans to file in the second half of 2018 (www.alderbio.com). If eptinezumab is approved, it will become the first CGRP antibody for migraine prevention that is administered as an infusion that allows for 100% of the dose to inhibit CGRP (www.alderbio.com).

4 Summary

Table 1 summarizes the history of the key compounds. The first gepant, olcegepant, was the first CGRP antagonist and showed that migraine could, in principle, be treated using CGRP antagonists. Telcagepant, the first orally active CGRP antagonist, was affected by concerns over liver toxicity under prolonged use, but some commentators think that these compounds were withdrawn too prematurely as the

Table 1 A selection of CGRP therapeutic entities, including those currently in clinical trial or due to enter clinical trial

Compound	Company	Progress
Small molecules		
Olcegepant	Boehringer	Phase II trials terminated; poor bioavailability
Telcagepant	Merck	Phase III trials terminated over concerns for liver tox
BI 44370 TA	Boehringer	Phase II trials terminated for unknown reasons
MK-2918	Merck	Preclinical candidate, development status unknown
MK-3207	Merck	Phase II trials terminated over concerns for liver tox
Rimegepant	Biohaven	Phase III ongoing
BHV-3500	Biohaven	Phase I on healthy volunteers to start in 2018
Ubrogepant	Allergan	Phase III
Atogepant	Allergan	Phase II ongoing
HTL0022562	Heptares	Phase I on healthy volunteers to start in 2018
Therapeutic antibodies		
Erenumab	Amgen	Approved
Fremanezumab	Teva	Phase III
Galcanezumab	Lilly	Phase III
Eptinezumab	Alder	Phase III

adverse effects were not detected with intermittent use (Holland and Goadsby 2018). Subsequent compounds, namely, ubrogepant and rimegepant, have progressed to phase III clinical trials, atogepant has progressed to phase II, and BHV-3500 and HTL0022562 are due to progress to phase I later this year. Encouragingly, liver toxicity has not been reported for these small molecule compounds (or for the antibodies), indicating that the liver toxicity observed for telcagepant and MK-3207 was related to the individual molecular structures rather than the strategy of targeting CGRP. Moreover, in contrast to the triptan alternatives, these compounds appear to lack vasoconstrictor properties (Bell 2014).

Regarding the antibody drugs, erenumab, which targets the CGRP receptor, has been approved while fremanezumab, eptinezumab, and galcanezumab, which target the CGRP peptide, are in phase III clinical trial (Table 1). The small molecules and the antibodies are complementary to each other, with both classes offering distinct therapeutic advantages. The advantage of antibodies is that they are highly specific for their target, and the use of humanized antibodies can minimize the risks of autoantibodies (which have been observed for the antibodies). Small molecules can be made highly specific by careful design, but off-target interactions are more likely to occur with small molecules. Small molecule CGRP antagonists are more likely to offer rapid relief at the onset of a migraine, but antibodies, with their longer duration of action following approximately monthly injections, potentially offer greater prophylaxis. The CGRP peptide is vasoactive and so is cardioprotective. While it is encouraging that a number of studies have noted no adverse cardiovascular effects, MaassenVanDenBrink et al. have suggested that the long-term removal of CGRP or blockage of the CGRP receptor, particularly by antibodies, may raise concerns for certain patient groups, including pregnant women and those with heart disease (MaassenVanDenBrink et al. 2016; Deen et al. 2017). On the other hand, unlike small molecules, antibodies are likely to be metabolized to their constitutive amino acids, and so adverse liver toxicity is highly unlikely.

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