CALCITONIN GENE-RELATED PEPTIDE (CGRP): NOVEL BIOLOGY AND TREATMENTS

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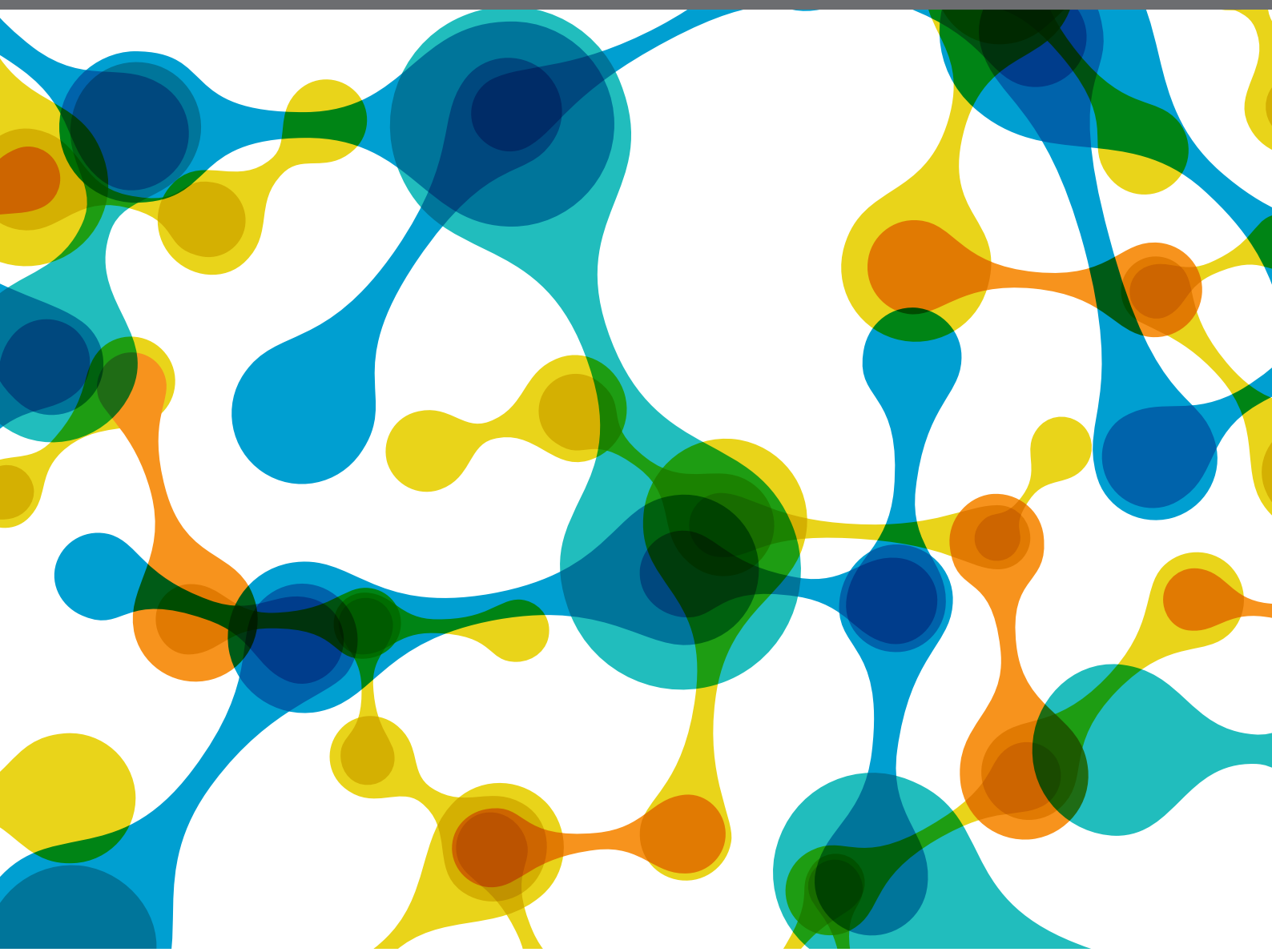

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CALCITONIN GENE-RELATED PEPTIDE (CGRP): NOVEL BIOLOGY AND TREATMENTS

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EDITORIAL
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Editorial: Calcitonin Gene-Related Peptide: Novel Biology and Treatments

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Keywords: CGRP–Calcitonin Gene-Related Peptide, migraine, adverse (side) effects, receptor, cardiovascular disease, antibodies and antagonists, receptor (biochemistry)

Editorial on the Research Topic

Calcitonin Gene-Related Peptide: Novel Biology and Treatments

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide originally discovered in 1982 as a product of alternative splicing of the calcitonin gene. It was realised over the next few years that CGRP is primarily localised to sensory nerves and is a potent microvascular vasodilator. During these times evidence started to emerge that CGRP may be of functional importance in the cerebral circulation and consequently of potential relevance to migraine. By comparison it took a surprisingly long time to decipher the unique structure of the CGRP receptor family. This was finally achieved in 1998. So, by the start of the 21st century, CGRP was known to have a range of potent biological activities and to be of potential relevance to the treatment of migraine (Russell et al., 2014). Since then, much has happened in terms of new science and drug discovery. CGRP and CGRP receptor antibodies have been used to benefit those living with migraine for several years and now small molecule CGRP receptor antagonists are beginning to play a role in the therapeutic landscape for migraine. This Frontiers theme showcases and overviews the broader biological discoveries in recent years. Additionally, we report how use of the CGRP antibodies and antagonists for migraine has allowed us to further decipher the wider biological importance of CGRP.

It is perhaps surprising that the expression and localization of these peptides and their receptors are still under study. However, it is only now becoming widely acknowledged that an amylin receptor (calcitonin receptor [CTR]-receptor activity-modifying protein 1 [RAMP1]; AMY_1), in addition to the CGRP receptor (calcitonin receptor-like receptor [CLR]-RAMP1), is potently activated by CGRP and therefore, knowledge of their expression is of importance in trigeminal ganglia neurons. Rees et al. show in this theme that unlike CLR, CTR is co-localised in trigeminal C- rather than A-fibres, including in human. Thus, CTR and CGRP are co-localised in a site where CGRP may autoregulate its own expression by a positive feedback loop to influence migraine and other cerebral conditions. Indeed, following along this line Edvinsson et al. discuss signalling within the trigeminovascular system and how important this is in our search for novel ways to treat migraine. Moreover, a review by Balcziak and Russo, emphasises the close link that CGRP has with dural structures including immune cells and genes and discusses their potential to be involved in migraine pathology.

Today, knowledge is continuing to increase concerning the Class B G protein-coupled receptor CGRP receptor family and ways in which the receptors may be targeted in the future. A study by Pearce et al. uses cutting edge technologies to demonstrate biased CLR-RAMP1 receptor signalling. They investigated receptor desensitisation and the downstream pathways to reveal the role of

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arrestins and associated kinases. There are a number of CGRP blocking antibodies and CGRP receptor antagonists available for the treatment of migraine. Current antagonists are either antibodies or small molecules but peptide antagonists offer a potential alternative. In this theme there is an article by Jamaluddin et al. on their approach to lipidate peptide antagonists to increase their half-life. The results show that whilst the study is at an early stage, there are positive aspects for applying lipidation to create novel peptide antagonists.

The precise mechanisms *via* which CGRP influences migraine remain unclear. However, the vasodilator effect of CGRP on cranial arteries and link with migraine is established. One mechanism *via* which CGRP dilates is *via* K_{ATP} channels. Coskun et al. shows that the K_{ATP} channel antagonist glibencamide had no effect on CGRP-induced headache or CGRP-induced vasodilation. Whilst interesting, there are questions concerning the dose and selectivity, or whether the CGRP-induced K_{ATP} activation is observed in humans. On the other hand, it is striking that whilst CGRP is a potent vasodilator, the use of CGRP antibodies and antagonists for migraine has met with relatively few adverse effects on the cardiovascular system. However, it should be noted that at least one CGRP blocking antibody is linked to an FDA hypertension warning. This could mean that insufficient CGRP is released in humans. An interesting manuscript by Skaria and Vogel suggests that CGRP, known to be released in exercising humans, may be influential in mediating cardiovascular protective effects in both physiological and pathological situations. Furthermore, CGRP antibodies and antagonists, were suggested by these authors to remove the long-term benefit of exercise. Their original data was obtained in exercising WT and CGRP knockout mice. This is complemented by reviews presented in the theme from Argunhan and Brain and the Kumar et al. that emphasise the potential importance of CGRP agonists as therapeutic agents to treat cardiovascular disease. CGRP is also involved as a protective factor in immune disease such as discussed in this theme by Mariotton et al. from Ganor's lab.

One of the most interesting aspects of CGRP biology is learning about its pathophysiological importance through administration of the CGRP blockers in migraine. Whilst the low incidence of side effects has been acknowledged worldwide, a surprising side effect in some has been constipation. In this issue Holzer and Holzer-Petsche, uses their long-standing experience in this research area to attempt to unravel the mechanisms involved. It is suggested that blocking the role of CGRP in peristalsis and secretion in the intestine underlies this side-effect of CGRP-targeted therapeutics.

The collective works presented in this research theme allow a better understanding of CGRP, both with respect to biological activities and as a druggable target. There are still many answers to find and research on CGRP remains at a high level of interest today.

AUTHOR CONTRIBUTIONS

SB wrote the draft and all authors made a contribution to the final version and approved it for publication.

REFERENCE

Russell, F. A., King, R., Smillie, S.-J., Kodji, X., and Brain, S. D. (2014). Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. *Physiol. Rev.* 94 (4), 1099–1142. doi:10.1152/physrev.00034.2013

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ORIGINAL RESEARCH
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The Effect of K_{ATP} Channel Blocker Glibenclamide on CGRP-Induced Headache and Hemodynamic in Healthy Volunteers

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Background: Calcitonin gene-related peptide (CGRP) dilates cranial arteries and triggers headache. The CGRP signaling pathway is partly dependent on activation of ATP-sensitive potassium (K_{ATP}) channels. Here, we investigated the effect of the K_{ATP} channel blocker glibenclamide on CGRP-induced headache and vascular changes in healthy volunteers.

Methods: In a randomized, double-blind, placebo-controlled, cross-over study, 20 healthy volunteers aged 18–27 years were randomly allocated to receive an intravenous infusion of 1.5 μg/min CGRP after oral pretreatment with glibenclamide (glibenclamide-CGRP day) or placebo (placebo-CGRP day). The primary endpoints were the difference in incidence of headache and the difference in area under the curve (AUC) for headache intensity scores (0–14 h) between glibenclamide and placebo. The secondary endpoints were the difference in AUC for middle cerebral artery blood flow velocity (V_{MCA}), superficial temporal artery (STA) and radial artery (RA) diameter, facial flushing, heart rate (HR) and mean arterial blood pressure (MAP) (0–4 h) between glibenclamide and placebo.

Results: We found no significant difference in the incidence of headache between glibenclamide-CGRP day (14/20, 70%) and placebo-CGRP day (19/20, 95%) ($P = 0.06$). The AUC for headache intensity, V_{MCA}, STA, RA, facial skin blood flow, HR, and MAP did not differ between glibenclamide-CGRP day compared to placebo-CGRP day ($P > 0.05$).

Conclusion: Pretreatment with a non-selective K_{ATP} channel inhibitor glibenclamide did not attenuate CGRP-induced headache and hemodynamic changes in healthy volunteers. We suggest that CGRP-induced responses could be mediated via activation of specific isoforms of sulfonylurea receptor subunits of K_{ATP} channel.

Keywords: humans, migraine, glyburide, calcitonin-gene related peptide, cranial arteries

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a potent vasodilator of cranial arteries (Brain et al., 1985; Falkenberg et al., 2020) and intravenous infusion of CGRP causes headache in healthy volunteers (Petersen et al., 2005b; Falkenberg et al., 2020) and migraine attacks in migraine patients (Lassen et al., 2002; Hansen et al., 2010; Ashina, 2020; Iljazi et al., 2020; Ashina et al., 2021). The molecular mechanisms by which CGRP mediates head pain are still unclear (Ashina et al., 2019). Recent studies suggested K_{ATP} channels as downstream effectors in the CGRP signaling pathway (Nelson et al., 1990; Kitazono et al., 1993; Quayle et al., 1994; Kleppisch and Nelson, 1995). K_{ATP} channels are expressed in neurons, vascular endothelium, and smooth muscle cells. These channels are involved in diverse physiological processes including insulin secretion, regulation of vascular tone, and protecting against metabolic stress (Al-Karagholi et al., 2017, 2019a, 2020b). In human provocation studies, K_{ATP} channel opener levcromakalim induces headache and migraine (Al-Karagholi et al., 2019b,c, 2020a, 2021a).

The widely used antidiabetic drug, glibenclamide, is a non-selective K_{ATP} channel inhibitor that belongs to the second-generation of sulfonylurea (Luzi and Pozza, 1997; Hibino et al., 2010). Preclinical evidence has demonstrated that glibenclamide attenuates CGRP-induced cranial artery dilation (Gozalov et al., 2005, 2008; Al-karagholi et al., 2019d) and trigeminal pain transmission (Christensen et al., 2020). In this study, we hypothesized that glibenclamide would inhibit CGRP-induced headache and vascular changes. To investigate this, we conducted a double-blind, randomized, placebo-controlled, crossover study in healthy volunteers.

MATERIALS AND METHODS

Twenty healthy volunteers were recruited through the Danish website[1]. Written informed consent was obtained from all participants after detailed oral and written study information. The female participants were required to have sufficient contraception [contraceptive pill or intrauterine device/system (IUD/IUS)]. Exclusion criteria were: (1) history of cardiovascular or cerebrovascular disease, diabetes mellitus, or hypercholesterolemia, (2) electrocardiogram (ECG) changes indicative of myocardial ischemia or hypertrophy, (3) hypertension at baseline on an experimental day (defined as a systolic blood pressure above 150 mmHg or a diastolic blood pressure above 100 mmHg), (4) current pregnancy or breastfeeding, (5) daily intake of medication (except oral contraceptives), (6) daily smoking within last 5 years, (7) first-degree relatives with a history of diabetes mellitus, and (8) history of any primary headache disorders (except episodic tension-type headache for <2 days per month during the last year) or first-degree family members with migraine as defined by the third International Classification of Headache Disorders (ICHD) (Vincent and Wang, 2018). A full medical examination and ECG were performed on the day of recruitment.

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-19065735) and the Danish Data Protection Agency, and was conducted according to the Declaration of Helsinki of 1964, with later revisions. The study was also registered at ClinicalTrials.gov (NCT04231617).

Experimental Design and Randomization

In a double-blind, placebo-controlled, crossover design, the participants were in a balanced order randomly allocated to receive an intravenous infusion of 1.5 μg/min CGRP over 20 min, 2 h after oral pretreatment with either 10 mg glibenclamide (Hexaglucon, Sandoz) or placebo (calcium supplement tablet) on 2 days separated by at least 1 week (**Figure 1**). Preparation of the study drug was performed by the Capital Region Central Pharmacy. Randomization and drug allocation were carried out by personal unrelated to the study to ensure study staff, participants and investigator remained blinded. Investigators had no access to the randomization code until the study was completed.

Participants arrived non-fasting at the clinic between 8:00 and 9:00 AM on each study day. The participants were placed in the supine position and a venous catheter was inserted into the left and right antecubital vein for drug (CGRP) and 20% glucose infusion. Then, participants rested for at least 30 min before baseline measurements of vital signs were performed. The infusion started using a time and volume-controlled infusion pump. Vital signs including mean arterial blood pressure (MAP), heart rate (HR), respiratory rate, blood oxygen saturation and nasal end-tidal CO_2 tension were continuously monitored and recorded every 10 min (ProPac Encore; Welch Allyn Protocol) (**Figure 2**). Room temperature was continuously monitored and recorded every 5 min. Facial flushing was measured by speckle contrast imager (moorFLPI; Full Laser Perfusion Imager). The contrast imager was placed 30 cm perpendicularly above the face of the participants and measured the skin blood flow automatically every 5 s as previously described (Ghanizada et al., 2020a). Middle cerebral artery blood flow velocity (V_{MCA}), left superficial temporal artery (STA) diameter, left radial artery (RA) diameter, and end-tidal partial pressure of CO_2 ($PetCO_2$) were recorded as previously described (Petersen et al., 2005a).

MA and HG evaluated eligibility, obtained informed consent, and enrolled the participants. Experiments were carried out at the Danish Headache Center, Department of Neurology, Rigshospitalet Glostrup from February 01, 2020 to September 01, 2020.

Headache and Accompanying Symptoms

Immediately before oral glibenclamide or placebo administration, and every 10 min after the administration, we asked participants specifically about the presence of headache, nausea, photophobia, and phonophobia. The features of headache including intensity, location,

[1] www.forsogsperson.dk

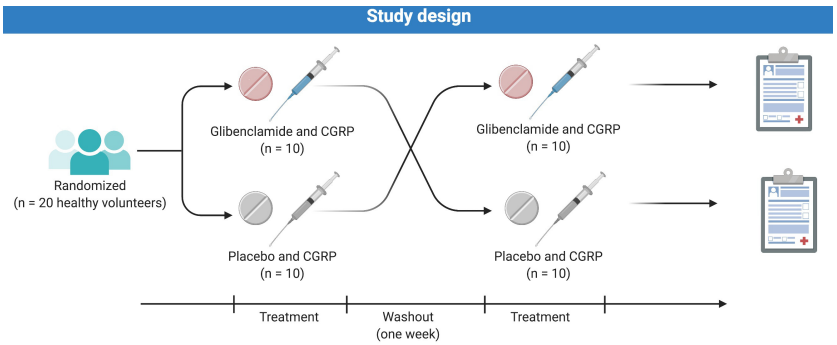

FIGURE 1 | Twenty healthy volunteers (11 women and 9 men) were randomly allocated to receive oral glibenclamide or placebo followed by a 20 min CGRP-infusion. The two study days were separated by at least 7 days to ensure proper wash-out.

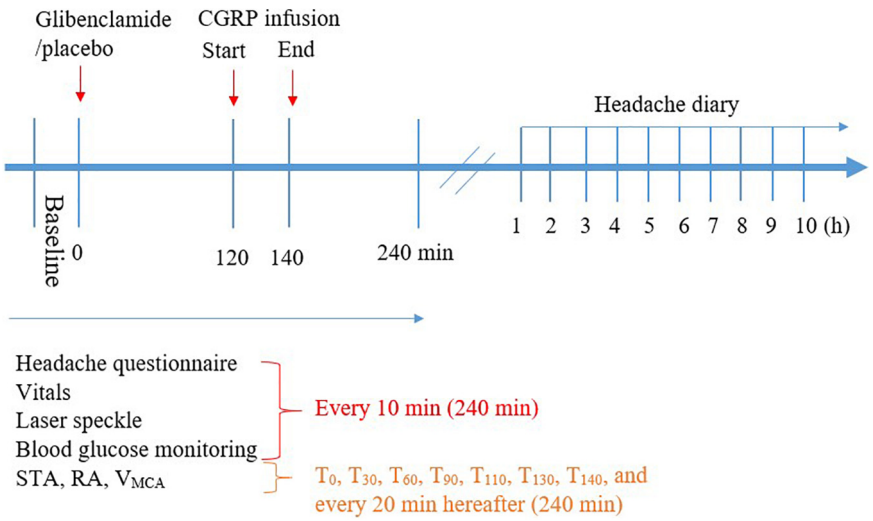

FIGURE 2 | Timeline of the procedure during hospital phase (0–240 min) and post-hospital phase (1–10 h).

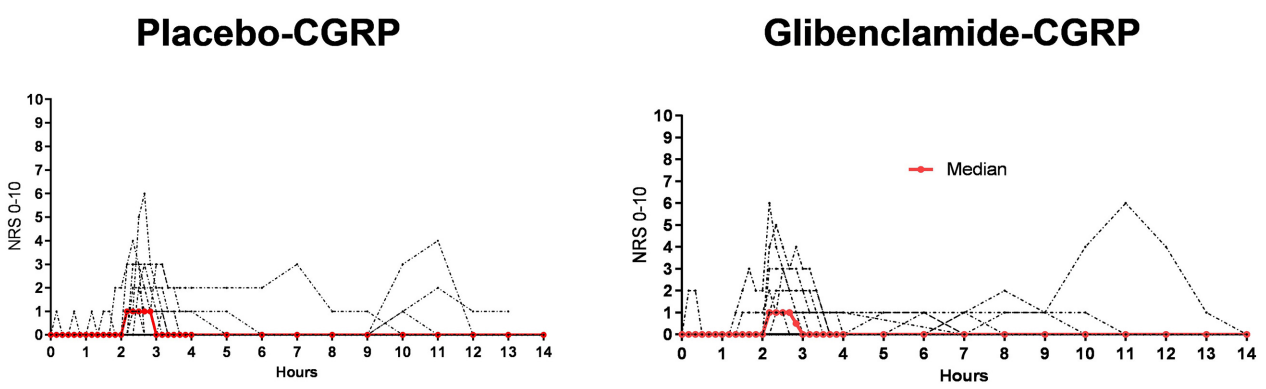

FIGURE 3 | Individual (black lines) and median (red line) headache intensity after glibenclamide and placebo, n = 20. All participants received CGRP-infusion at 120 min. On glibenclamide-CGRP day 14 reported headache and on placebo-CGRP day 19 participants reported headache. We found no difference in headache incidence and intensity between glibenclamide-CGRP and placebo-CGRP day (P = 0.06).

TABLE 1 | Clinical characteristics of headache and associated symptoms in healthy volunteers after glibenclamide and placebo (0–14 h observation period).

Participants	Peak headache (duration of headache)	Headache characteristics	Associated symptoms	Migraine-like attacks (onset)	Treatment (time)/efficacy
1					
Glibenclamide-CGRP	None				
Placebo-CGRP	140 min (40 min)	Bilat/1/press/−	− / − / −	no	None
2					
Glibenclamide-CGRP	130 min (420 min)	Bilat/3/press/+	− / − /−	no	None
Placebo-CGRP	150 min (310 min)	Bilat/2/press/−	− / − /−	no	None
3					
Glibenclamide-CGRP	140 min (420 min)	Bilat/2/press+throb/+	− / − /−	no	Paracetamol 1 g (9 h/ yes)
Placebo-CGRP	130 min (440 min)	Bilat/3/press+throb/+	− / − /−	no	Paracetamol 1 g (7 h/ yes)
4					
Glibenclamide-CGRP	140 min (80 min)	Bilat/1/press/−	+ / − /−	no	None
Placebo-CGRP	130 min (50 min)	Bilat/1/press+throb/−	+ / − /−	no	None
5					
Glibenclamide-CGRP	130 min (250 min)	Bilat/6/throb/+	+/ − /−	Yes	None
Placebo-CGRP	10 min (90 min)	Bilat/1/throb+ press/+	−/ − /−	no	None
6					
Glibenclamide-CGRP	None				
Placebo-CGRP	130 min (30 min)	Bilat/1/throb/−	− / − /−	no	None
7					
Glibenclamide-CGRP	none				
Placebo-CGRP	180 min (170 min)	unileft/3/press+throb/−	− / + /−	no	None
8					
Glibenclamide-CGRP	540 min (550 min)	Bilat/6/press+throb/−	− / − /+	no	Paracetamol 1 g (11 h/ yes)
Placebo-CGRP	540 min (150 min)	Bilat/4/press+throb/−	− / − /+	no	None
9					
Glibenclamide-CGRP	130 min (50 min)	Bilat/1/press+throb/−	− / − /−	no	None
Placebo-CGRP	130 min (30 min	Bilat/1/throb/−	− / − /−	no	None
10					
Glibenclamide-CGRP	None				
Placebo-CGRP	140 min (20 min)	bilat/3/press/−	− / − /−	no	None
11					
Glibenclamide-CGRP	None				
Placebo-CGRP	None				
12					
Glibenclamide-CGRP	130 min (120 min)	bilat/1/press+throb/+	+/ + /+	Yes	None
Placebo-CGRP	130 min (120 min)	bilat/1/press+throb/+	+/ + /+	Yes	None
13					
Glibenclamide-CGRP	140 min (170 min)	bilat/5/press+throb/−	−/ + /+	Yes	None
Placebo-CGRP	160 min (70 min)	bilat/6/press+throb/−	+/ + /−	Yes	None
14					
Glibenclamide-CGRP	150 min (140 min)	bilat/3/press+throb/+	−/ − /−	no	None
Placebo-CGRP	140 min (100 min)	bilat/4/press+throb/+	−/ + /−	no	None
15					
Glibenclamide-CGRP	130 min (30 min)	bilat/1/press/+	−/ − /−	no	None
Placebo-CGRP	130 min (30 min)	bilat/1/press/−	−/ − /−	no	None
16					
Glibenclamide-CGRP	540 min (440 min)	bilat/8/press/−	+/ − /+	no	None
Placebo-CGRP	150 min (450 min)	bilat/2/press/+	+/ + /−	no	None

(Continued)

TABLE 1 | Continued

Participants	Peak headache (duration of headache)	Headache characteristics[a]	Associated symptoms[b]	Migraine-like attacks (onset)[c]	Treatment (time)/efficacy[d]
17					
Glibenclamide-CGRP	240 min (430 min)	bilat/2/press+throb/–	–/ – /–	no	None
Placebo-CGRP	140 min (20 min)	bilat/1/throb/–	+/ – /–	no	None
18					
Glibenclamide-CGRP	none				
Placebo-CGRP	160 min (410 min)	bilat/2/press+throb/+	– / – /–	no	None
19					
Glibenclamide-CGRP	150 min (90 min)	bilat/1/ throb/–	+ / – /+	no	None
Placebo-CGRP	130 min (610 min)	bilat/1/ press+throb /+	– / – /–	no	None
20					
Glibenclamide-CGRP	540 min (180 min)	bilat/1/ press /–	+/ – /–	no	None
Placebo-CGRP	130 min (70 min)	diffus/1/ throb /+	–/ – /–	no	None

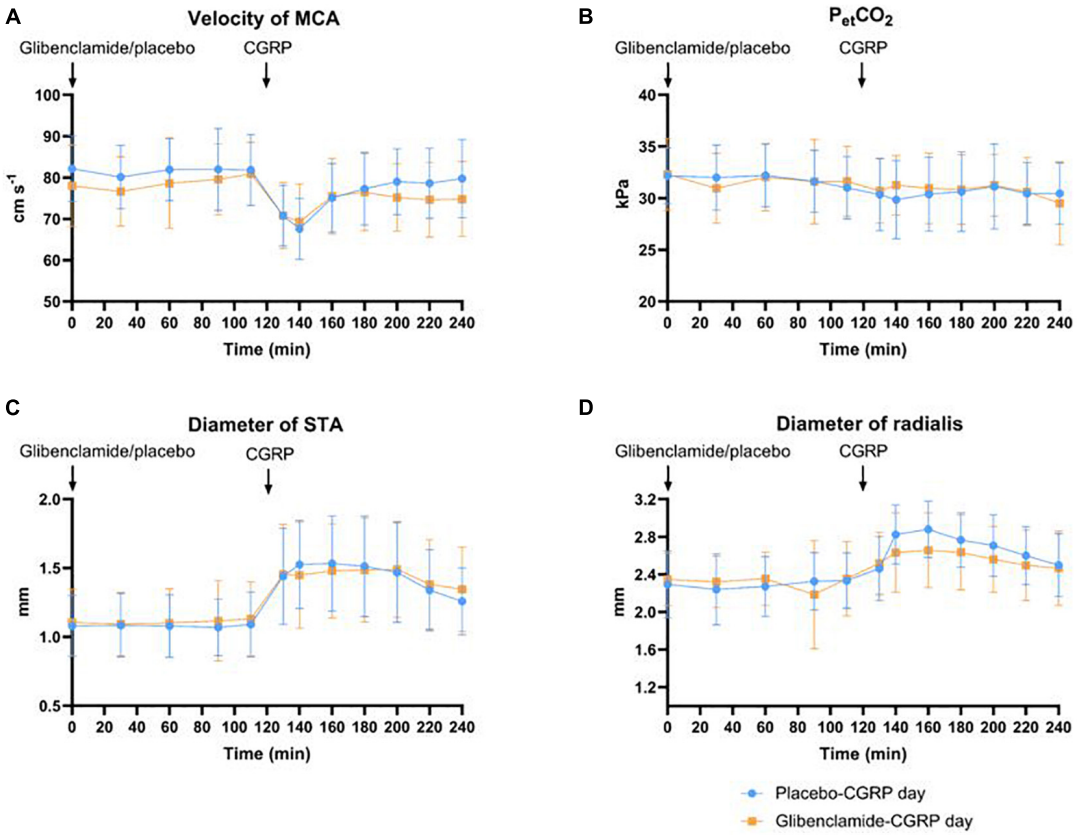

FIGURE 4 | (A) Effect of glibenclamide and placebo on middle cerebral artery (MCA) as changes in velocity (cm/s), $n = 20$. There was no difference in $AUC_{0-240\ min}$ for V_{MCA} between glibenclamide-CGRP and placebo-CGRP day. **(B)** Changes in end-tidal pCO_2 were monitored during MCA-measurements. There was no difference in changes in end-tidal PCO_2 ($PetCO_2$) between two experimental days. **(C)** Changes in superficial temporal artery (STA) in diameter (mm). There was no difference in $AUC_{0-240\ min}$ for STA between glibenclamide-CGRP and placebo-CGRP day. **(D)** Changes in radial artery (RA) diameter (mm). No changes in $AUC_{0-240\ min}$ for RA diameter was observed between glibenclamide-CGRP and placebo-CGRP day.

throbbing/pressing and aggravation by activity were recorded using a standardized questionnaire. Headache intensity was recorded on a numerical rating scale (NRS 0–10) rating pain from none (NRS 0) to maximum imaginable (NRS 10). The participants completed the headache questionnaires hourly until 10 h (waking hours) after discharge from the clinic (**Figure 2**). If the symptoms fulfilled ICHD-3 beta criteria C and D for migraine without aura (Vincent and Wang, 2018), they were characterized as migraine-like attacks.

Glucose Infusion

Plasma glucose concentrations were monitored during a 20 min baseline period before the administration of oral glibenclamide/placebo. After the start, and when initial fasting glycaemia had declined by 10%, blood glucose concentrations were clamped around this level (4–7 mmol/L) by 20% glucose infusion. Glucose infusion rates (GIRs) is based on a previous study (Ampudia-Blasco et al., 1994). Infusion rates (IRs) necessary to maintain stable blood glucose after drug intake were registered and adjusted throughout the ensuing 240 min. The following standard formula was used to calculate the IR taking the participants weight into account and ignoring factors such as gender, age and basal metabolic rate:

$$\text{Infusion rate}\left(\frac{\text{ml}}{\text{hr}}\right)\ \frac{\text{GIR}\frac{\text{mg}}{\text{kg}\times\text{min}}\times\text{weight}\,(\text{kg})\times 60\frac{\text{min}}{\text{hr}}}{\text{concentration}\frac{\text{g}}{100\text{ml}}\times 1,000\frac{\text{mg}}{\text{g}}/100}$$

Twenty-nine blood samples were obtained for the determination of glucose during the experiment period. Blood samples were drawn at 5 min intervals between 30 and 90 min, and at 10 min intervals thereafter. The venous blood samples were drawn from the intravenous catheter using a blood gas aspirator (Radiometer, SafePICO, self-filling blood gas syringe) and the blood glucose concentrations were determined with a blood gas analyzer (Radiometer, ABL90 FLEX).

Statistics and Data Analysis

Baseline was defined as T_0 before the start of oral glibenclamide or placebo administration. For glucose measurement, the baseline was defined as $T_{-20\ to\ 0}$ before the start. Calculation of sample size was based on previous similar studies (Vollesen et al., 2019; Ghanizada et al., 2020b). We aimed to detect a 50% difference at 5% significance with 90% power between glibenclamide compared to placebo. We estimated that 17 participants are needed, and we included 20 subjects to ensure power. Headache intensity scores are presented as median (range). The primary endpoints were the difference in incidence of headache and the difference in area under the curve (AUC) for headache intensity scores between two experimental days: glibenclamide-CGRP day (oral glibenclamide followed by infusion of CGRP) versus placebo-CGRP day (oral placebo followed by infusion of CGRP). The secondary endpoints included: the difference in AUC for V_{MCA}, STA, RA, HR, MAP, and facial skin blood flow between two experimental days.

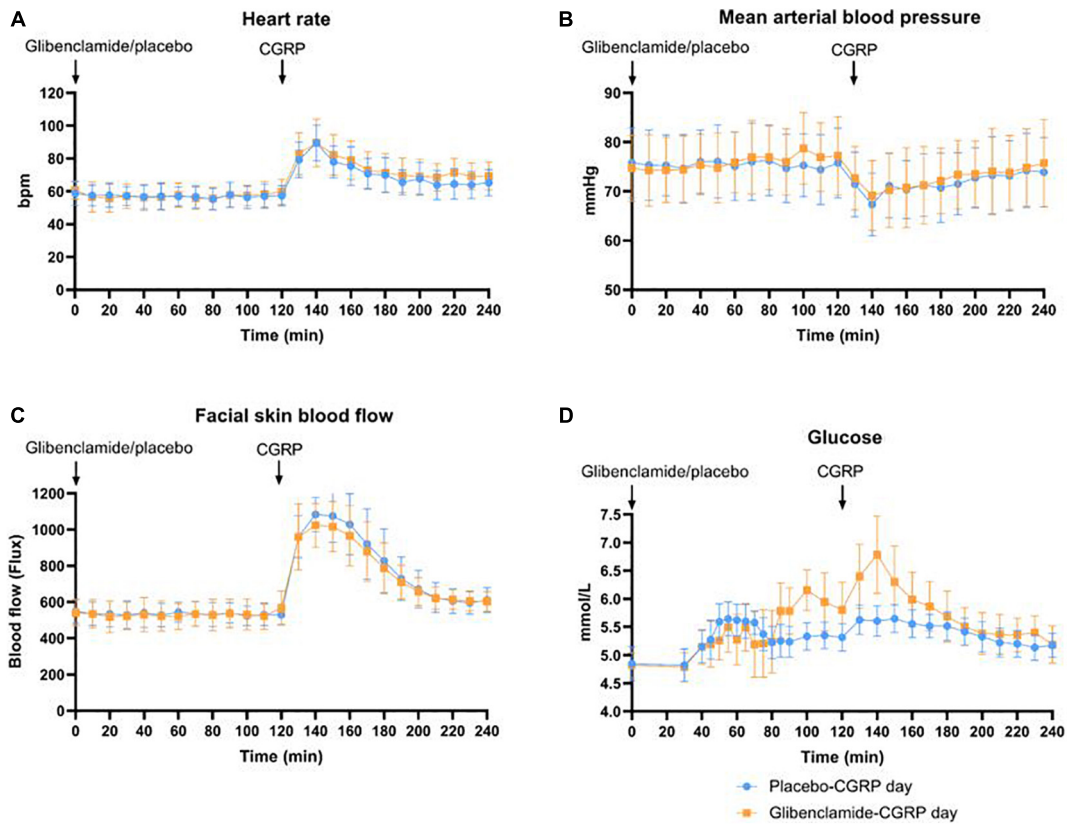

FIGURE 5 | (A) Changes in heart rate (bpm) were registered every 10 min. Heart rate did not differ between glibenclamide-CGRP and placebo-CGRP day. **(B)** Changes in mean arterial blood pressure (MAP) in mmHg. MAP did not differ between glibenclamide-CGRP and placebo-CGRP day. **(C)** Facial skin blood flow measured with Laser Speckle as changes in flux. No changes in $AUC_{0-240\ min}$ for facial skin blood flow were observed between glibenclamide-CGRP (157.5 ± 15.4) and placebo-CGRP day (160.8 ± 14.5; $P = 0.3$). **(D)** Blood glucose samples were drawn at 5 min intervals between 30 and 90 min, and at 10 min intervals hereafter. Blood glucose was clamped between 4 and 7 mmol/L.

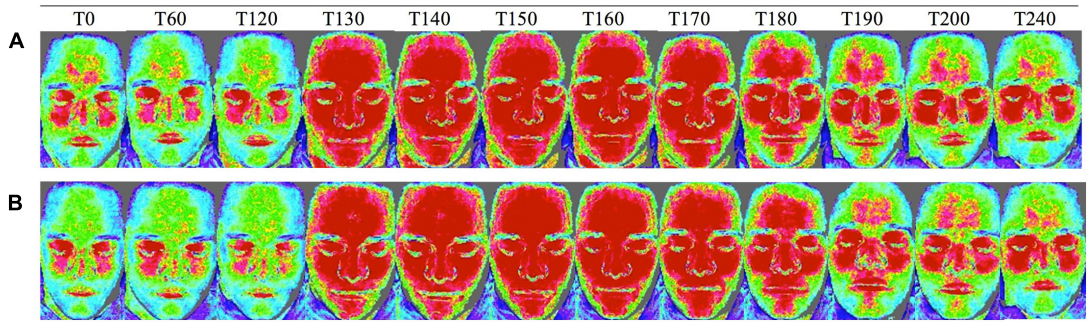

FIGURE 6 | Facial skin blood flow changes measured with laser speckle on **(A)** glibenclamide-CGRP day and **(B)** placebo-CGRP day between T_0 and $T_{240\ min}$. Blue areas indicate low blood flow, green moderate blood flow, and red high blood flow (Kazmi et al., 2015; Ghanizada et al., 2020a). Upon CGRP infusion at $T_{120\ min}$ facial skin blood flow increased on both days.

We used the trapezoidal rule to calculate AUC to analyse the differences in response between glibenclamide and placebo. We used Wilcoxon signed rank test to analyse headache intensity scores, paired two-way *t*-test to analyse other variables, and Mann–Whitney *U* test and independent *t*-test to assess period and carry-over effects for all variables. Binary categorical data including the incidence of headache, accompanying symptoms and adverse events were analyzed with McNemar's test. All analyses were performed with SPSS Statistics version 23 for Windows and a *P*-value < 0.05 was considered as the level of significance.

Data Availability

The data supporting the findings in the present study are available from the corresponding author, upon reasonable request.

RESULTS

Twenty healthy volunteers (11 women and 9 men) completed both days of the study. The mean age was 23 years (range 18–27) and mean bodyweight was 72 kg (range 51–94, body mass index range 18–25). There was no difference between the right and left-sided MCA, and therefore the average of both sides was used. There was no carry-over or period effect for values of headache, HR, MAP, V_{MCA}, STA, or RA.

Headache and Associated Symptoms

Fourteen participants reported headache on glibenclamide-CGRP day (14/20, 70%) compared to 19 on placebo-CGRP day (19/20, 95%; $P = 0.06$). The median peak headache intensity score was 1 (range 1–8) (**Figure 3**) and the median time to peak headache score was 140 min (range 10 min–9 h) on both study days (**Table 1**). Median headache duration was 105 min on glibenclamide-CGRP day and 95 min on placebo-CGRP day. We found no difference in the $AUC_{0-14\ h}$ for headache intensity after glibenclamide (5.08 ± 7.4) compared with placebo (6.18 ± 10.5; $P = 0.69$). Three participants reported migraine-like attacks after glibenclamide compared with two after placebo (**Table 1**).

The Hemodynamic Effects

We observed a 21% decrease in V_{MCA}, 41% dilation of STA and 23% dilation of RA at the end of CGRP infusion (20 min after infusion start) on both days. We found no difference in the $AUC_{0-240\ min}$ for V_{MCA} between glibenclamide-CGRP day (83.4 ± 8.5) and placebo-CGRP day (85.5 ± 7.7; $P = 0.13$) (**Figures 4A,B**). There was no difference in the $AUC_{0-240\ min}$ for STA after glibenclamide (1.4 ± 0.33) compared with placebo (1.4 ± 0.29; $P = 0.75$) and in the $AUC_{0-240\ min}$ for RA after glibenclamide (2.7 ± 0.36) compared with placebo (2.8 ± 0.31; $P = 0.24$) (**Figures 4C,D**). Pretreatment with glibenclamide caused no change in HR and MAP compared with placebo (**Figures 5A,B**). Glucose fluctuations due to glibenclamide treatment were avoided by clamping glucose between 4 and 7 mmol/L (**Figure 5D**). CGRP increased facial skin blood flow on both days (**Figures 5C**, **6**). We found no difference in the $AUC_{0-240\ min}$ for facial skin blood flow after glibenclamide (157.5 ± 15.4) compared with placebo (160.8 ± 14.5; $P = 0.3$).

Adverse Events

The number of participants who reported flushing, heat sensation and palpitation after glibenclamide did not differ from placebo ($P > 0.05$). Eleven participants experienced fatigue after glibenclamide compared to 4 participants after placebo, and 5 participants experienced thirst after glibenclamide compared to none after placebo (**Table 2**).

TABLE 2 | Incidence of adverse events 0–14 h after glibenclamide or placebo.

Adverse event	Glibenclamide-CGRP	Placebo-CGRP
Fatigue	11	4
Flushing	20	20
Warm sensation	19	20
Yawn urge	2	1
Thirst	5	0
Neck stiffness	3	4
Palpitation	18	17

DISCUSSION

The major outcome of the present study was that K_{ATP} channel inhibitor glibenclamide did not affect CGRP-induced headache and vascular changes. The CGRP-induced headache and vascular changes reported in the present study were consistent with previous studies (Petersen et al., 2005b; Hansen et al., 2010; Ashina et al., 2018; Falkenberg et al., 2020). Petersen et al. showed that pretreatment with the CGRP receptor antagonist BIBN4096BS inhibited headache and extracranial vasodilation after intravenous infusion of 1.5μg/min CGRP over 20 min.

In vivo studies showed that glibenclamide attenuated CGRP-induced vasodilation in basilar (Kitazono et al., 1993; Faraci and Sobey, 1998), pial (Hong et al., 1996), and dural arteries (Gozalov et al., 2008). The inhibitory effect of glibenclamide on CGRP-induced vasodilation was dose-dependent, and high doses were necessary to observe an effect (Gozalov et al., 2008). Intravenous glibenclamide administration at concentrations of 20–30 mg/kg attenuated CGRP-induced vasodilation (Gozalov et al., 2008), and i.p. injection of 1 and 10 mg/kg glibenclamide attenuated trigeminal pain transmission in rats with spontaneous trigeminal allodynia (Christensen et al., 2020). Since we observed the participants for 5 h, we used 10 mg per oral glibenclamide which is the maximal single dose tested in humans (Williams et al., 1998; Bayerle-Eder et al., 2000; Gori et al., 2005; Hougaard et al., 2020). This corresponds to 0.14 mg/kg, which is substantially lower than doses used in preclinical models. This might explain the insufficient glibenclamide effect to counteract the CGRP-induced vascular changes. However, using equivalent doses in humans would cause severe hypoglycemia and cannot be reasoned.

Interestingly, *in vitro* studies showed that glibenclamide did not affect CGRP-induced dilation of dural arteries and MCA (Kageyama et al., 1993; Gao et al., 1994; Gozalov et al., 2008). In rat coronary arteries, only the combination of glibenclamide and a calcium-activated K^+ (BK_{Ca}) channel blocker charybdotoxin could attenuate the effect of CGRP on arteries *in vitro* (Sheykhzade and Berg Nyborg, 2001) suggesting a dual action of K_{ATP} and BK_{Ca} channels in CGRP-induced vasodilation. The role of BKCa is recently implicated in headache and migraine patophysiology (Al-Karagholi et al., 2020d, 2021b). Possible involvement of other type of potassium channels in CGRP-induced vasodilation cannot be ruled out. Altogether, these data suggest that interspecies differences are likely to contribute to the discrepancy in the findings of glibenclamide on CGRP-induced vascular effects.

Binding of CGRP to the CGRP-receptor complex on vascular smooth muscle cells (VSMCs) (Eftekhari et al., 2013; Edvinsson et al., 2018) mediates an activation of the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway resulting in potassium efflux through K_{ATP} channels, hyperpolarization, and eventually relaxation and vasodilation (Hong et al., 1996; Williams et al., 1998; Ashina et al., 2021). K_{ATP} channels consist of different isoforms of sulfonylurea receptor subunits (SUR1, SUR2A, and SUR2B) (Kokoti et al., 2020). SUR1 are expressed in the pancreas (Babenko et al., 2000), the TG and the trigeminal nucleus caudalis (TNC) (Ploug et al., 2012). The SUR2B isoform is of vascular origin and highly expressed in cranial arteries (Ploug et al., 2008a,b, 2012). Glibenclamide is a non-specific SUR subunit blocker shown to have high affinity for SUR1 and low affinity for SUR2B (Inagaki et al., 1996; Stephan et al., 2006). SUR subunits are downstream molecules in CGRP signaling cascades, and whether CGRP receptors and SUR subunits have a cross-reactivity at the same cellular level is yet to be demonstrated. Thus, the results of the present study might be explained by different affinity of glibenclamide and CGRP signaling pathway to SUR isoforms.

In conclusion, we found that pretreatment with glibenclamide did not affect CGRP-induced headache and vascular responses. Our findings could suggest that: (1) the potency of used glibenclamide concentration is too low to result in any measurable effects on CGRP-induced headache and vasodilation, (2) CGRP-induced headache and vascular responses could be initiated by activation of SUR2B K_{ATP} channel (Al-Karagholi et al., 2017), and/or (3) interspecies differences might influence the role of K_{ATP} channel in CGRP signaling pathway. To clarify the molecular interaction in the CGRP signaling pathway, more studies on specific isoforms of sulfonylurea receptor subunits of K_{ATP} channel in humans are needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Capital Region of Denmark (H-19065735). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Al-Karagholi, M. A., Ghanizada, H., Nielsen, C. A. W., Hougaard, A., and Ashina, M. (2021a). Opening of ATP sensitive potassium channels causes migraine attacks with aura. *Brain*:awab136. doi: 10.1093/brain/awab136 [Epub ahead of print].

Al-karagholi, M. A., Sode, M., Gozalov, A., and Ashina, M. (2019d). The vascular effect of glibenclamide?: A systematic review. *Cephalalgia Rep.* 2:251581631988493. doi: 10.1177/2515816319884937

Al-Karagholi, M. A. M., Aghazadeh, S., Skovgaard, L. T., and Olesen, J. (2019a). Extracranial activation of ATP-sensitive potassium channels induces vasodilation without nociceptive effects. *Cephalalgia* 39, 1789-1797.

Al-Karagholi, M. A. M., Ghanizada, H., Hansen, J. M., Skovgaard, L. T., Olesen, J., Larsson, H. B. W., et al. (2019b). Levcromakalim, an adenosine triphosphate-sensitive potassium channel opener, dilates extracerebral but not cerebral arteries. *Headache* 59, 1468–1480. doi: 10.1111/head.13634

Al-Karagholi, M. A.-M., Ghanizada, H., Nielsen, C. A. W., Skandarioon, C., Snellman, J., Lopez, C. L., et al. (2020d). Opening of BK_{Ca} channels alters cerebral hemodynamic and causes headache in healthy volunteers. *Cephalalgia* 40, 1145–1154. doi: 10.1177/0333102420940681

Al-Karagholi, M. A.-M, Ghanizada, H., Nielsen, C. A. W., Skandarioon, C., Snellman, J., Lopez-Lopez, C., et al. (2021b). Opening of BK_{Ca} channels causes migraine attacks. *PAIN* doi: 10.1097/j.pain.0000000000002238

Al-Karagholi, M. A. M., Ghanizada, H., Kokoti, L., Paulsen, J. S., Hansen, J. M., and Ashina, M. (2020a). Effect of KATP channel blocker glibenclamide on levcromakalim-induced headache. *Cephalalgia* 40, 1045–1054. doi: 10.1177/0333102420949863

Al-Karagholi, M. A. M., Ghanizada, H., Nielsen, C. A. W., Ansari, A., Gram, C., Younis, S., et al. (2020b). Cerebrovascular effects of glibenclamide investigated using high-resolution magnetic resonance imaging in healthy volunteers. *J. Cereb. Blood Flow Metab.* 7:271678X20959294.

Al-Karagholi, M. A. M., Hansen, J. M., Guo, S., Olesen, J., and Ashina, M. (2019c). Opening of ATP-sensitive potassium channels causes migraine attacks: a new target for the treatment of migraine. *Brain* 142, 2644–2654. doi: 10.1093/brain/awz199

Al-Karagholi, M. A. M., Hansen, J. M., Severinsen, J., Jansen-Olesen, I., and Ashina, M. (2017). The KATP channel in migraine pathophysiology: a novel therapeutic target for migraine. *J. Headache Pain* 18:90.

Ampudia-Blasco, F. J., Heinemann, L., Bender, R., Schmidt, A., Heise, T., Berger, M., et al. (1994). Comparative dose-related time-action profiles of glibenclamide and a new non-sulphonylurea drug, AG-EE 623 ZW, during euglycaemic clamp in healthy subjects. *Diabetologia* 37, 703–707. doi: 10.1007/s001250050167

Ashina, H., Schytz, H. W., and Ashina, M. (2018). CGRP in human models of primary headaches. *Cephalalgia* 38, 353–360. doi: 10.1177/0333102416684344

Ashina, H., Schytz, H. W., and Ashina, M. (2019). CGRP in human models of migraine. *Handb. Exp. Pharmacol.* 255, 109–120. doi: 10.1007/164_2018_128

Ashina, M. (2020). Migraine . *N. Engl. J. Med.* 383, 215–227.

Ashina, M., Terwindt, G. M., Al-Karagholi, M. A.-M., de Boer, I., Lee, M. J., Hay, D. L., et al. (2021). Migraine: disease characterisation, biomarkers, and precision medicine. *Lancet* 397, 1496–1504. doi: 10.1016/s0140-6736(20)32162-0

Babenko, A. P., Gonzalez, G. and Bryan, J (2000)Pharmaco-topology of sulfonylurea receptors. Separate domains of the regulatory subunits of K(ATP) channel isoforms are required for selective interaction with K(+) channel openers. *J. Biol. Chem.* 275, 717–720. doi: 10.1074/jbc.275.2.717

Bayerle-Eder, M., Wolzt, M., Langenberger, H., Pleiner, J., Rainer, G., Eichler, H. G., et al. (2000). Hypercapnia-induced cerebral and ocular vasodilation is not altered by glibenclamide in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278, 1667–1673. doi: 10.1152/ajpregu.2000.278.6.R1667

Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R., and MacIntyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313, 54–56. doi: 10.1038/313054a0

Christensen, S. L., Munro, G., Petersen, S., Shabir, A., Jansen-Olesen, I., Kristensen, D. M., et al. (2020). ATP sensitive potassium (KATP) channel inhibition: a promising new drug target for migraine. *Cephalalgia* 40, 650–664. doi: 10.1177/0333102420925513

Edvinsson, L., Haanes, K. A., and Warfvinge, K. (2018). Krause DiN. CGRP as the target of new migraine therapies - Successful translation from bench to clinic. *Nat. Rev. Neurol.* 14, 338–350. doi: 10.1038/s41582-018-0003-1

Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *J. Pain* 14, 1289–1303. doi: 10.1016/j.jpain.2013.03.010

Falkenberg, K., Rønde Bjerg, H., Yamani, N., and Olesen, J. (2020). Sumatriptan does not antagonize CGRP-induced symptoms in healthy volunteers. *Headache* 60, 665–676. doi: 10.1111/head.13747

Faraci, F. M., and Sobey, C. G. (1998). Role of potassium channels in regulation of cerebral vascular tone. *J. Cereb. Blood Flow Metab.* 18, 1047–1063. doi: 10.1097/00004647-199810000-00001

Gao, Y., Nishimura, Y., Suzuki, A., and Yoshida, K. (1994). Relaxant isolated effects small of calcitonin renal arteries in stroke-prone rats peptide on spontaneously hypertensive of rats. *J. Smooth Muscle Res.* 30, 9–19. doi: 10.1540/jsmr.30.9

Ghanizada, H., Al-Karagholi, M. A. M., Arngrim, N., Olesen, J., and Ashina, M. (2020a). PACAP27 induces migraine-like attacks in migraine patients. *Cephalalgia* 40, 57–67. doi: 10.1177/0333102419864507

Ghanizada, H., Iljazi, A., Ashina, H., Do, T. P., Al-Karagholi, M. A. M., Amin, F. M., et al. (2020b). Nocebo response in human models of migraine: a systematic review and meta-analysis of randomized, double-blind, placebo-controlled, two-way crossover trials in migraine without aura and healthy volunteers. *Cephalalgia* 41, 99–111. doi: 10.1177/0333102420970489

Gori, T., Sicuro, S., Dragoni, S., Donati, G., Forconi, S., and Parker, J. D. (2005). Sildenafil prevents endothelial dysfunction induced by ischemia and reperfusion via opening of adenosine triphosphate-sensitive potassium channels: a human in vivo study. *Circulation* 111, 742–746. doi: 10.1161/01.cir.0000155252.23933.2d

Gozalov, A., Jansen-Olesen, I., Klaerke, D., and Olesen, J. (2008). Role of KATP channels in cephalic vasodilatation induced by calcitonin gene-related peptide, nitric oxide, and transcranial electrical stimulation in the rat. *Headache* 48, 1202–1213. doi: 10.1111/j.1526-4610.2008.01205.x

Gozalov, A., Petersen, K. A., Mortensen, C., Jansen-Olesen, I., Klaerke, D., and Olesen, J. (2005). Role of KATP channels in the regulation of rat dura and pia artery diameter. *Cephalalgia* 25, 249–260. doi: 10.1111/j.1468-2982.2004.00848.x

Hansen, J. M., Hauge, A. W., Olesen, J., and Ashina, M. (2010). Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. *Cephalalgia* 30, 1179–1186. doi: 10.1177/0333102410368444

Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I., and Kurachi, Y. (2010). Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol. Rev.* 90, 291–366. doi: 10.1152/physrev.00021.2009

Hong, K. W., Yoo, S. E., Suk, S. S., Lee, J. Y., and Rhim, B. Y. (1996). Pharmacological coupling and functional role for CGRP receptors in the vasodilation of rat pial arterioles. *Am. J. Physiol.* 270, 39–31.

Hougaard, A., Younis, S., Iljazi, A., Haanes, K. A., Lindberg, U., Vestergaard, M. B., et al. (2020). Cerebrovascular effects of endothelin-1 investigated using high-resolution magnetic resonance imaging in healthy volunteers. *J. Cereb. Blood Flow Metab.* 40, 1685–1694. doi: 10.1177/0271678x19874295

Iljazi, A., Ashina, H., Zhuang, Z. A., Lopez Lopez, C., Snellman, J., Ashina, M., et al. (2020). Hypersensitivity to calcitonin gene-related peptide in chronic migraine. *Cephalalgia* 41, 701–710. doi: 10.1177/0333102420981666

Inagaki, N., Gonoi, T., Clement, I. V. J. P., Wang, C. Z., Aguilar-Bryan, L., Bryan, J., et al. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K+ channels. *Neuron* 16, 1011–1017. doi: 10.1016/s0896-6273(00)80124-5

Kageyama, M., Yanagisawa, T., and Taira, N. (1993). Calcitonin gene-related peptide relaxes porcine coronary arteries via cyclic AMP-dependent mechanisms, but not activation of ATP-sensitive potassium channels. *J. Pharmacol. Exp. Ther.* 265, 490–497.

Kazmi, S. M. S., Richards, L. M., Schrandt, C. J., Davis, M. A., and Dunn, A. K. (2015). Expanding applications, accuracy, and interpretation of laser speckle contrast imaging of cerebral blood flow. *J. Cereb. Blood Flow Metab.* 35, 1076–1084. doi: 10.1038/jcbfm.2015.84

Kitazono, T., Heistad, D. D., and Faraci, F. M. (1993). Role of ATP-sensitive K+ channels in CGRP-induced dilatation of basilar artery in vivo. *Am. J. Physiol.* 265, 581–585.

Kleppisch, T., and Nelson, M. T. (1995). ATP-sensitive K+ currents in cerebral arterial smooth muscle: pharmacological and hormonal modulation. *Am. J. Physiol.* 269, 38–35.

Kokoti, L., Al-Karagholi, M. A. M., and Ashina, M. (2020). Latest insights into the pathophysiology of migraine: the ATP-sensitive potassium channels. *Curr. Pain Headache Rep.* 24, 77.

Lassen, L. H., Haderslev, P. A., Jacobsen, V. B., Iversen, H. K., Sperling, B., and Olesen, J. (2002). CGRP may play a causative role in migraine. *Cephalalgia* 22, 54–61. doi: 10.1046/j.1468-2982.2002.00310.x

Luzi, L., and Pozza, G. (1997). Glibenclamide: an old drug with a novel mechanism of action? *Acta Diabetol.* 34, 239–244. doi: 10.1007/s005920050081

Nelson, M. T., Huang, Y., Brayden, J. E., Hescheler, J., and Standen, N. B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K+ channels. *Nature* 344, 770–773. doi: 10.1038/344770a0

Petersen, K. A., Birk, S., Lassen, L. H., Kruuse, C., Jonassen, O., Lesko, L., et al. (2005a). The CGRP-antagonist, BIBN4096BS does not affect cerebral or systemic haemodynamics in healthy volunteers. *Cephalalgia* 25, 139–147. doi: 10.1111/j.1468-2982.2004.00830.x

Petersen, K. A., Lassen, L. H., Birk, S., Lesko, L., and Olesen, J. (2005b). BIBN4096BS antagonizes human α-calcitonin gene related peptide-induced headache and extracerebral artery dilatation. *Clin Pharmacol Ther.* 77, 202–213. doi: 10.1016/j.clpt.2004.10.001

Ploug, K. B., Amrutkar, D. V., Baun, M., Ramachandran, R., Iversen, A., Lund, T. M., et al. (2012). K ATP channel openers in the trigeminovascular system. *Cephalalgia* 32, 55–65.

Ploug, K. B., Boni, L. J., Baun, M., Hay-Schmidt, A., Olesen, J., and Jansen-Olesen, I. (2008a). K ATP channel expression and pharmacological in vivo and in vitro studies of the K ATP channel blocker PNU-37883A in rat middle meningeal arteries. *Br. J. Pharmacol.* 154, 72–81. doi: 10.1038/bjp.2008.86

Ploug, K. B., Sørensen, M. A., Strøbech, L., Klaerke, D. A., Hay-Schmidt, A., Sheykhzade, M., et al. (2008b). KATP channels in pig and human intracranial arteries. *Eur. J. Pharmacol.* 601, 43–49. doi: 10.1016/j.ejphar.2008.10.041

Quayle, J. M., Bonev, A. D., Brayden, J. E., and Nelson, M. T. (1994). Calcitonin gene-related peptide activated ATP-sensitive K+ currents in rabbit arterial smooth muscle via protein kinase A. *J. Physiol.* 475, 9–13. doi: 10.1113/jphysiol.1994.sp020045

Sheykhzade, M., and Berg Nyborg, N. C. (2001). Mechanism of CGRP-induced relaxation in rat intramural coronary arteries. *Br. J. Pharmacol.* 132, 1235–1246. doi: 10.1038/sj.bjp.0703936

Stephan, D., Winkler, M., Kühner, P., Russ, U., and Quast, U. (2006). Selectivity of repaglinide and glibenclamide for the pancreatic over the cardiovascular KATP channels. *Diabetologia* 49, 2039–2048. doi: 10.1007/s00125-006-0307-3

Vincent, M., and Wang, S. (2018). Headache classification committee of the international headache society (IHS) the international classification of headache disorders, 3rd edition. *Cephalalgia* 38, 1–211. doi: 10.1177/0333102417738202

Vollesen, L. H., Guo, S., Andersen, M. R., and Ashina, M. (2019). Effect of the H 1 -antihistamine clemastine on PACAP38 induced migraine. *Cephalalgia* 39, 597–607. doi: 10.1177/0333102418798611

Williams, S., Abbott, D., Morfis, L., Manwaring, P., Diamond, T., and Howes, L. G. (1998). Effects of glibenclamide on blood pressure and cardiovascular responsiveness in non-insulin dependent diabetes mellitus. *J. Hypertens.* 16, 705–711. doi: 10.1097/00004872-199816050-00019

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Native CGRP Neuropeptide and Its Stable Analogue SAX, But Not CGRP Peptide Fragments, Inhibit Mucosal HIV-1 Transmission

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Background: The vasodilator neuropeptide calcitonin gene-related peptide (CGRP) plays both detrimental and protective roles in different pathologies. CGRP is also an essential component of the neuro-immune dialogue between nociceptors and mucosal immune cells. We previously discovered that CGRP is endowed with anti-viral activity and strongly inhibits human immunodeficiency virus type 1 (HIV-1) infection, by suppressing Langerhans cells (LCs)-mediated HIV-1 trans-infection *in-vitro* and mucosal HIV-1 transmission *ex-vivo*. This inhibition is mediated *via* activation of the CGRP receptor non-canonical NFκB/STAT4 signaling pathway that induces a variety of cooperative mechanisms. These include CGRP-mediated increase in the expression of the LC-specific pathogen recognition C-type lectin langerin and decrease in LC-T-cell conjugates formation. The clinical utility of CGRP and modalities of CGRP receptor activation, for inhibition of mucosal HIV-1 transmission, remain elusive.

Methods: We tested the capacity of CGRP to inhibit HIV-1 infection *in-vivo* in humanized mice. We further compared the anti-HIV-1 activities of full-length native CGRP, its metabolically stable analogue SAX, and several CGRP peptide fragments containing its binding C-terminal and activating N-terminal regions. These agonists were evaluated for their capacity to inhibit LCs-mediated HIV-1 trans-infection *in-vitro* and mucosal HIV-1 transmission in human mucosal tissues *ex-vivo*.

Results: A single CGRP intravaginal topical treatment of humanized mice, followed by HIV-1 vaginal challenge, transiently restricts the increase in HIV-1 plasma viral loads but maintains long-lasting higher CD4+ T-cell counts. Similarly to CGRP, SAX inhibits LCs-mediated HIV-1 trans-infection *in-vitro*, but with lower potency. This inhibition is mediated *via* CGRP receptor activation, leading to increased expression of both langerin and STAT4

in LCs. In contrast, several N-terminal and N+C-terminal bivalent CGRP peptide fragments fail to increase langerin and STAT4, and accordingly lack anti-HIV-1 activities. Finally, like CGRP, treatment of human inner foreskin tissue explants with SAX, followed by polarized inoculation with cell-associated HIV-1, completely blocks formation of LC-T-cell conjugates and HIV-1 infection of T-cells.

Conclusion: Our results show that CGRP receptor activation by full-length CGRP or SAX is required for efficient inhibition of LCs-mediated mucosal HIV-1 transmission. These findings suggest that formulations containing CGRP, SAX and/or their optimized agonists/analogues could be harnessed for HIV-1 prevention.

Keywords: CGRP, HIV-1, humanized BLT mice, Langerhans cells, SAX, STAT4

INTRODUCTION

CGRP is a 37 amino acid potent vasodilator neuropeptide secreted from peripheral sensory nerves, such as pain nociceptors, which plays important physiological and pathophysiological roles (1). The CGRP receptor is a heteromeric complex, composed of calcitonin receptor-like receptor (CLR), the transmembrane receptor activity-modifying protein 1 (RAMP1), and the intracellular receptor component protein (RCP) that is important for signaling (2). CGRP receptor antagonism has been proven effective against migraine, in which CGRP is detrimental, and several CGRP receptor antagonists and neutralizing antibodies (Abs) are used clinically (3, 4).

However, CGRP-mediated vasodilation is potentially protective, at least during hypertension and cardiovascular complications (5). Indeed, both CGRP and its long-acting metabolically stable analogue SAX (serinyl-$CGRP_{2-37}$-amide with an albumin binding fatty acid moiety in the N-terminus) (6, 7), exert protective vascular pharmacological effects *in-vitro* (7) and *in-vivo* (8). Compared to CGRP, SAX has a longer half-life (6), but decreased potency (6, 7).

CGRP also directly modulates immune function in a vasodilator-independent manner, as part of the neuro-immune dialogue between CGRP-secreting mucosa-innervating nociceptors and resident mucosal immune cells (9). For instance, nociceptors associate with LCs and CGRP shifts LCs-mediated antigen presentation and cytokine secretion from Th1 to Th2/Th17 (10).

We previously reported that LCs are the early cellular targets of HIV-1 upon its mucosal entry in the inner foreskin, and subsequently transfer infectious virus to CD4+ T-cells (11, 12) in a process termed trans-infection. We further discovered that LCs express the components of the CGRP receptor (i.e., CLR, RAMP1 and RCP) (13, 14), and that CGRP modulates a multitude of cellular processes in LCs, which cooperate together to significantly inhibit LCs-mediated HIV-1 trans-infection *in-vitro* and mucosal HIV-1 transmission *ex-vivo* (13–15). Accordingly, CGRP increases expression of the LC-specific pathogen recognition C-type lectin langerin, and facilitates efficient viral degradation by diverting HIV-1 from endo-lysosomes towards faster viral proteasomal degradation. CGRP also decreases LCs surface expression of several adhesion molecules, leading to reduced conjugate formation with CD4+ T-cells. Importantly, although CGRP activates the canonical CGRP receptor cAMP/PKA signaling pathway in LCs (16), we found that the anti-HIV-1 effects of CGRP in LCs are mediated *via* non-canonical NFκB/STAT4 signaling, as pharmacological inhibitors of both NFκB (13) and STAT4 (14) completely abrogate CGRP-induced inhibition of HIV-1 trans-infection. Based on these observations, we suggested that CGRP agonists/analogues might be useful for prevention of mucosal HIV-1 transmission.

The N-terminus (residues 1–7, containing a disulfide bond between the cysteines at positions 2 and 7) and amidated C-terminus (residues 27–37) of CGRP interact independently with the CGRP receptor in a two-domain model, whereby the C-terminus first binds the receptor, facilitating subsequent binding and activation by the N-terminus (17). The N-terminal disulfide loop is crucial for agonistic activity, as the peptide fragment $CGRP_{8-37}$ is an antagonist, and as several N-terminal peptide fragments of CGRP are low-potency agonists with anti-hypertensive function (18). Other CGRP peptide fragments, containing constrained N-terminus (i.e., truncated loop with only three residues) and/or introduced disulfide bridge in the C-terminus, yield analogues with affinities comparable to native CGRP (19).

Here we evaluated the inhibitory activity of CGRP in pre-clinical experiments, using a mucosal model of HIV-1 infection in humanized mice. We further determined the requirements of CGRP receptor activation for inhibition of HIV-1 trans-infection *in-vitro*, by comparing the anti-HIV-1 activities of full-length native CGRP, its analogue SAX, and several CGRP N-terminal fragments and N+C-terminal bivalent fragments. Finally, we compared CGRP and SAX for their capacity to inhibit mucosal HIV-1 transmission in human mucosal tissues *ex-vivo*.

MATERIALS AND METHODS

Agonists and Antagonists

We used the following molecules: CGRP (1 mM stock solution in water; Sigma), biotinylated CGRP (1 mM stock solution in water; AnaSpec), SAX (1 mM stock solution in DMSO) prepared as we described (7), custom synthesized CGRP peptide fragments

(1 mM or 10 mM stock solutions in water, for N-terminal or N+C-terminal fragments, respectively; United Biosystems), and the CGRP receptor antagonist BIBN4096 (10 mM stock solution in DMSO; Sigma).

Cells and Tissues

Peripheral blood mononuclear cells (PBMCs) from healthy HIV-1 seronegative individuals were separated from whole blood by standard Ficoll gradient. CD4+ T-cells and CD14+ monocytes were purified from PBMCs using appropriate negative magnetic selection kits (Stemcell Technologies), according to the manufacturer's instructions. Monocytes (10^6 cells/well in 12-well plates) were differentiated into monocyte-derived LCs (MDLCs) in complete RPMI medium [RPMI 1640 medium, 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco Invitrogen)], supplemented with 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/ml interleukin 4 (IL4), and 10 ng/ml transforming growth factor beta 1 (TGFβ1) (R&D systems) as described (20), and used 7–9 days after differentiation.

Normal foreskin tissues were obtained from healthy adults undergoing circumcision (Urology Service, Cochin Hospital, Paris). Human penile tissues were obtained as part of our previous study (21).

Virus and Infected Cells

Viral stocks of the HIV-1 molecular clones JRCSF and ADA, and the primary isolate 93BR029 (V29), both clade B with R5 tropism (NIH AIDS reagent program), were prepared by transfection of 293T cells or by amplification on phytohemagglutinin (PHA)/IL2-activated PBMCs, respectively, and quantified using the p24 Innotest HIV-1 ELISA (Fujirebio). HIV-1 V29-infected PBMCs were prepared as we reported (11).

CGRP and HIV-1 Infection in Mice

CGRP (10 nM, 100 nM or 1 µM) was diluted in 30 µl sterile phosphate-buffered saline (PBS), alone or in combination with 1% hydrocortisone, and applied intravaginally for 6 h in normal female BALB/c mice (10 weeks old, 25–30 g, synchronized in estrous cycle). Spleen, lymph nodes, gut, liver, kidneys, and female reproductive system were then collected, and hematoxylin and eosin stained 3 µm paraffin sections were examined for histopathological analysis. Selected slides were stained after antigen retrieval with monoclonal Abs (Bio-Rd), including rat-anti-human CD3, rat-anti-mouse B220/CD45R (clone RA3-6B2), and rat-anti-mouse F4/80 (clone CI:A31), followed by rat on rodent horseradish peroxidase (HRP)-polymer, 3,3'-diaminobenzidine (DAB) as chromogen (Biocare Medical) and counterstaining with hematoxylin. Images were acquired with the AxioCam HRc using the AxioVision System SE64 (Zeiss).

Humanized female bone-marrow/liver/thymus (BLT) mice were prepared, inoculated and examined for HIV-1 infection at the Ragon Institute Human Immune System Mouse Program, according to their established protocols (see https://www.ragoninstitute.org/research/services/humanized-mouse/). Expression of human langerin was determined by immunohistochemistry of 4 µm vaginal tissue paraffin sections as we described (12), using goat-anti-human langerin Ab (R&D), the LSAB2-HRP System with DAB as substrate (Dako) and counterstaining with hematoxylin. For infection experiments, CGRP (500 nM or 5 µM) in 30 µl sterile PBS or PBS alone were topically applied onto the vaginal epithelium for 4 h, followed by topical vaginal challenge with 2×10^4 $TCID_{50}$ HIV-1 JRCSF.

HIV-1 Trans-Infection and Langerin Expression

MDLCs (10^5/well in 96 round-bottom well plates) were treated for 24 h at 37°C (200 µl/well final) with the indicated molar concentrations of CGRP, SAX or CGRP fragments. The CGRP receptor antagonist BIBN4096 was added 15 min before agonists. For langerin surface expression, MDLCs were washed and stained for 20 min on ice in a final volume of 50 µl PBS with a phycoerythrin (PE)-conjugated mouse monoclonal Ab against human langerin (clone DCGM4, Beckman Coulter), or matched isotype control. For HIV-1 trans-infection, MDLCs were washed and pulsed with HIV-1 ADA (1ng p24 corresponding to multiplicity of infection of 0.2) for 4 h. MDLCs were next incubated with autologous CD4+ T-cells or with green fluorescent protein (GFP)-reporter T-cells, and HIV-1 replication was measured in the co-culture supernatants using p24 ELISA (Fujirebio) or by evaluating GFP fluorescent by flow cytometry, as we described (13–15). Fluorescent profiles were acquired using a Guava easyCite and analyzed with the InCyte software (Merck-Millipore).

STAT4 Western Blot (WB)

For PBMCs, cells (2×10^6/sample) were activated with PHA (5 µg/ml) + IL2 (100 U/ml) for 48 h at 37°C, serum-starved overnight, and stimulated for 30 min at 37°C with either IL12 (10 ng/ml; R&D systems) or interferon alpha (IFNα, 5×10^4 U/ml; pbl Assay Science). For MDLCs, cells (2×10^6/sample) were re-suspended in complete RPMI medium without cytokines and rested overnight at 37°C. MDLCs were next treated for 24 h at 37°C with CGRP (0.1 µM), SAX (0.1 µM), $CGRP_{1-8}$ (10 µM), or lipopolysaccharide (LPS, 10 µg/ml). The CGRP receptor antagonist BIBN4096 (1 µM) was added 15 min before agonists. The cells were then washed and stimulated for 30 min at 37°C with combination of IL12 + IFNα at the concentrations indicated above. PBMCs/MDLCs were subsequently lysed for 30 min on ice with 100 µl lysis buffer [50 mM Tris buffer pH = 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X100, 0.1% SDS, 1:100 dilutions of phosphatase inhibitors II/III and protease inhibitor cocktail (Sigma)], followed by three cycles of 10 s vortex and 10 min incubation on ice. Lysates were centrifuged for 10 min at 4°C/13,200 rpm, and supernatants were collected and stored at −80°C. Protein contents in cell lysates were quantified using the BCA kit (Thermo Fisher) according to the manufacturer's instructions, and 20 µg proteins were mixed with loading buffer (100 mM Tris pH 7.2, 5% β-mercaptoethanol, 12% glycerol, 5 mM EDTA, 5% SDS, 0.01% bromophenol blue), heated for 5 min at 95°C, run over a 10% SDS-PAGE, and transferred onto nitrocellulose membranes.

Blocking was performed for 1 h at room temperature with blocking buffer (Tris-buffered saline (TBS), 0.5% Tween 20, and 0.5% dry milk). The blots were next incubated overnight at 4°C with commercial rabbit polyclonal Abs suitable for WB, directed against human STAT4 (Proteintech #13028-1AP, 0.5 µg/ml) or phosphorylated STAT4 (pSTAT4; R&D systems, #AF4319, 1 µg/ml), followed by 1:1,000 dilution of HRP-conjugated donkey-anti-rabbit IgG Ab (Southern Biotech) for 1 h at room temperature. Loading control was verified by incubation with goat polyclonal Ab to beta actin (abcam, 0.4 µg/ml), followed by 1:5,000 dilution of HRP-conjugated donkey-anti-goat IgG Ab (Promega). All Abs were diluted in blocking buffer, and pre-stained SDS-PAGE standard markers (ThermoFisher) were applied to determine molecular weights. Revelation was performed for 1–10 s with ECL-Prime chemiluminescence detection kit (Amersham). Images were acquired with the Fusion FX camera platform (Vilber Lournmat) and protein expression was quantified with ImageJ software (NIH).

CGRP Entry and HIV-1 Transmission in Human Mucosal Tissue Explants

Polarized penile *fossa navicularis* tissue explants were prepared as we previously described (21), and exposed for 3 h to 500 nM or 5 µM biotinylated CGRP in 100 µl RPMI 1640 added to the apical side. After incubation, tissue penetration of biotinylated CGRP was examined by histochemistry of 4 µm paraffin sections as we described (21), using HRP-coupled streptavidin (Vector), followed by the red 3-amino-9-ethylcarbazole (AEC) HRP substrate (Dako) and counterstaining with hematoxylin. Images were acquired with an Olympus BX63F microscope using MetaMorph (Molecular Devices) and analyzed with ImageJ software.

For infection experiments, round (8 mm diameter) inner foreskin tissue pieces were placed in 24-well plates and incubated submerged for 24 h at 37°C with 1 ml complete RPMI medium, alone or supplemented with CGRP or SAX (1 µM, four explants per condition). The tissues were next washed, transferred to two-chamber transwell inserts (Sigma), and inoculated in a polarized manner for 4 h at 37°c with either non-infected or HIV-1 (V29)-infected PBMCs (in duplicates), as we described (11, 12).

Epidermal cell suspensions were prepared immediately after inoculation, using enzymatic digestion with dispase/trypsin as we described (11, 12). Pooled cells of each duplicate were resuspended in PBS, transferred to 96 round-bottom well plates and stained for 30 min on ice with 10 µl of fluorescein isothiocyanate (FITC)-conjugated mouse-anti-human CD1a, PE-conjugated mouse-anti-human CD8 and allophycocyanin (APC)-conjugated mouse-anti-human CD3 (BD Pharmingen) Abs, diluted in PBS in a final volume of 50 µl/well.

Dermal cell suspensions were prepared following washing of the explants, additional incubation for three days at 37°C submerged in 1 ml fresh medium, and subsequent enzymatic digestion with collagenase/DNase as we described (11, 12). Pooled cells of each duplicate were surface stained as above using FITC-conjugated mouse-anti-human CD3 Ab (Pharmingen), fixed, permeabilized, and stained for 30 min at room temperature with 1:160 dilution of PE-conjugated mouse-anti-human Ab to HIV-1 p24 and core antigens (Beckman Coulter). Fluorescent profiles were recorded using a Guava easyCyte and InCyte software.

Data and Statistical Analysis

Data was analyzed using Prism software (GraphPad). Concentration-response curves were analyzed with the [log (agonist) vs. response (three parameters)] model for langrin upregulation and the [log(inhibitor) vs. normalized response – variable slope] model for HIV-1 trans-infection inhibition. The -log molar concentrations of agonists generating 50% response represented potencies (i.e., pEC_{50} and pIC_{50}). Statistical significance was analyzed with the two-tailed Student's t-test.

RESULTS

CGRP Limits HIV-1 Infection *In-Vivo*

To test for the possible clinical utility of CGRP receptor agonism, we investigated the effects of CGRP in normal mice and in a mucosal model of HIV-1 infection in humanized mice.

First, as CGRP mediates vasodilator-dependent neurogenic inflammation that can result in immune cell recruitment, we topically applied CGRP onto the vagina of normal BALB/c mice for 6 h and examined potential toxicity and immune cells modulation. These experiments showed that CGRP, tested at 10 nM, 100 nM or 1µM, did not induce signs of toxicity and did not induce overt inflammation. Of note, in our routine experiments, treatment of MDLCs *in-vitro* with up to 10 µM CGRP for 24 h did not affect cell viability. At its highest concentration tested *in-vivo* of 1 µM, CGRP did not modify the distribution and/or density of T cells, B cells, and macrophages, neither in the epithelium nor in the stroma (**Figure 1A**).

Second, we used humanized BLT mice, which are suitable to study many aspects of HIV-1 infection, prevention and mucosal/vaginal transmission (22). We confirmed the reconstitution and presence of human langerin-expressing LCs within the vaginal epithelium (**Figure 1B**). We then topically applied CGRP onto the vagina of BLT mice for 4 h, followed by a vaginal challenge with high dose of cell-free HIV-1, as routinely used in this model (23). Such cell-free viral challenge permits to achieve productive infection, which would have been obtained using a much lower inoculum of cell-associated HIV-1 [i.e., that is transmitted more efficiently due to the formation of viral synapses between cell-associated HIV-1 and apical epithelial cells, leading to polarized budding of HIV-1, such as we reported in the inner foreskin (11, 12)]. Subsequently, we sampled blood at different time points for quantification of HIV-1 viral loads and CD4+ T-cell counts (**Figure 1C**).

These experiments showed that a single CGRP application dose dependently and significantly restricted the increase in plasma viral loads at two early time points (weeks 4 and 6, **Figure 1D**). CGRP treatment also significantly maintained

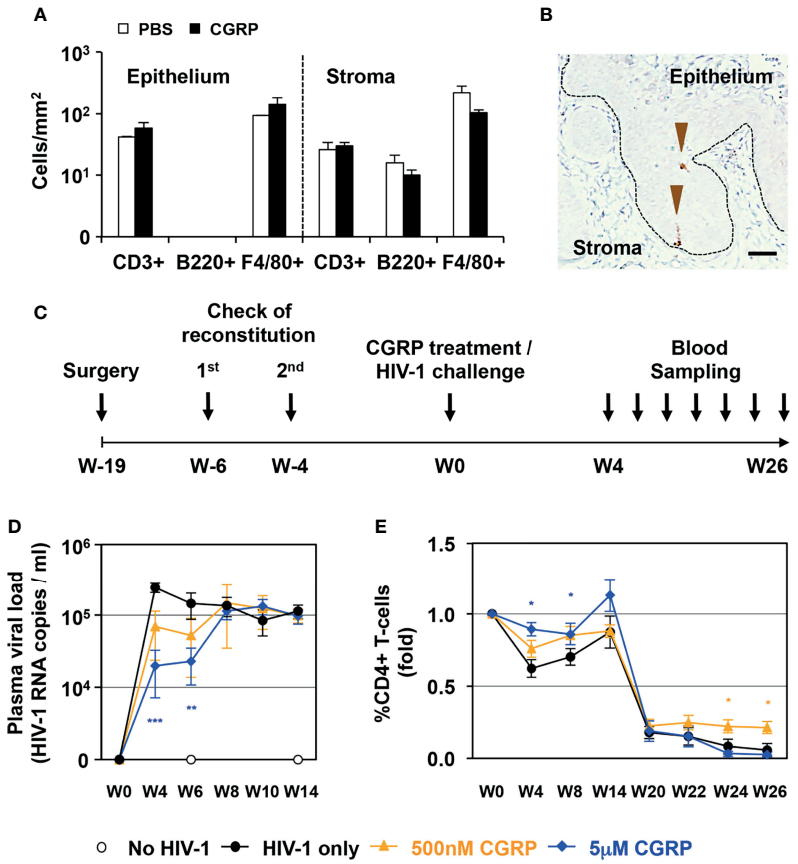

FIGURE 1 | CGRP limits mucosal HIV-1 transmission *in-vivo*. **(A)** CGRP (1 μM) or PBS were topically applied intravaginally in normal female BALB/c mice (n = 3 animals per group). Vaginal tissue sections were examined by immunohistochemistry for the presence of CD3+ T-cells, B220+ B cells and F4/80+ macrophages. Shown are mean ± SEM cell densities expressed as cells/mm^2 (of n = 3 independent experiments) in either the vaginal epithelium or stroma. **(B)** Representative image of the vaginal tissue of humanized BLT mice, showing expression of human langerin (arrowheads) in the epithelium; broken line denotes the basement membrane and scale bar = 20 μm. **(C)** Experimental schedule for preparing humanized female BLT mice, intravaginally applying CGRP or PBS followed by vaginal challenge with high-dose HIV-1 JRCSF, and subsequent blood sampling for measurement of plasma viral loads and CD4+ T-cells counts. **(D, E)** Shown are mean ± SEM (n = 5 BLT mice per group) of plasma viral loads (HIV-1 RNA copies/ml) or CD4+ T-cells percentages (fold). *p < 0.0500, **p < 0.0050, ***p < 0.0005; two-sided Student's t-test.

higher CD4+ T-cells counts, both at the same early time points and also at the latest time points examined (weeks 24 and 26, **Figure 1E**).

These results show no signs of local CGRP-mediated toxicity *in-vivo*. In addition, CGRP exerts transient protection against the increase in HIV-1 viral loads, but long-lasting maintenance of higher CD4+ T-cell counts in HIV-1-infected BLT mice, providing proof-of-concept for the utility of CGRP *in-vivo*.

CGRP and SAX, But Not CGRP Peptide Fragments, Inhibit HIV-1 Trans-Infection and Increase Langerin Surface Expression

To further determine the functional activities of CGRP receptor agonists, we compared the anti-HIV-1 inhibitory potential of CGRP, SAX, and several CGRP peptide fragments (**Figure 2**). As several CGRP N-terminal fragments are biologically active and exert anti-hypertensive functions (24), we tested the previously described $CGRP_{1-8}$ (18) and $CGRP_{1-18}$ (19) fragments, as well as the negative control mutated $[Ala^2]CGRP_{1-18}$ fragment (**Figure 2**, left). We also designed novel bivalent CGRP peptide fragments (**Figure 2**, right), by linking the previously described constrained CGRP N- and C-terminal regions (19), containing disulfide bonds either at both N/C-terminal regions or only at the N-terminal, with a tri-glycine spacer. These bivalent fragments were termed according to the number of their cystetin residues, namely 4C, 2C and the control $2C_{lin}$ devoid of disulfide bonds.

MDLCs were treated for 24 h with CGRP, SAX or the different CGRP peptide fragments, pulsed with HIV-1, washed and co-cultured with autologous or GFP-reporter CD4+ T-cells. HIV-1 replication was next determined by measuring the content of the HIV-1 capsid protein p24 in the co-culture supernatant by ELISA or by evaluating GFP fluorescence using flow cytometry. In line with our previous results (13–15), CGRP strongly inhibited MDLCs-mediated HIV-1 trans-infection in a dose-dependent manner (**Figure 3A**). SAX also significantly inhibited HIV-1 trans-infection in a dose-dependent manner (**Figure 3A**), but had

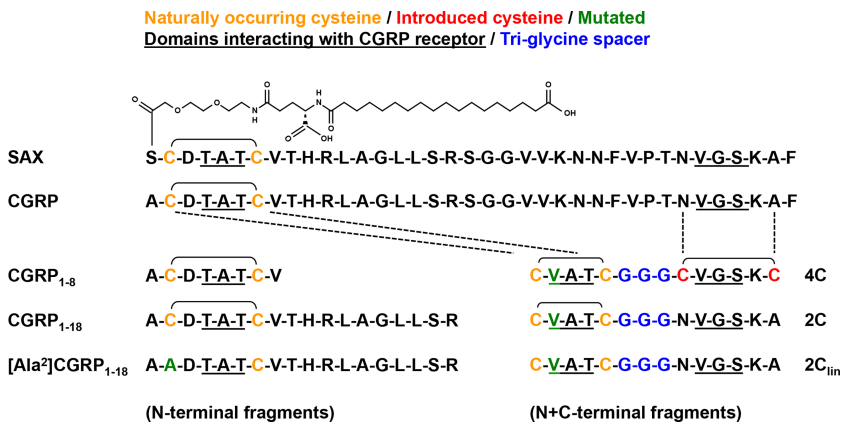

FIGURE 2 | Sequences of CGRP, SAX and CGRP fragments. Amino acid sequences of the different agonists used in the current study, including full-length native CGRP; the long-acting metabolically stable CGRP analogue SAX; the N-terminal fragments $CGRP_{1-8}$, $CGRP_{1-18}$, and control mutated $[Ala^2]CGRP_{1-18}$; the bivalent N+C-terminal fragments 4C, 2C, and control $2C_{lin}$ lacking disulfide bonds.

lower potency than CGRP, with pIC_{50} values [95% confidence intervals (CIs)] of 8.9 [9.9–7.9] compared to 10.2 [10.9–9.4], respectively. Of note, vehicle control for SAX treatment, i.e., 0.1% DMSO, had no significant effect. These inhibitory effects were mediated *via* activation of the CGRP receptor, as pre-incubation with the CGRP receptor antagonist BIBN4096 completely abrogated both CGRP- and SAX-mediated inhibition (**Figure 3B**). In contrast, none of the CGRP N-terminal (**Figure 3C**) and bivalent (**Figure 3D**) fragments significantly inhibited MDLCs-mediated HIV-1 trans-infection.

We previously showed that one of the functional effects of CGRP during inhibition of HIV-1 trans-infection is related to upregulation of langerin surface expression in LCs (13–15). MDLCs were therefore treated with CGRP, SAX or CGRP peptide fragments for 24 h, and langerin surface expression was evaluated by flow cytometry. These experiments showed that both CGRP and SAX increased langerin expression in MDLCs in a dose-dependent manner (**Figure 4A**). As for inhibition of MDLC-mediated HIV-1 trans-infection, SAX had lower potency than CGRP, with pEC_{50} values of 8.3 [9.6–6.5] compared to 10.6 [11.5–9.7], respectively. Langerin upregulation was mediated *via* CGRP receptor activation, as the CGRP receptor antagonist BIBN4096 completely abrogated CGRP- and SAX-mediated increase in langerin expression (**Figure 4B**). In contrast, all CGRP fragments lacked agonistic activity and did not significantly increase langerin surface expression (**Figures 4C, D**).

These results show that CGRP and SAX inhibit HIV-1 trans-infection, which correlate with their ability to activate the CGRP receptor and increase langerin expression in MDLCs. In contrast, CGRP peptide fragments fail to increase langerin and inhibit HIV-1 trans-infection.

CGRP and SAX, But Not $CGRP_{1–8}$, Increase STAT4 Expression

We previously discovered that CGRP inhibits MDLCs-mediated HIV-1 trans-infection *via* STAT4 (14), and therefore performed WB experiments to quantify STAT4 levels directly. Using activated PBMCs as positive control, we first confirmed the suitability of our Abs for detection of total STAT4 following cell activation (**Figure 5A** showing PBMCs from one representative individual of n = 4 tested; **Supplementary Figure 1** showing PBMCs from two of the additional individuals), as well as pSTAT4 following cell activation and additional 30 min cytokine stimulation (**Figure 5B**), using IL12 and IFNα that induce STAT4 phosphorylation (25). Next, we measured total STAT4 in MDLCs treated with CGRP, SAX or $CGRP_{1–8}$, as well as with LPS as positive control [i.e., LPS increases STAT4 in dendritic cells (26)]. These experiments showed that like LPS, both CGRP and SAX significantly increased total STAT4 expression (**Figures 5C, D**). In contrast, the CGRP N-terminal fragment $CGRP_{1–8}$ failed to increase STAT4 expression, which remained comparable to that in untreated MDLCs (**Figures 5C, D**). As for HIV-1 trans-infection and langerin expression described above, the CGRP receptor antagonist BIBN4096 abrogated CGRP- and SAX-mediated increase in STAT4 (**Figure 5E**). Finally, we confirmed that CGRP- and SAX-induced increased STAT4 was functional, as it could be readily phosphorylated upon subsequent cytokine stimulation with IL12 + IFNα (**Figure 5F**).

These results show that CGRP and SAX, but not $CGRP_{1–8}$, increase expression of STAT4 that is implicated in inhibition of HIV-1 trans-infection in MDLCs.

CGRP and SAX Inhibit Mucosal HIV-1 Transmission in Human Mucosal Tissues *Ex-Vivo*

We further tested the anti-HIV-1 activities of CGRP and SAX using our previously described models of human penile and inner foreskin tissue explants (11, 21). In these models, small pieces of human mucosal tissues are placed in two-chamber transwell inserts, and hollow cloning ring cylinders are adhered to their apical side using surgical glue, permitting for subsequent

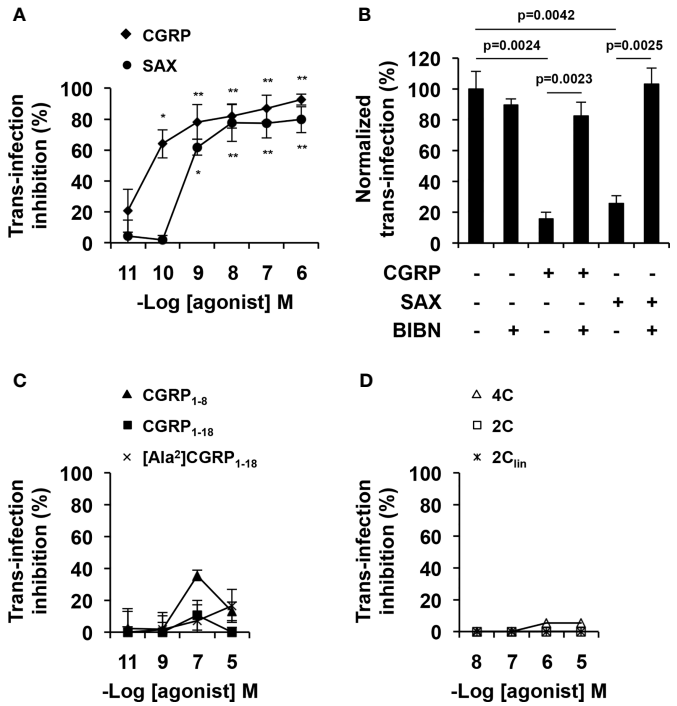

FIGURE 3 | CGRP and SAX, but not CGRP peptide fragments, inhibit MDLCs-mediated HIV-1 trans-infection. MDLCs were left untreated or treated for 24 h with the indicated molar concentrations of CGRP, SAX or CGRP peptide fragments. In panel **(B)**, the CGRP receptor antagonist BIBN4096 (BIBN, 1 μM) was added 15 min before addition of agonists (0.1 μM). The cells were then pulsed with HIV-1 ADA for 4 h, washed, and incubated for 7 days with autologous CD4+ T-cells **(A–C)** or 3 days with HIV-1 GFP-reporter T-cells **(D)**. HIV-1 trans-infection and replication in T-cells was evaluated by p24 ELISA in the co-culture supernatants or by GFP expression and flow cytometry. Shown are mean- ± SEM percentages of HIV-1 trans-infection inhibition **(A, C, D)** or normalized **(B)**, derived from n = 5 **(A)** and n = 3 **(B–D)** independent experiments using MDLCs from different individuals. *p < 0.0500, **p < 0.0050, two-sided Student's t-test.

polarized exposure to HIV-1 that mimics viral transmission *in-vivo*. Of note, HIV-1 entry in these mucosal sites is induced by polarized exposure to HIV-1-infected cells, which form viral synapses with apical epithelial cells that lead to polarized HIV-1 budding. In contrast, cell-free HIV-1 inefficiently enters these epithelia (11, 21).

To test for CGRP mucosal penetration, we prepared tissue explants from the stratified and non-keratinized penile *fossa navicularis* region that structurally resembles the vaginal epithelium, and added biotinylated CGRP to the apical side for 3 h, followed by histochemistry. These experiments showed that CGRP readily penetrated the epithelium, but not the stroma (**Figure 6A**).

We next pre-treated inner foreskin tissue explants for 24 h with CGRP or SAX at 1 μM, i.e., their molar concentration inducing maximal responses at similar efficiencies *in-vitro* (see **Figures 3A** and **4A**). Explants were next inoculated in a polarized manner with non-infected or HIV-1-infected PBMCs for 4 h. Epidermal cell suspensions were then immediately prepared and the percentages of high forward scatter (FSC) conjugates between LCs and T-cells were determined by flow cytometry. Of note, we focused on $CD1a^{high}$ cells that represent the LC1 population, as $CD1a^{+}$ cells in the inner foreskin include both the LC2 population and epidermal dendritic cells (27–29). In agreement with our previous results (11, 12), polarized exposure to HIV-1-infected PBMCs increased the percentages of $FSC^{high}CD1a^{high}CD3^{+}CD8^{-}$ conjugates (**Figure 6B**). As before, CGRP pre-treatment completely abrogated this increase (13), and a similar complete inhibitory effect was observed following SAX pre-treatment (**Figure 6C**).

In other experiments, dermal cell suspensions were prepared following additional incubation of explants in fresh medium for three days, and the percentages of HIV-1-infected T-cells were determined by flow cytometry. These experiments confirmed that polarized exposure to HIV-1-infected PBMCs resulted in HIV-1 infection of a small proportion of T-cells in the dermis (**Figure 6D**), and that CGRP completely blocked such infection (**Figure 6E**), as we reported (13). Similarly, SAX pre-treatment resulted in undetectable levels of HIV-1 $p24^{+}CD3^{+}$ dermal T-cells (**Figure 6E**).

These results indicate that both CGRP and SAX are highly effective in preventing mucosal HIV-1 transmission and infection within human mucosal tissues *ex-vivo*.

DISCUSSION

In the present study, we determined the requirements and utility of CGRP receptor activation, by CGRP receptor agonists, for the

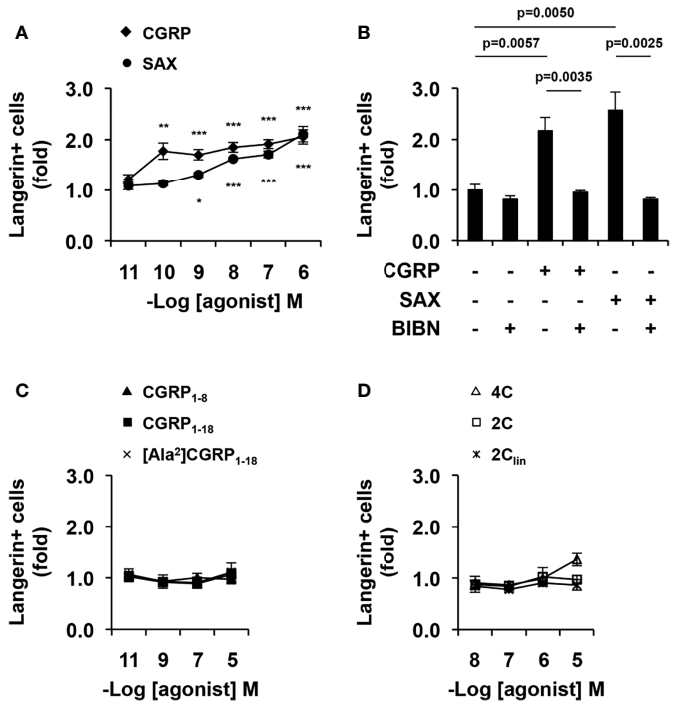

FIGURE 4 | CGRP and SAX, but not CGRP peptide fragments, increase langerin expression in MDLCs. MDLCs were left untreated or treated for 24 h with the indicated molar concentrations of CGRP, SAX or CGRP peptide fragments. In panel **(B)**, the CGRP receptor antagonist BIBN4096 (BIBN, 1 μM) was added 15 min before addition of agonists (0.1 μM). The cells were then stained for surface langerin and examined by flow cytometry. Shown are mean ± SEM folds increase in the percentages of langerin+ cells, derived from n = 5 **(A, C)**, n = 4 **(B)** and n = 3 **(D)** independent experiments using MDLCs from different individuals. *p < 0.0500, **p < 0.0050, ***p < 0.0005, two-sided Student's t-test.

inhibition of mucosal HIV-1 transmission. These findings are schematically summarized in **Figure 7**.

Our results show that in order to block langerin-mediated HIV-1 trans-infection in MDLCs, CGRP receptor activation requires full-length CGRP or SAX, in contrast to CGRP peptide fragments that are ineffective. While SAX has >10-fold lower potency than CGRP, both agonists are similarly effective at their highest micromolar concentrations tested. We speculate that CGRP peptide fragments, previously reported to be functional (18, 19, 24), are ineffective in our experimental settings due to potential 'biased signaling' (30) across the CGRP receptor, which similar to other G-protein coupled receptors, activates multiple downstream signaling pathways (31). Hence, compared to the full-length native CGRP ligand and the analogue SAX, CGRP fragments might have allosteric bias for preferential activation of particular signaling pathways, which are not the ones mediating inhibition of HIV-1 trans-infection. In support of this hypothesis, our results show that activation of the CGRP receptor by CGRP and SAX results in an increased expression of langerin and STAT4, which are involved in the inhibition of HIV-1 trans-infection (14). In contrast, $CGRP_{1-8}$ fails to increase langerin and STAT4 expression, and accordingly lacks anti-HIV-1 inhibitory activity. We also speculate that our bivalent CGRP peptide fragments might require further optimization to be rendered functional. For instance, the N+C-terminal fragments could be re-designed to better fit into the CGRP receptor binding pockets with higher affinity, by using longer, different and/or more flexible spacer regions.

In the current study we used our previously described model of human inner foreskin tissue explants *ex-vivo*, which represents an early snapshot of mucosal HIV-1 entry (11, 12). In this model, polarized exposure to cell-associated HIV-1 increases the formation of LC-T-cell conjugates in the epithelium (11, 12). Our results show that CGRP penetration is restricted to the epithelium, and that both CGRP and SAX completely inhibit the increase in LC-T-cell conjugates formation mediated by cell-associated HIV-1. Importantly, although CD4+ T-cells express the CGRP receptor and are CGRP-responsive (32, 33), we previously showed that CGRP treatment of CD4+ T-cells has no effect on HIV-1 trans-infection (13). We therefore speculate that CGRP- and SAX-mediated inhibition of CD4+ T-cells infection with HIV-1, which we further observed *ex-vivo*, is mediated by CGRP and SAX acting on inner foreskin LCs and reducing their capacity to disseminate HIV-1 to CD4+ T-cells across cellular conjugates.

We also tested the effects of CGRP *in-vivo* in normal and humanized BLT mice. The latter represents a complimentary

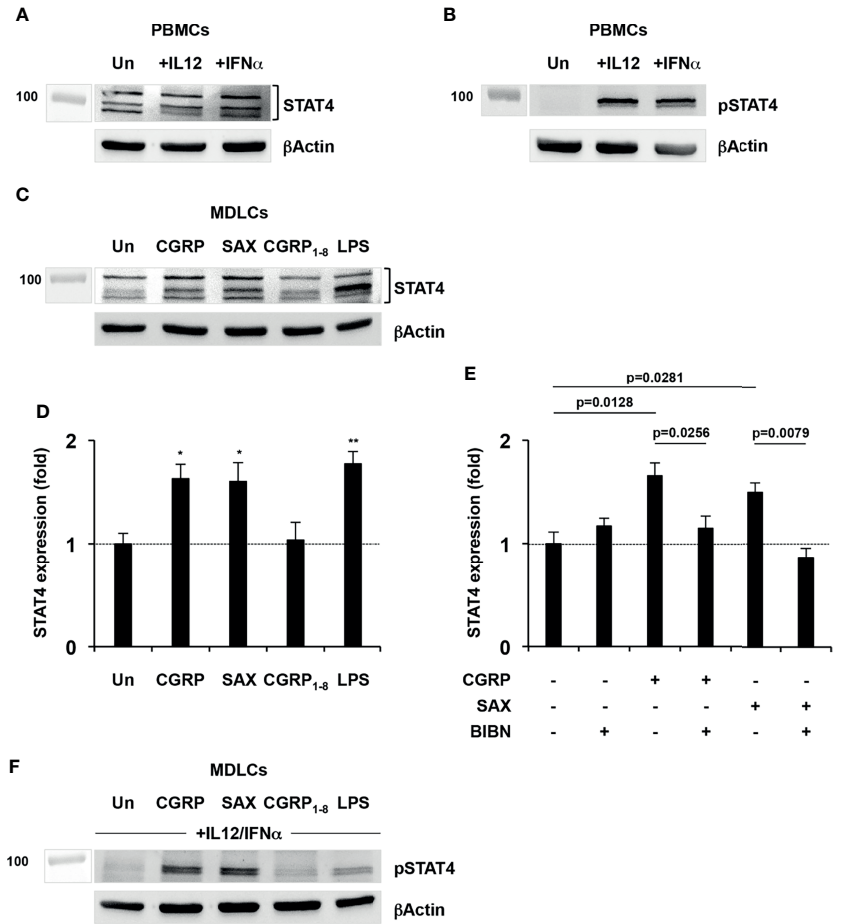

FIGURE 5 | CGRP and SAX, but not $CGRP_{1-8}$, increase STAT4 expression in MDLCs. **(A, B)** PHA/IL2-activated PBMCs were serum-starved overnight at 37°C, and left untreated (Un) or stimulated for 30 min with either IL12 or IFNα. Shown are representative Western blots (of n = 4 independent experiments using PBMCs from different individuals) of total STAT4 **(A)** and pSTAT4 **(B)** expression. **(C, D)** MDLCs were cytokine-starved overnight at 37°C, and treated with CGRP (0.1 μM), SAX (0.1 μM), $CGRP_{1-8}$ (10 μM) or LPS (10 μg/ml) as positive control. In panel **(C)**, shown is a representative Western blot (of n = 4 independent experiments using MDLCs from different individuals) of total STAT4 expression. In panel **(D)**, shown are mean ± SEM folds expression of total STAT4, normalized to that of beta actin. *p < 0.0500, **p < 0.0050, two-sided Student's t-test. **(E)** MDLCs were cytokine-starved overnight at 37°C and treated with CGRP (0.1 μM) or SAX (0.1 μM). The CGRP receptor antagonist BIBN4096 (BIBN, 1 μM) was added 15 min before addition of agonists. Shown are mean ± SEM (of n = 4 independent experiments using MDLCs from different individuals) folds expression of total STAT4, normalized to that of beta actin. **(F)** MDLCs were treated as described in panels **(C, D)** above, and further stimulated for 30 min with combination of IL12 + IFNα. Shown is a representative Western blot (of n = 4 independent experiments using MDLCs from different individuals) of pSTAT4 expression.

model to our *ex-vivo* tissue explants, as it permits to follow mucosal HIV-1 transmission over time. By combining the results obtained in these different models, we propose potential explanations for CGRP-mediated long-term maintenance of CD4+ T-cells *in-vivo*. Hence, CGRP transiently controls the increase in viral loads and could inhibit the previously reported process of HIV-1 dissemination from vaginal LCs to CD4+ T-cells (34). In turn, these effects would result in limited HIV-1 infection and elimination of CD4+ T-cells, permitting their long-term maintenance in the BLT model. In contrast, CGRP-mediated effects are not mediated *via* T-cells recruitment (i.e., as we observed in normal mice), or direct inhibition of CD4+ T-cells infection with HIV-1 [that is one of the reported mechanisms mediating HIV-1 transmission in the vagina (34). Interestingly, both CGRP and SAX completely block HIV-1 transmission in inner foreskin tissue explants *ex-vivo*, but CGRP exerts only partial protection in BLT mice *in-vivo*. Such differences might be related to the duration of agonist pre-treatment, i.e., 24 h in tissue explants vs. 6 h in BLT mice.

Pre-exposure prophylaxis (PrEP) is currently available and is highly effective for the prevention of HIV-1 transmission. Yet, important barriers still limit PrEP efficacy and usage, such as adherence, cost, access, stigma, adverse side effects, and drug resistance (35). Therefore, alternative approaches are being developed to increase the range of biomedical HIV-1 prevention options, such as long-acting injectable formulations,

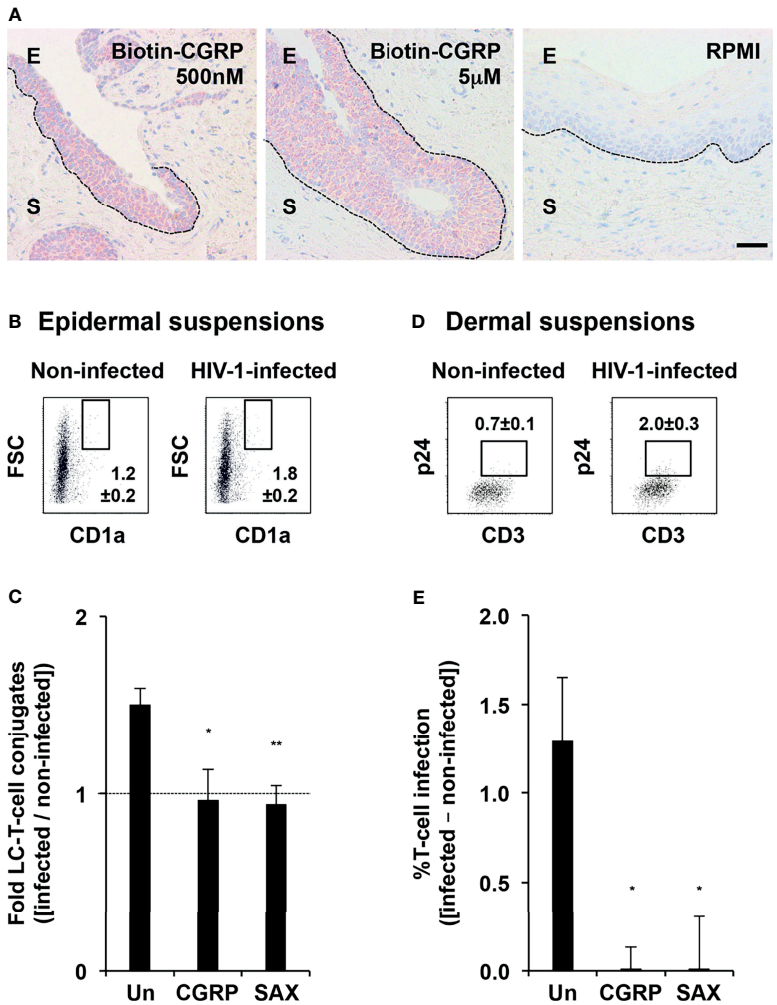

FIGURE 6 | CGRP and SAX inhibit mucosal HIV-1 transmission in human mucosal tissues *ex-vivo*. **(A)** Entry of biotinylated CGRP into the epithelium of penile *fossa navicularis* explants, revealed with streptavidin-HRP, AEC peroxidase substrate (red), and hematoxyline counterstaining (blue). Images are representative of n = 3 tissues; broken lines denote the basement membranes and scale bar = 20 μm; E, epithelium and S, stroma. **(B, C)** Inner foreskin tissue explants were left untreated or pre-treated with CGRP or SAX (1 μM) for 24 h at 37°C. Explants were next inoculated in a polarized manner with either non-infected or HIV-1-infected PBMCs for 4 h, and immediately digested with dispase/trypsin. In panel **(B)**, shown are representative flow cytometry dot plots of epidermal cell suspensions triple stained for surface expression of CD3, CD8, and CD1a and examined by flow cytometry. Cells were gated on CD3+CD8- T-cells, and numbers represent mean ± SEM (of n = 4 independent experiments using tissues from different individuals) percentages of $FSC^{high}CD1a^{high}$ conjugates following inoculation with either non-infected or HIV-1-infected PBMCs. In panel **(C)**, graph shows mean ± SEM folds increase in conjugate percentages, calculated as [(% conjugates following inoculation with HIV-1-infected PBMCs)/(% conjugates following inoculation with non-infected PBMCs)]. *p = 0.0252 and **p = 0.0071 for CGRP or SAX vs. untreated, two-sided Student's t-test. **(D, E)** Other explants were further incubated for additional three days and digested with collagenase/DNase. In panel **(D)**, shown are representative flow cytometry dot plots of dermal cell suspensions double stained for surface CD3 and intracellular p24 and examined by flow cytometry. Cells were gated on $FSC^{low}SSC^{low}$ lymphocytes, and numbers represent mean ± SEM (n = 4) percentages of CD3+p24+ cells following inoculation with either non-infected or HIV-1-infected PBMCs. In panel **(E)**, graph shows mean ± SEM percentages of HIV-1-infected T-cells, calculated as [(%CD3+p24+ cells following inoculation with HIV-1-infected PBMCs) – (%CD3+p24+ cells following inoculation with non-infected PBMCs)]; *p = 0.0249 and 0.0209 for CGRP or SAX vs. untreated, two-sided Student's t-test.

broadly neutralizing Abs, vaginal rings, implants, dermal patches, and topical microbicides (36).

Collectively, our results provide proof-of-concept that CGRP receptor agonists are useful in blocking HIV-1 transmission in complex mucosal settings. We suggest that in order to achieve better and long-lasting viremia and CD4+ T-cell control, treatment with CGRP receptor agonists should be longer, with repeated and continuous applications. In parallel, novel HIV-1 prophylactic formulations/devices could be developed, which would permit a slow release of optimized agonists of CGRP and/or higher potency mucosal metabolically stable derivatives. As such, HIV-1 infection should be included within the different pathologies and inflammatory conditions, in which CGRP is beneficial and could be harnessed to exert protective clinical effects.

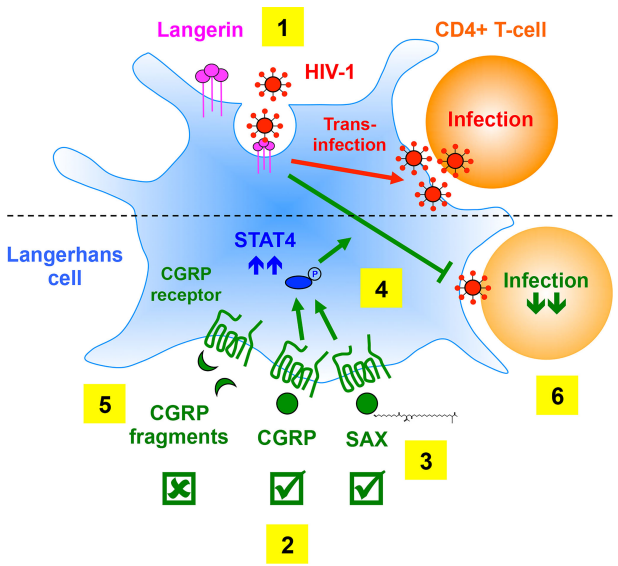

FIGURE 7 | Summary of the requirements of CGRP receptor activation for inhibition of mucosal HIV-1 transmission. **(1)** In LCs, HIV-1 binding to langerin induces viral internalization and subsequent degradation, while virions escaping degradation trans-infect CD4+ T-cells. **(2)** We previously showed that CGRP activates its cognate receptor expressed by LCs and affects a multitude of cellular and molecular process (not shown), resulting in significant inhibition of mucosal HIV-1 trans-infection *in-vitro* and *ex-vivo*. **(3)** We show in the present study that SAX, a long-acting metabolically stable analogue of CGRP, also activates the CGRP receptor. **(4)** Both CGRP and SAX increase expression of langerin (not shown) and STAT4 (that can be readily phosphorylated upon subsequent cytokine stimulation), which result in inhibition of HIV-1 trans-infection *in-vitro* and *ex-vivo*. **(5)** In contrast, several CGRP peptide fragments fail to activate the CGRP receptor and to increase langerin/STAT4 expression, and accordingly lack anti-HIV-1 activity. **(6)** CGRP-mediated inhibition of HIV-1 dissemination from LCs to CD4+ T-cells might permit their long-term maintenance in the BLT model of mucosal HIV-1 infection *in-vivo*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comités de Protection des Personnes (CPP Paris-IdF XI, N.11016). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the institutional review board of the San Raffaele Scientific Institute (IACUC no. 599).

AUTHOR CONTRIBUTIONS

YG and MB conceived the study and designed the experiments. YG, JM, EC, and ASe performed the experiments. ASa provided SAX and its experimental requirements. GS, FS, and LL designed and performed experiments using normal mice. ND and MZ provided foreskin tissues. YG wrote the paper. All authors made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.785072/full#supplementary-material

REFERENCES

1. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. *Physiol Rev* (2014) 94:1099–142. doi: 10.1152/physrev.00034.2013
2. Hay DL, Garelja ML, Poyner DR, Walker CS. Update on the Pharmacology of Calcitonin/CGRP Family of Peptides: IUPHAR Review 25. *Br J Pharmacol* (2018) 175:3–17. doi: 10.1111/bph.14075
3. Moore EL, Salvatore CA. Targeting a Family B GPCR/RAMP Receptor Complex: CGRP Receptor Antagonists and Migraine. *Br J Pharmacol* (2012) 166:66–78. doi: 10.1111/j.1476-5381.2011.01633.x
4. Tso AR, Goadsby PJ. Anti-CGRP Monoclonal Antibodies: The Next Era of Migraine Prevention? *Curr Treat Options Neurol* (2017) 19:27. doi: 10.1007/s11940-017-0463-4
5. Kumar A, Potts JD, Dipette DJ. Protective Role of Alpha-Calcitonin Gene-Related Peptide in Cardiovascular Diseases. *Front Physiol* (2019) 10:821. doi: 10.3389/fphys.2019.00821

6. Nilsson C, Hansen TK, Rosenquist C, Hartmann B, Kodra JT, Lau JF, et al. Long Acting Analogue of the Calcitonin Gene-Related Peptide Induces Positive Metabolic Effects and Secretion of the Glucagon-Like Peptide-1. *Eur J Pharmacol* (2016) 773:24–31. doi: 10.1016/j.ejphar.2016.01.003
7. Sheykhzade M, Abdolalizadeh B, Koole C, Pickering DS, Dreisig K, Johansson SE, et al. Vascular and Molecular Pharmacology of the Metabolically Stable CGRP Analogue, SAX. *Eur J Pharmacol* (2018) 829:85–92. doi: 10.1016/j.ejphar.2018.04.007
8. Aubdool AA, Thakore P, Argunhan F, Smillie SJ, Schnelle M, Srivastava S, et al. A Novel Alpha-Calcitonin Gene-Related Peptide Analogue Protects Against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure. *Circulation* (2017) 136:367–83. doi: 10.1161/CIRCULATIONAHA.117.028388
9. Baral P, Udit S, Chiu IM. Pain and Immunity: Implications for Host Defence. *Nat Rev Immunol* (2019) 19:433–47. doi: 10.1038/s41577-019-0147-2
10. Granstein RD, Wagner JA, Stohl LL, Ding W. Calcitonin Gene-Related Peptide: Key Regulator of Cutaneous Immunity. *Acta Physiol (Oxf)* (2015) 213:586–94. doi: 10.1111/apha.12442
11. Ganor Y, Zhou Z, Tudor D, Schmitt A, Vacher-Lavenu MC, Gibault L, et al. Within 1 H, HIV-1 Uses Viral Synapses to Enter Efficiently the Inner, But Not Outer, Foreskin Mucosa and Engages Langerhans-T Cell Conjugates. *Mucosal Immunol* (2010) 3:506–22. doi: 10.1038/mi.2010.32
12. Zhou Z, Barry De Longchamps N, Schmitt A, Zerbib M, Vacher-Lavenu MC, Bomsel M, et al. HIV-1 Efficient Entry in Inner Foreskin Is Mediated by Elevated CCL5/RANTES That Recruits T Cells and Fuels Conjugate Formation With Langerhans Cells. *PloS Pathog* (2011) 7:e1002100. doi: 10.1371/journal.ppat.1002100
13. Ganor Y, Drillet-Dangeard AS, Lopalco L, Tudor D, Tambussi G, Delongchamps NB, et al. Calcitonin Gene-Related Peptide Inhibits Langerhans Cell-Mediated HIV-1 Transmission. *J Exp Med* (2013) 210:2161–70. doi: 10.1084/jem.20122349
14. Ganor Y, Drillet-Dangeard AS, Bomsel M. Calcitonin Gene-Related Peptide Inhibits Human Immunodeficiency Type 1 Transmission by Langerhans Cells *via* an Autocrine/Paracrine Feedback Mechanism. *Acta Physiol (Oxf)* (2015) 213:432–41. doi: 10.1111/apha.12366
15. Bomsel M, Ganor Y. Calcitonin Gene-Related Peptide Induces HIV-1 Proteasomal Degradation in Mucosal Langerhans Cells. *J Virol* (2017) 91: e01205–01217. doi: 10.1128/JVI.01205-17
16. Asahina A, Moro O, Hosoi J, Lerner EA, Xu S, Takashima A, et al. Specific Induction of cAMP in Langerhans Cells by Calcitonin Gene-Related Peptide: Relevance to Functional Effects. *Proc Natl Acad Sci USA* (1995) 92:8323–7. doi: 10.1073/pnas.92.18.8323
17. Watkins HA, Rathbone DL, Barwell J, Hay DL, Poyner DR. Structure-Activity Relationships for Alpha-Calcitonin Gene-Related Peptide. *Br J Pharmacol* (2013) 170:1308–22. doi: 10.1111/bph.12072
18. Qing X, Wimalawansa SJ, Keith IM. Specific N-Terminal CGRP Fragments Mitigate Chronic Hypoxic Pulmonary Hypertension in Rats. *Regul Pept* (2003) 110:93–9. doi: 10.1016/S0167-0115(02)00211-2
19. Hakala JM, Lindvall M, Koskinen AM. Modelling Constrained Calcitonin Gene-Related Peptide Analogues. *Protein Eng* (1996) 9:143–8. doi: 10.1093/protein/9.2.143
20. Geissmann F, Prost C, Monnet JP, Dy M, Brousse N, Hermine O. Transforming Growth Factor Beta1, in the Presence of Granulocyte/Macrophage Colony-Stimulating Factor and Interleukin 4, Induces Differentiation of Human Peripheral Blood Monocytes Into Dendritic Langerhans Cells. *J Exp Med* (1998) 187:961–6. doi: 10.1084/jem.187.6.961
21. Ganor Y, Zhou Z, Bodo J, Tudor D, Leibowitch J, Mathez D, et al. The Adult Penile Urethra Is a Novel Entry Site for HIV-1 That Preferentially Targets Resident Urethral Macrophages. *Mucosal Immunol* (2013) 6:776–86. doi: 10.1038/mi.2012.116
22. Karpel ME, Boutwell CL, Allen TM. BLT Humanized Mice as a Small Animal Model of HIV Infection. *Curr Opin Virol* (2015) 13:75–80. doi: 10.1016/j.coviro.2015.05.002
23. Nixon CC, Mavigner M, Sampey GC, Brooks AD, Spagnuolo RA, Irlbeck DM, et al. Systemic HIV and SIV Latency Reversal *via* Non-Canonical NF-kappaB Signalling *In Vivo*. *Nature* (2020) 578:160–5. doi: 10.1038/s41586-020-1951-3
24. Maggi CA, Rovero P, Giuliani S, Evangelista S, Regoli D, Meli A. Biological Activity of N-Terminal Fragments of Calcitonin Gene-Related Peptide. *Eur J Pharmacol* (1990) 179:217–9. doi: 10.1016/0014-2999(90)90422-3
25. Yang C, Mai H, Peng J, Zhou B, Hou J, Jiang D. STAT4: An Immunoregulator Contributing to Diverse Human Diseases. *Int J Biol Sci* (2020) 16:1575–85. doi: 10.7150/ijbs.41852
26. Remoli ME, Ragimbeau J, Giacomini E, Gafa V, Severa M, Lande R, et al. NF-{Kappa}B Is Required for STAT-4 Expression During Dendritic Cell Maturation. *J Leukoc Biol* (2007) 81:355–63. doi: 10.1189/jlb.0506319
27. Bertram KM, Botting RA, Baharlou H, Rhodes JW, Rana H, Graham JD, et al. Identification of HIV Transmitting CD11c(+) Human Epidermal Dendritic Cells. *Nat Commun* (2019) 10:2759. doi: 10.1038/s41467-019-10697-w
28. Bertram KM, Truong NR, Smith JB, Kim M, Sandgren KJ, Feng KL, et al. Herpes Simplex Virus Type 1 Infects Langerhans Cells and the Novel Epidermal Dendritic Cell, Epi-Cdc2s, *via* Different Entry Pathways. *PloS Pathog* (2021) 17:e1009536. doi: 10.1371/journal.ppat.1009536
29. Liu X, Zhu R, Luo Y, Wang S, Zhao Y, Qiu Z, et al. Distinct Human Langerhans Cell Subsets Orchestrate Reciprocal Functions and Require Different Developmental Regulation. *Immunity* (2021) 54:2305–2320 e2311. doi: 10.1016/j.immuni.2021.08.012
30. Wingler LM, Lefkowitz RJ. Conformational Basis of G Protein-Coupled Receptor Signaling Versatility. *Trends Cell Biol* (2020) 30(9):736–47. doi: 10.1016/j.tcb.2020.06.002
31. Walker CS, Conner AC, Poyner DR, Hay DL. Regulation of Signal Transduction by Calcitonin Gene-Related Peptide Receptors. *Trends Pharmacol Sci* (2010) 31:476–83. doi: 10.1016/j.tips.2010.06.006
32. Holzmann B. Modulation of Immune Responses by the Neuropeptide CGRP. *Amino Acids* (2013) 45:1–7. doi: 10.1007/s00726-011-1161-2
33. Assas BM, Pennock JI, Miyan JA. Calcitonin Gene-Related Peptide Is a Key Neurotransmitter in the Neuro-Immune Axis. *Front Neurosci* (2014) 8:23. doi: 10.3389/fnins.2014.00023
34. Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D, et al. Initial Events in Establishing Vaginal Entry and Infection by Human Immunodeficiency Virus Type-1. *Immunity* (2007) 26:257–70. doi: 10.1016/j.immuni.2007.01.007
35. Mayer KH, Agwu A, Malebranche D. Barriers to the Wider Use of Pre-Exposure Prophylaxis in the United States: A Narrative Review. *Adv Ther* (2020) 37:1778–811. doi: 10.1007/s12325-020-01295-0
36. Coelho LE, Torres TS, Veloso VG, Landovitz RJ, Grinsztejn B. Pre-Exposure Prophylaxis 2.0: New Drugs and Technologies in the Pipeline. *Lancet HIV* (2019) 6(11):e788–99. doi: 10.1016/S2352-3018(19)30238-3

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frontiers in Physiology

REVIEW
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Constipation Caused by Anti-calcitonin Gene-Related Peptide Migraine Therapeutics Explained by Antagonism of Calcitonin Gene-Related Peptide's Motor-Stimulating and Prosecretory Function in the Intestine

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The development of small-molecule calcitonin gene-related peptide (CGRP) receptor antagonists (gepants) and of monoclonal antibodies targeting the CGRP system has been a major advance in the management of migraine. In the randomized controlled trials before regulatory approval, the safety of these anti-CGRP migraine therapeutics was considered favorable and to stay within the expected profile. Post-approval real-world surveys reveal, however, constipation to be a major adverse event which may affect more than 50% of patients treated with erenumab (an antibody targeting the CGRP receptor), fremanezumab or galcanezumab (antibodies targeting CGRP). In this review article we address the question whether constipation caused by inhibition of CGRP signaling can be mechanistically deduced from the known pharmacological actions and pathophysiological implications of CGRP in the digestive tract. CGRP in the gut is expressed by two distinct neuronal populations: extrinsic primary afferent nerve fibers and distinct neurons of the intrinsic enteric nervous system. In particular, CGRP is a major messenger of enteric sensory neurons which in response to mucosal stimulation activate both ascending excitatory and descending inhibitory neuronal pathways that enable propulsive (peristaltic) motor activity to take place. In addition, CGRP is able to stimulate ion and water secretion into the intestinal lumen. The motor-stimulating and prosecretory actions of CGRP combine in accelerating intestinal transit, an activity profile that has been confirmed by the ability of CGRP to induce diarrhea in mice, dogs and humans. We therefore conclude that the constipation elicited by antibodies targeting CGRP or its receptor results from interference with the physiological function of CGRP in the small and large intestine in which it contributes to the maintenance of peristaltic motor activity, ion and water secretion and intestinal transit.

Keywords: calcitonin gene-related peptide (CGRP), CGRP receptor antagonists (gepants), CGRP antibodies, CGRP receptor antibodies, migraine, constipation, peristaltic motor activity, diarrhea

INTRODUCTION

Calcitonin gene-related peptide was overtaking many a neuropeptide in its path to clinical exploitation, given that only some 20 years after its discovery (Rosenfeld et al., 1983) a landmark paper (Olesen et al., 2004) provided clinical proof of the concept that a small molecule antagonist of CGRP (code name BIBN4096BS, later given the international non-proprietary name olcegepant) is able to prevent and treat migraine. Since then several small molecule antagonists (as a group termed *gepants*) have been developed for use in migraine management, followed by the successful introduction of monoclonal antibodies targeting CGRP or its receptor (for reviews see Edvinsson, 2019; Hargreaves and Olesen, 2019; Omaer et al., 2021). The results of the randomized controlled trials (RCTs) in phase 3 of clinical development attributed not only satisfactory efficacy but also acceptable safety and tolerability to these CGRP-directed migraine therapeutics. The profile of treatment-emergent adverse effects did not expose any major concern with those compounds that were advanced to regulatory approval. Unexpectedly, though, post-approval real-world surveys revealed constipation to be a major adverse effect of the monoclonal antibodies targeting the CGRP system.

Constipation caused by CGRP antagonism/neutralization is the prime focus of this review article which seeks to provide a mechanistic explanation of this adverse effect which in hindsight was not totally unforeseen. In addressing this issue, we first survey the gastrointestinal adverse effect profile of gepants and monoclonal antibodies targeting either CGRP or the CGRP receptor, collectively termed anti-CGRP migraine therapeutics, that came to light in the phase 3 trials prior to marketing authorization. We then summarize the available data showing constipation to be associated with the use of antibodies targeting CGRP or its receptor in the post-approval stage. Considering the post-approval data we go on to examine the question whether constipation caused by inhibition of CGRP signaling can mechanistically be deduced from the known actions and pathophysiological implications of CGRP in the gastrointestinal tract of laboratory animals and humans. Although we notice that the effects of CGRP in the human gut have insufficiently been studied, we are able to gather plausible evidence that CGRP can stimulate propulsive motility and secretion in the human intestine. If the pharmacological actions of CGRP in the human intestine were to reflect the physiological implications of the peptide, antagonism of CGRP signaling would be expected to cause constipation as shown by the adverse effect of anti-CGRP therapeutics. In addition, we take into account that certain neuroendocrine tumors expressing and releasing CGRP are known to cause watery diarrhea, which indirectly ascribes CGRP a role in promoting propulsive motility in the human intestine. With these considerations in mind we provide a rational explanation why long-term blockade of CGRP signaling results in constipation as a major adverse effect of monoclonal antibodies targeting CGRP or its receptor.

GASTROINTESTINAL SIDE EFFECT PROFILE OF ANTI-CALCITONIN GENE-RELATED PEPTIDE MIGRAINE THERAPEUTICS IN PRE-APPROVAL CLINICAL TRIALS

Although they were efficacious in migraine management, the first gepants including olcegepant (code name BIBN4096BS), MK-3207 and telcagepant (code name MK-0974) did not make it to approval by regulatory authorities for various reasons. One issue was that MK-3207 and telcagepant were liable to enhance serum levels of hepatic aminotransferases and carry a risk of hepatotoxicity (Ho et al., 2008). Concerns about drug-induced liver injury also accompanied the development of further gepants. However, ubrogepant, rimegepant and atogepant have meanwhile been approved as migraine therapeutics by the US Food and Drug Administration (FDA), and other regulatory authorities including the European Medicines Agency (EMA) were following suit in licensing these gepants. The phase 3 trials of these small molecule CGRP receptor antagonists revealed only rare and transient elevations of serum aminotransferase levels (Croop et al., 2019; Dodick et al., 2019; Lipton et al., 2019a,b; Ailani et al., 2020; Omaer et al., 2021) that did not lead to clinically manifest liver injury.

The gepants in clinical use are in general well tolerated as proved in the RCTs during clinical development. Apart from infections of the upper respiratory, urinary and/or gastrointestinal tract, nausea (1.8–4.7% of patients on gepants) was the most common gastrointestinal complaint reported in phase 3 trials of ubrogepant, rimegepant and atogepant (Croop et al., 2019; Lipton et al., 2019a,b; Ailani et al., 2020, 2021; Omaer et al., 2021). Diarrhea was noted in 2.5–2.7% of patients on ubrogepant (Ailani et al., 2020) while constipation was reported in 6.9–7.7% of subjects taking atogepant (Ailani et al., 2021), but were not noticed in the trials of the other gepants. The proven efficacy of gepants in migraine prevention and treatment provided ample evidence for an implication of CGRP in the pathophysiology of migraine and fostered efforts to explore other routes to target the CGRP system.

To avoid the potential hepatic toxicity of some of the gepants, monoclonal antibodies targeting the CGRP system were envisaged as an alternative therapeutic approach. Meanwhile, a number of monoclonal antibodies targeting either the CGRP receptor (erenumab) or CGRP itself (fremanezumab, galcanezumab and eptinezumab) have entered the therapeutic arena. The safety of these monoclonal antibodies during clinical development has been regarded as favorable and to stay within a profile expected for this class of therapeutics (production of anti-drug antibodies, local injection responses, hypersensitivity reactions, infections of the upper respiratory tract). The results of the phase 3 trials also showed that their gastrointestinal adverse effect profile posed no particular concern, although some distinct differences between the antibodies targeting the CGRP receptor and those targeting CGRP emerged.

Fremanezumab appeared to be largely devoid of adverse effects on the gut as the frequency of nausea observed in the antibody-treated patients did not exceed that noted in placebo-treated subjects (Silberstein et al., 2017; Dodick et al., 2018b; Ferrari et al., 2019). No mention of constipation was made in three phase 3 trials (Silberstein et al., 2017; Dodick et al., 2018b; Sakai et al., 2021), whereas one phase 3 study reported constipation occurring in 3% of subjects receiving fremanezumab (675 mg) once every 3 months but not in those receiving fremanezumab (225 mg) monthly (Ferrari et al., 2019). Similarly, constipation and other gastrointestinal adverse effects in individuals on galcanezumab were either not reported or their rate did not exceed that seen in placebo-treated patients (Detke et al., 2018; Skljarevski et al., 2018; Stauffer et al., 2018; Mulleners et al., 2020). Only one phase 3 trial showed nausea to occur in 6.4–7.8% of patients on galcanezumab (Camporeale et al., 2018). Constipation was likewise found to be absent or negligible in five phase 3 studies of eptinezumab (Ashina M. et al., 2020; Lipton et al., 2020; Silberstein et al., 2020; Kudrow et al., 2021; Winner et al., 2021) while low rates of eptinezumab-associated nausea were noted in two phase 3 trials (Lipton et al., 2020; Silberstein et al., 2020).

Unlike the antibodies targeting CGRP (fremanezumab, galcanezumab, eptinezumab), erenumab, an antibody targeting the CGRP receptor, emerged to have some potential to cause constipation in the pre-approval RCTs. Thus, constipation in 1.6–5.1% of patients on erenumab was reported in three phase 3 trials (Goadsby et al., 2017; Tepper et al., 2017; Takeshima et al., 2021) whereas in five other phase 3 trials (Sun et al., 2016; Dodick et al., 2018a; Reuter et al., 2018; Goadsby et al., 2020; Hirata et al., 2021) no mention of constipation was made. Occasional nausea and diarrhea were noted in one phase 3 trial (Goadsby et al., 2020). Taken together, the intestinal adverse effect profile of erenumab was considered to be without substantial concern based on the data collected in the pre-approval trials of the antibody. It is worth mentioning, though, that a constipation rate of 11% was observed when erenumab, but not galcanezumab, was co-administered with ubrogepant in an open-label phase 1b trial of migraine patients (Jakate et al., 2021).

CONSTIPATION AS AN ADVERSE EFFECT OF CALCITONIN GENE-RELATED PEPTIDE AND CALCITONIN GENE-RELATED PEPTIDE RECEPTOR ANTIBODIES IN POST-APPROVAL SURVEYS

As the development of anti-CGRP migraine therapeutics gained momentum, concerns were raised that blockade of CGRP signaling may have adverse effects, especially if used for long term periods that were not covered by phase 3 trials. In view of CGRP's vasodilator action (for a review see Russell et al., 2014) it was, for instance, argued that comorbidities in the cardiovascular system may enhance the risk of cerebral and cardiac ischemia (MaassenVanDenBrink et al., 2016; Deen et al., 2017). Since CGRP also plays a role in safeguarding gastrointestinal mucosal blood flow in the face of pending injury (Holzer, 1998; Akiba et al., 2002), antagonism of CGRP's vasodilator action was regarded to potentially compromise gastrointestinal mucosal homeostasis. Indeed, neutralization of CGRP with an antibody or CGRP knockout prevents the protective effect of capsaicin, a stimulant of extrinsic sensory neurons releasing CGRP, against ethanol-induced damage in the rat gastric mucosa (Peskar et al., 1993; Ohno et al., 2008). Likewise, experimental colitis evoked by trinitrobenzene sulfonic acid in rodents is aggravated by the CGRP receptor antagonist $CGRP_{8-37}$, a CGRP antibody (Reinshagen et al., 1998), and genetic deletion of CGRP (Engel et al., 2011). CGRP antagonism may thus interfere with the protective function of CGRP released from sensory nerve fibers in the gastrointestinal mucosa (Holzer, 1998; Akiba et al., 2002), a function that may include facilitated wound healing and neovascularization (Ohno et al., 2008). Fortunately, this adverse event potential of anti-CGRP therapeutics in the gastrointestinal mucosa has thus far not been substantiated in the RCTs and post-approval surveys.

The concerns raised by Deen et al. (2017) and Hargreaves and Olesen (2019) that CGRP antagonism/neutralization may bear a risk of constipation, though, has been corroborated to a remarkable degree in post-approval studies and surveys. The predictive validity of this concern was uncertain at that time, since any long-term effects of CGRP-targeting migraine therapy were not yet known and the pathophysiological implications of CGRP in humans remained largely unexplored. In addition, the ongoing phase 3 trials did not disclose any substantial gastrointestinal adverse effect potential of the anti-CGRP migraine therapeutics under clinical development. It therefore came as an unforeseen surprise that in post-approval real-world surveys constipation emerged as a major unwanted side effect of monoclonal antibodies targeting the CGRP system. **Table 1** summarizes the pertaining reports that have been published to date and shows that the available real-world evidence for constipation relates, at the present time, to the monoclonal antibodies targeting the CGRP system.

The data put together in **Table 1** also demonstrate that constipation is a troubling adverse effect independently of whether the antibodies neutralize the CGRP receptor or CGRP itself. As the surveys show, constipation may affect more than 50% of patients receiving erenumab. It is worth noting that the lowest rate of erenumab-associated constipation (7.6%) was found in Spanish patients (Belvís et al., 2021), while the highest rates (32.6–65%) were found in Dutch (de Vries Lentsch et al., 2021) and US (Alex et al., 2020; Kanaan et al., 2020) patients as well as in a Danish cohort of patients with headache attributed to mild traumatic brain injury (Ashina H. et al., 2020). A more detailed analysis indicates that constipation due to erenumab depends on dose (Alex et al., 2020) and is most prominent at the start of treatment (Lambru et al., 2020). Antibodies targeting CGRP itself appear to be equally liable to cause constipation as erenumab

TABLE 1 | Constipation rates in migraine patients treated with CGRP receptor antagonists or monoclonal antibodies targeting CGRP or its receptor as reported in post-approval surveys (real-world evidence).

Compound	Target	Recommended dosing	Treatment duration	Constipation rate	References
Ubrogepant	Small molecule CGRP receptor antagonist	50 or 100 mg p.o. twice daily	1–3 Months after prescription (50 or 100 mg)	4.7%	Chiang et al., 2021
Erenumab	Monoclonal anti-CGRP receptor antibody	70 or 140 mg s.c. once a month	6-month follow-up (70 mg optionally followed by 140 mg after 3 months)	65%	de Vries Lentsch et al., 2021
			8-month survey period (70 or 140 mg)	43%	Kanaan et al., 2020
			3-month survey period (140 mg)	34%	Ashina H. et al., 2020
			6-month follow-up (70 or 140 mg)	20% (70 mg erenumab), 32.6% (140 mg erenumab)	Alex et al., 2020
			6-month follow-up (70 mg optionally followed by 140 mg after 3 months)	23. 9%	Russo et al., 2020
			3-month treatment (70 mg)	23.8%	Scheffler et al., 2020
			6-month survey period (70 or 140 mg)	23.6%	Robblee et al., 2020
			16-month survey period (data combined for erenumab, galcanezumab and fremanezumab, doses not specified)	23%	Robbins and Phenicie, 2020
			13-month survey period (at least 3 months on erenumab, 70 or 140 mg)	21%	Faust et al., 2021
			3-month treatment (data combined for erenumab, 70 mg, galcanezumab and fremanezumab)	21%	Scheffler et al., 2021
			Duration and specific dose not specified	20%	Robbins, 2019
			6-month follow-up (70 mg, with the option to increase dose to 140 mg in months 4–6)	20% at month 1 11% at month 3 5% at month 6	Lambru et al., 2020
			3-month survey period (data combined for erenumab, 140 mg, and galcanezumab, 120 mg, after loading dose of 240 mg)	20%	Torres-Ferrús et al., 2021
			6-month follow-up (70 mg optionally followed by 140 mg after 3 months)	20%	Silvestro et al., 2021
			12-month survey period (70 or 140 mg)	16.7%	Eghtesadi et al., 2021
			6-month survey period (70 mg optionally followed by 140 mg after 3 months)	13.5%	Ornello et al., 2020
			18-month survey period (70 or 140 mg)	7.6%	Belvís et al., 2021
Galcanezumab	Monoclonal anti-CGRP antibody	120 mg s.c. once a month, with a first loading dose of 240 mg s.c.	16-month survey period (data combined for erenumab, galcanezumab and fremanezumab, doses not specified)	23%	Robbins and Phenicie, 2020
			3-month survey period (data combined for erenumab, 140 mg, and galcanezumab, 120 mg, after loading dose of 240 mg)	20%	Torres-Ferrús et al., 2021
			6-month follow-up (240 mg loading dose followed by 120 mg once a month)	17.4%	Alex et al., 2020
Fremanezumab	Monoclonal anti-CGRP antibody	225 mg s.c. once a month or 675 mg s.c. every 3 months	6-month follow-up (225 mg once a month)	25%	Alex et al., 2020
			16-month survey period (data combined for erenumab, galcanezumab and fremanezumab, doses not specified)	23%	Robbins and Phenicie, 2020

(**Table 1**). Thus, constipation rates of 17.4–23% were reported for galcanezumab and rates of 23–25% for fremanezumab (Alex et al., 2020; Robbins and Phenicie, 2020; Torres-Ferrús et al., 2021).

As regards the small molecule CGRP receptor antagonists, only one post-approval survey has been published, showing that 4.7% of patients on ubrogepant complained about constipation (Chiang et al., 2021). As described in the previous section of

this article, no mention of constipation as an adverse effect was made in the phase 3 trials of ubrogepant. Whether the real world constipation rates for other gepants will also be higher than those in the pre-approval RCTs remains to be examined. Such a post-approval evaluation is in particular awaited for atogepant which in one phase 3 trial was reported to cause constipation in 6.9–7.7% of patients (Ailani et al., 2021).

The pre- and post-approval studies/surveys considered here were selected in order to provide valid evidence for constipation being an unwanted action of CGRP migraine therapeutics and to underline the need to explain this unforeseen adverse effect. There are, however, some limitations inherent in these studies. Firstly, gepants are indicated for the acute migraine treatment while antibodies targeting the CGRP system are used for preventive migraine management. Secondly, the post-approval studies/surveys of CGRP-directed antibodies have typically been conducted in migraine patients that had a history of multiple treatment failures. Thirdly, in many studies the concomitant use of one or more preventive headache medications was permitted. If the migraine patients continue treatment by a polypharmacy approach including, for instance, opioid receptor agonists, the rate of constipation may be equally high before and after erenumab therapy (Faust et al., 2021).

The high rates of constipation disclosed by real world evaluation of the efficacy and safety of antibodies targeting the CGRP receptor or CGRP itself emphasize the need to closely monitor their intestinal adverse effect profile and in this way safeguard the success in migraine treatment. In this context it is also important to address the question in which way self-rated constipation can be classified in a clinically meaningful manner. Assessment of drug-induced constipation is not a trivial task because it is important to differentiate between self-reported constipation and constipation assessed by objective criteria as, for instance, proposed for opioid-induced constipation (Lacy et al., 2016; Farmer et al., 2019). Self-assessed constipation is dominated by individual perceptions such as ease of defecation or incomplete bowel emptying and individual expectations in the frequency of bowel movements. This may also in part explain why the rates of self-reported constipation vary considerably among the different surveys listed in **Table 1**. The use of diagnostic procedures for objective assessment of constipation, e.g., the Rome IV criteria (Lacy et al., 2016) or the functional bowel index (Farmer et al., 2019), may yield results appreciably different from self-reported constipation rates. Apart from differences in individual perception and expectation, patients may also overlook gradual changes in bowel movements that could have taken place before a CGRP-targeting therapy was begun. It should not go unnoticed in this context that migraine as well as non-migrainous headache can be comorbid with gastrointestinal disturbances including constipation (Kelman, 2004; Aamodt et al., 2008). Notwithstanding these considerations, the post-approval survey data indicate that a considerable part of migraine patients treated with antibodies against CGRP or the CGRP receptor suffer from constipation severe enough to curtail their quality of life.

EVIDENCE FOR A ROLE OF CALCITONIN GENE-RELATED PEPTIDE IN STIMULATING INTESTINAL PROPULSION AND SECRETION IN LABORATORY ANIMALS

Innervation of the Gut by Calcitonin Gene-Related Peptide-Expressing Neurons

In our attempt to explain the potential of CGRP antagonism/neutralization to induce constipation we first review experimental studies in laboratory animals for relevant effects of CGRP and CGRP antagonism on gastrointestinal motor activity and secretory processes. The major sources of CGRP in the gut of rodents are extrinsic afferent nerve fibers and intrinsic enteric neurons (for early reviews see Taché et al., 1991; Sternini, 1992; Holzer, 1994, 2000; Maggi, 1995), and this dual CGRP innervation holds also true for the digestive tract of dogs and pigs (Sternini et al., 1992; Rasmussen et al., 2001). **Figure 1** provides a schematic diagram of the neuronal sources of CGRP in the rodent gut. In the rat, most of the CGRP expressed in extrinsic sensory neurons is α-CGRP, whereas the peptide found in enteric neurons is β-CGRP (Mulderry et al., 1988; Sternini and Anderson, 1992). In the mouse, β-CGRP appears to be the only form of the peptide occurring in the intestine (Schütz et al., 2004). While the major source of CGRP in the foregut of rat, mouse and guinea-pig are extrinsic afferent neurons, intrinsic enteric neurons constitute the major source of the peptide in the small and large intestine (Sternini et al., 1987; Su et al., 1987; Green and Dockray, 1988; Mitsui, 2011). CGRP-immunoreactive neurons of the myenteric and submucosal plexus in the guinea-pig intestine extend abundant processes to the mucosa (Furness et al., 1985). Similarly, CGRP has been localized to cholinergic secretomotor neurons in the submucosal plexus of the mouse ileum (Mongardi Fantaguzzi et al., 2009). In the rat intestine CGRP-expressing neurons have been found to issue oral or caudal projections within the nerve plexus as well as to the muscle layers and the mucosa (Ekblad et al., 1987; Sternini et al., 1987). CGRP is a particular chemical code of intrinsic primary afferent neurons (IPANs) which originate from the enteric nerve plexus of the mouse, rat and guinea-pig intestine (**Figure 2**) and have direct connections with both excitatory and inhibitory enteric motor pathways (Furness et al., 2004; Mitsui, 2009, 2011; Hibberd et al., 2018; Smolilo et al., 2020). Likewise, CGRP occurs in more than 90% of IPANs (neurons with a Dogiel type II morphology) in the myenteric plexus of the porcine small intestine, but is also expressed in other types of neuron (Brehmer et al., 2002; Wolf et al., 2007).

The CGRP-expressing extrinsic afferent neurons in the rodent gut (**Figure 1**) originate primarily from cell bodies in the dorsal root ganglia (Sternini et al., 1987; Green and Dockray, 1988; Mulderry et al., 1988; Sternini and Anderson, 1992; Robinson et al., 2004; Tan et al., 2008; Chen et al., 2016). Within the gastrointestinal tract, they supply primarily the arterial system but also project to the submucosal and myenteric nerve plexus,

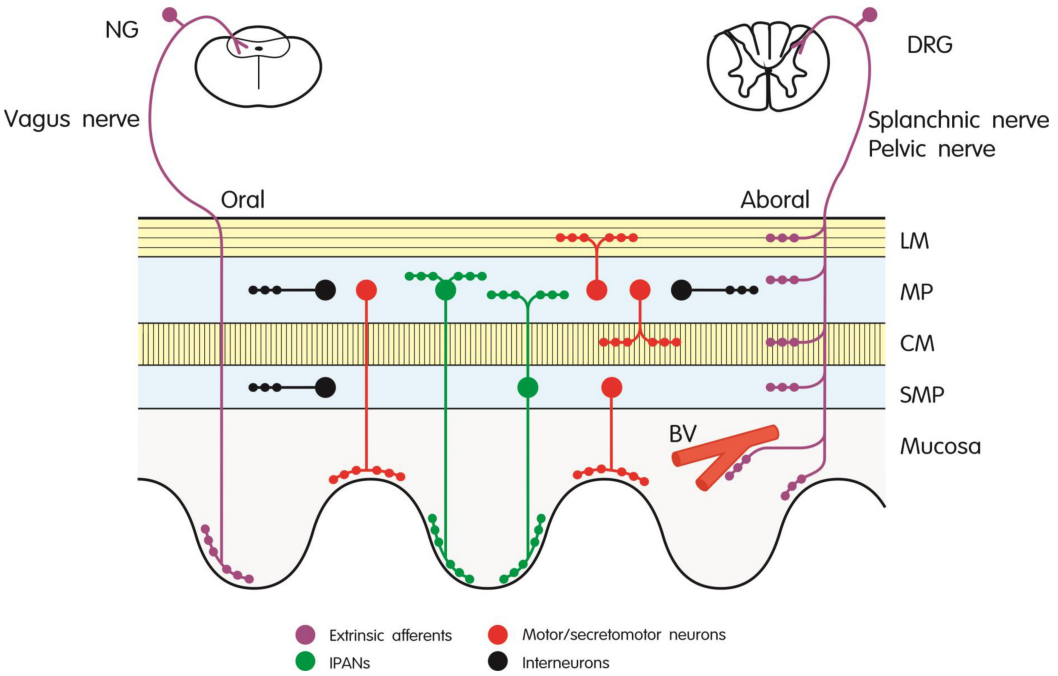

FIGURE 1 | Innervation of the rodent gut by extrinsic afferent and intrinsic enteric neurons expressing CGRP. The different types of neuron are depicted by different color codes as explained in the graph. BV, blood vessel; CM, circular muscle; DRG, dorsal root ganglion; IPANs, intrinsic primary afferent neurons; LM, longitudinal muscle; MP, myenteric plexus; NG, nodose ganglion; SMP, submucosal plexus.

to the circular and longitudinal muscle layers and the lamina propria of the mucosa (Sternini et al., 1987; Su et al., 1987; Green and Dockray, 1988). CGRP-positive vagal afferents originating from the nodose ganglia make a limited contribution to the CGRP content of the rat foregut, supplying primarily the mucosa but not the muscle layers (Sternini and Anderson, 1992; Bäck et al., 1994; Suzuki et al., 1997; Tan et al., 2009).

Expression of Calcitonin Gene-Related Peptide Receptors in the Gut and Functional Evidence for a Role of Calcitonin Gene-Related Peptide in Stimulating Propulsive Motility

CGRP has been reported to have multiple actions in the gastrointestinal tract, influencing motor, secretory, vascular and immune functions (for a review see Holzer, 2000), these implications depending on the innervation of the target tissues by CGRP-releasing neurons and the expression of CGRP receptors by the effector cells. The localization of specific CGRP binding sites to the enteric nerve plexus and other tissues in the canine and rat digestive tract (Gates et al., 1989; Ozdemir et al., 1999) has been confirmed by the presence of CGRP receptors in the myenteric nerve plexus of the human gastrointestinal tract (Cottrell et al., 2012). The functional implications of CGRP receptors cannot only be studied by the use of CGRP receptor antagonists and antibodies targeting CGRP or the CGRP receptor but also by (genetic) modification of the expression of CGRP receptors. With regard to the elucidation of gastrointestinal CGRP functions, the latter approach has not yet been employed to any significant extent. This may in part be related to the fact that functional CGRP receptors are heterodimers of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein-1 (RAMP1) (for a review on CGRP receptor pharmacology see Hay et al., 2018). There is still a lack of studies localizing functional CGRP (CLR/RAMP1) receptors to particular effector systems and matching the presence of these receptors with the pharmacological actions of CGRP in the gut.

In explaining the potential of CGRP antagonism/neutralization to cause constipation it is of prime relevance to consider the known pharmacological and physiological roles that CGRP plays in the regulation of gastrointestinal motor activity. Given the multiple sources of CGRP in the gut it is to be expected that the pharmacological actions of CGRP are complex, comprising both excitatory and inhibitory actions on gastrointestinal motility. CGRP causes muscle relaxation in the guinea-pig and rat small and large intestine by a direct action on the muscle (Holzer et al., 1989; Barthó et al., 1991; Barthó and Holzer, 1995; Sun and Benishin, 1995; Maggi et al., 1996; Rekik et al., 1997; Clifton et al., 2007), an effect that is also seen when CGRP is released from extrinsic sensory neurons stimulated by capsaicin (Barthó et al., 1991; Barthó and Holzer, 1995). Defunctionalization of extrinsic CGRP-releasing neurons by capsaicin, however, as well as CGRP antagonism by $CGRP_{8-37}$ do not modify distension-induced peristalsis in the isolated guinea-pig small intestine (Barthó and Holzer, 1995). In contrast, release of CGRP from extrinsic sensory neurons slows gastric emptying

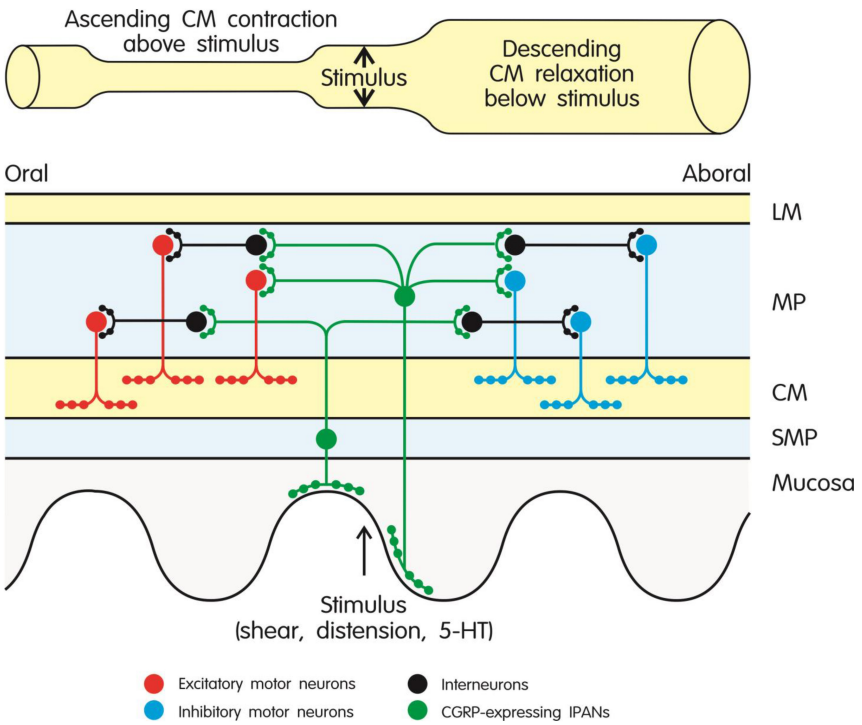

FIGURE 2 | Role of CGRP-expressing intrinsic primary afferent neurons (IPANs) in the peristaltic motor activity of the intestine through activation of ascending excitatory and descending inhibitory pathways in the enteric nerve plexus. CGRP-expressing IPANs are stimulated by mechanical or chemical stimulation of the mucosa and intestinal wall. Ascending contraction of the circular muscle above the stimulus is mediated by interneurons and excitatory cholinergic motor neurons, while descending relaxation of the circular muscle below the stimulus is brought about by interneurons and inhibitory motor neurons (expressing nitric oxide synthase and various relaxant mediators). The different types of neuron are depicted by different color codes as explained in the graph. The model shown in the graph takes particular account of the findings reported by Grider et al. (1998) and Smolilo et al. (2020). CM, circular muscle; 5-HT, 5-hydroxytryptamine; LM, longitudinal muscle; MP, myenteric plexus; SMP, submucosal plexus.

in the rat (Forster and Dockray, 1991) as does intravenous or intraperitoneal administration of CGRP to dogs (L'Heureux et al., 2000) and rats (Lenz, 1988; Raybould et al., 1988; Plourde et al., 1993; Julia and Buéno, 1997) through inhibition of gastric corpus motility (Zittel et al., 1994). The inhibitory action of CGRP released from extrinsic sensory neurons on gastrointestinal motility plays an important role in the ileus following abdominal surgery. Capsaicin-induced ablation of extrinsic spinal sensory neurons, CGRP antagonism by $CGRP_{8-37}$ or olcegepant, and CGRP immunoneutralization in the mouse, rat and dog are all able to reverse postoperative ileus in terms of inhibition of gastric corpus motility (Zittel et al., 1994), inhibition of gastric emptying (Plourde et al., 1993; Freeman et al., 1999; Trudel et al., 2003) and inhibition of transit through the small and large intestine (Zittel et al., 1998; Freeman et al., 1999; Glowka et al., 2015). The delay in gastric emptying and intestinal transit caused by endotoxin in mice and experimental peritonitis in rats is likewise mitigated by $CGRP_{8-37}$ (Julia and Buéno, 1997; De Winter et al., 2009).

While CGRP released from extrinsic sensory neurons participates in gastrointestinal motor inhibition under particular pathological conditions, CGRP released from intrinsic enteric neurons plays a physiological role in initiating peristaltic (propulsive) motility in the intestine. This role is at first glance difficult to extract from a multitude of reports describing both excitatory (contractile) and inhibitory (relaxant) motor actions of CGRP in isolated segments or strips of the intestine. However, local effects of the peptide such as muscle relaxation (Holzer et al., 1989; Barthó et al., 1991; Barthó and Holzer, 1995; Sun and Benishin, 1995; Maggi et al., 1996; Rekik et al., 1997; Clifton et al., 2007), inhibition of acetylcholine release from myenteric neurons (Schwörer et al., 1991) or activation of inhibitory neural pathways (Holzer et al., 1989) reflect only stationary aspects of coordinated propulsive motility. Experiments with a more integrated view on intestinal propulsion demonstrate that CGRP stimulates motor patterns that are relevant to the aboral movement of the intestinal contents. Thus, CGRP induces phasic contractions of the circular muscle of the guinea-pig small intestine due to stimulation of cholinergic neurons (Holzer et al., 1989). Close intraarterial administration of CGRP to the canine ileum evokes rhythmic phasic contractions at smaller doses and giant migrating contractions at higher doses, effects that are prevented by $CGRP_{8-37}$ (Sarna, 2003) although neither CGRP nor $CGRP_{8-37}$ infused to dogs modifies the duration of the migrating motor complex during the interdigestive period (L'Heureux et al., 2000).

Detailed analysis of the stimulant effect of CGRP on intestinal propulsion has disclosed that CGRP-expressing IPANs in the rodent intestine play a crucial role in initiating those enteric

reflexes (ascending contraction and descending relaxation of the circular muscle) that enable peristaltic movements to be triggered and to propagate in an aboral direction (**Figure 2**). In line with this implication, mechanical or chemical stimulation of the intestinal mucosa leads to release of CGRP from enteric sensory neurons (Grider, 1994; Foxx-Orenstein et al., 1996; Grider et al., 1998; Roza and Reeh, 2001). More specifically, when stimulated by mucosal stroking or 5-hydroxytryptamine (5-HT) released from enterochromaffin cells, CGRP-expressing IPANs lead to activation of ascending excitatory and descending inhibitory pathways in the enteric nerve plexus underlying peristalsis (**Figure 2**) in the rat, guinea-pig and mouse small and large intestine, this implication of CGRP being prevented by $CGRP_{8-37}$ (Grider, 1994, 2003; Foxx-Orenstein et al., 1996; Grider et al., 1998; Pan and Gershon, 2000). When muscle stretch is used to stimulate the peristaltic motor pathways, CGRP released from capsaicin-sensitive extrinsic sensory neurons also comes into play (Grider, 1994). The prokinetic action of bile acids in the mouse colon is mediated by a similar mechanism, given that the effect of deoxycholic acid to stimulate peristalsis is inhibited by $CGRP_{8-37}$ (Alemi et al., 2013). The stimulant effect of CGRP on peristalsis is consistent with the peptide's action to depolarize enteric neurons and enhance their excitability (Palmer et al., 1986) and to potentiate excitatory neurotransmission to the circular muscle of the guinea-pig colon (Kojima and Shimo, 1995; Maggi et al., 1997).

Functional Evidence for a Role of Calcitonin Gene-Related Peptide in Stimulating Intestinal Secretion and Causing Diarrhea

Motor activity in the gut is intimately related to intestinal ion and water secretion, given that an increase in the intraluminal volume of fluid and chyme will have a mechanical impact on the mucosa as well as gastrointestinal wall, in this way stimulate propulsive motility, and in combination with increased water secretion give rise to diarrhea. The intestinal mucosa is supplied by CGRP-positive intrinsic enteric as well as extrinsic sensory neurons which are strategically located to influence secretory processes. Thus, when these neurons are activated, epithelial ion transport in the mouse colon is enhanced by release of CGRP from submucosal enteric neurons (Tough et al., 2018). CGRP stimulates chloride secretion in the rat colonic mucosa by activating CGRP receptors that are preferentially located on the basolateral surface of epithelial cells (Cox, 1995). In contrast, CGRP's secretory action in the guinea-pig colon is mediated by enteric neurons (McCulloch and Cooke, 1989). It may be added that the distension-evoked release of CGRP in the mouse colon is accompanied by a release of prostaglandin E_2 (Roza and Reeh, 2001), which is known to play a role in intestinal secretion and diarrhea (Rask-Madsen et al., 1990). The prosecretory effect of CGRP is also seen *in vivo*, given that intravenous administration of the peptide enhances ion and water secretion into the rat colon (Rolston et al., 1989). In addition, evidence for a prosecretory role of CGRP comes from reports that inhibition of endogenous CGRP activity by knockdown of the CLR subunit of the CGRP receptor (Bhargava et al., 2013) or treatment with the receptor antagonist $CGRP_{8-37}$ (Keates et al., 1998) attenuates intestinal water secretion induced by *Clostridioides* (previously termed *Clostridium*) *difficile* toxin A. In addition, the accelerated gastrointestinal transit evoked by ovalbumin anaphylaxis is blunted in mice deficient in RAMP1, the other subunit of the CGRP receptor (Yoshikawa et al., 2011).

The implications of CGRP in intestinal ion and water secretion are confirmed by the ability of exogenous CGRP to induce overt diarrhea in dogs and mice. Specifically, intravenous infusion of CGRP to dogs causes jejunal electrolyte and water secretion and causes diarrhea in the majority of animals (Reasbeck et al., 1988). Intraperitoneal administration of CGRP to mice likewise triggers diarrhea as assessed by stool consistency and weight, an effect that is reproduced by intracerebroventricular administration of the peptide which appears to cause diarrhea only following leakage to the periphery (Kaiser et al., 2017). Intraperitoneal pretreatment of the animals with CGRP antibodies is able to prevent the CGRP-induced diarrhea, while olcegepant is less efficacious in blunting the laxative action of the peptide (Kaiser et al., 2017).

Taken together, there is substantial evidence from studies in laboratory animals that CGRP can stimulate propulsive motor activity and secretion in the intestine, which leads to diarrhea. If these effects were physiologically relevant, CGRP antagonism/neutralization would be expected to delay gastrointestinal transit and bring about a functional state resembling constipation. Unfortunately, there is only limited information as to whether CGRP receptor antagonists or CGRP antibodies interfere *per se* with physiological gut function in laboratory animals. Thus, gastric corpus motility (Zittel et al., 1994), gastric emptying (De Winter et al., 2009), gastrointestinal transit (Glowka et al., 2015), and colonic transit (Zittel et al., 1998) in rats and mice remain unaltered by a single administration of $CGRP_{8-37}$, olcegepant or a monoclonal CGRP antibody. One study, though, has shown that $CGRP_{8-37}$ delays gastrointestinal transit in mice (De Winter et al., 2009). Notwithstanding these inconclusive observations, the CGRP-induced diarrhea in mice (Kaiser et al., 2017) and dogs (Reasbeck et al., 1988) attests to the peptide's ability to stimulate intestinal transit and secretion, a contention that has been confirmed in humans (Falkenberg et al., 2020).

EVIDENCE FOR A ROLE OF CALCITONIN GENE-RELATED PEPTIDE IN STIMULATING INTESTINAL PROPULSION AND SECRETION IN HUMANS

Expression of Calcitonin Gene-Related Peptide and Calcitonin Gene-Related Peptide Receptors in the Human Intestine

Although the evidence gathered in experimental studies with laboratory animals attest to CGRP's activity in enhancing

intestinal peristalsis and secretion, species differences between laboratory animals and humans cannot *a priori* be neglected. Thus, it is important to review the available literature for information as to how CGRP impacts on intestinal motility and secretion in humans. As a matter of fact, however, the presence of CGRP and its physiological and pathophysiological implications in the human digestive tract have not as extensively been studied as in the gut of laboratory animals. The available information indicates that CGRP-like immunoreactivity in the human small intestine occurs not only in neuronal somata and fibers but also in mucosal endocrine cells (Timmermans et al., 1992; Brehmer et al., 2004; Cottrell et al., 2012). CGRP-positive enteric neurons are more abundant in the submucosal plexus than in the myenteric plexus, and some of the neuronal somata containing CGRP in the myenteric plexus of the human small intestine appear to be IPANs with a Dogiel type II morphology (Timmermans et al., 1992; Brehmer et al., 2004). Besides the peptide, CLR and RAMP1, the two subunits of functional CGRP receptors, have also been localized to the human stomach, small intestine and colon (Hagner et al., 2002; Cottrell et al., 2012). CLR-like immunoreactivity occurs in the myenteric and submucosal plexus, in nerve fibers within the circular and longitudinal muscle as well as in mucosal endocrine cells (Hagner et al., 2002; Cottrell et al., 2012). In addition, CLR and RAMP1 have been co-localized in the myenteric plexus where they are thought to form functional cell-surface receptors (Cottrell et al., 2012).

Functional Evidence for a Role of Calcitonin Gene-Related Peptide in Stimulating Intestinal Motility and Secretion and Causing Diarrhea

Only a limited number of studies has addressed the actions of CGRP on motor activity and secretory processes in isolated preparations of the human intestine. CGRP has been reported to have a weak relaxant effect in muscle strips of the human ileum and colon exposed to electrical field stimulation (Maggi et al., 1990; Smith and Smid, 2005). The ability of CGRP to instigate peristaltic motor activity has been studied in segments of the human jejunum. As seen in the rodent gut (**Figure 2**), mucosal stroking, 5-HT released from enterochromaffin cells or exposure to a 5-HT_4 receptor agonist causes the release of CGRP, presumably from IPANs, which gives rise to ascending contraction and descending relaxation of the circular muscle, these motor reflexes being prevented by $CGRP_{8-37}$ (Foxx-Orenstein et al., 1996; Grider et al., 1998). It is also likely that CGRP can stimulate chloride secretion in the mucosa of the human intestine as deduced from the prosecretory effect of CGRP in two human epithelial cell lines (Cox and Tough, 1994).

In view of the potential of erenumab to cause constipation (occasionally of a severe nature), as seen already in some pre-approval RCTs, Falkenberg et al. (2020) examined the effect of a 2-h intravenous infusion of CGRP (1.5 μg/min) to 30 healthy volunteers on 2 different days. CGRP was found to induce symptoms of gastrointestinal hypermotility in 93% of the participants on both study days. Rumbling, stomach pain, nausea, an urge to defecate and diarrhea were the most commonly experienced adverse effects during the infusion of the peptide. The symptoms were first noted after a delay of 20 min, on average reached a peak after 60 min and, with the exception of nausea, disappeared shortly after the infusion had been stopped. Since the symptoms were not prevented by sumatriptan, Falkenberg et al. (2020) classified the CGRP-induced disturbances of gastrointestinal function as a direct effect of the peptide on the gut.

With regard to the potential of CGRP antagonism/neutralization to cause constipation it is also relevant to ask whether idiopathic chronic constipation (ICC) may involve a change in the gastrointestinal CGRP system. However, this question is not yet possible to answer. While the number of CGRP-immunoreactive nerve fibers in the colonic myenteric ganglia of ICC patients was found to be markedly enhanced in one study (Dolk et al., 1990) it remained grossly unaltered in another study (Sjölund et al., 1997).

DIARRHEA ASSOCIATED WITH NEUROENDOCRINE TUMORS: POSSIBLE INVOLVEMENT OF CALCITONIN GENE-RELATED PEPTIDE

CGRP was discovered when it was realized that alternative processing (tissue-specific splicing) of the mRNA for calcitonin in the parafollicular (C) cells of the rat thyroid leads to production of CGRP, this peptide being widely expressed in neuronal tissue (Rosenfeld et al., 1983). Following the isolation of CGRP from patients with medullary thyroid carcinoma (MTC) (Morris et al., 1984), the peptide was, together with calcitonin, found to be expressed in the majority of MTCs and released into the circulation (Schifter et al., 1986; Takami et al., 1990; Hanna et al., 1997). Watery diarrhea has been reported to occur in some 30% of patients with MTC, this symptom being variously attributed to excess production of calcitonin and other factors (Hanna et al., 1997). CGRP doses administered to dogs mimicking the elevated plasma levels of CGRP found in human patients with MTC are able to stimulate water secretion in the small intestine and elicit diarrhea in 4 of 6 dogs (Reasbeck et al., 1988). The contention that high MTC-derived concentrations of CGRP in the circulation could contribute to the watery diarrhea accompanying MTC has likewise been put forward by Rolston et al. (1989) on the basis of the prosecretory effect of CGRP seen in rats. CGRP and calcitonin are also expressed in other neuroendocrine tumors associated with diarrhea such as carcinoid (Takami et al., 1990) and prostatic adenocarcinoma (Shulkes et al., 1991). The circulating levels of CGRP in prostatic adenocarcinoma correlate with the presence of diarrhea, and both the plasma levels of CGRP and the associated diarrhea have been found to be resistant to octreotide, a somatostatin analog commonly used in an attempt to mitigate tumor-associated symptoms (Shulkes et al., 1991).

Although no direct proof has yet been provided that MTC-associated diarrhea can be mitigated by CGRP antagonism or neutralization, the findings that elevated plasma levels of CGRP are associated with diarrhea indirectly support the contention that CGRP is able to stimulate intestinal motility and secretion. At the same time, this argument raises the possibility that

CGRP antagonism could be beneficial in controlling the diarrhea associated with CGRP-producing tumors.

MECHANISTIC BASIS OF CONSTIPATION CAUSED BY ANTI-CALCITONIN GENE-RELATED PEPTIDE MIGRAINE THERAPEUTICS

Evidence Indicating That Constipation Results From Inhibition of Calcitonin Gene-Related Peptide's Stimulant Effect on Intestinal Peristalsis and Secretion

The information reviewed herein attests CGRP both a motility-stimulating and prosecretory action in the intestine of various mammalian laboratory animals as well as humans (for a schematic summary see **Figure 3**). As there is evidence that CGRP is involved in the maintenance of peristaltic motor activity in the rodent and human gut, constipation evoked by CGRP antagonism/neutralization can be explained to result from interference with the physiological function of the peptide in the small and large intestine. In the gut, CGRP is expressed by distinct intrinsic enteric neurons and extrinsic primary afferent nerve fibers originating primarily in the dorsal root ganglia. The intrinsic enteric CGRP neurons participate in neuronal pathways that play a role in the initiation of propulsive motor activity and secretion of ions and water into the intestinal lumen (**Figure 3**). CGRP, in particular, is expressed in IPANs, intrinsic sensory neurons, of the mouse, rat, guinea-pig and pig (Brehmer et al., 2002; Furness et al., 2004; Wolf et al., 2007; Mitsui, 2009, 2011; Hibberd et al., 2018; Smolilo et al., 2020) and has also been localized to IPANs of the human small intestine (Timmermans et al., 1992; Brehmer et al., 2004). The CGRP-expressing IPANs contribute to the effect of mucosal stimulation to initiate peristaltic movements, given that both the ensuing ascending contraction and descending relaxation of the circular muscle in the mouse, rat, guinea-pig and human intestine *in vitro* are blunted by the CGRP receptor antagonist $CGRP_{8-37}$ (Foxx-Orenstein et al., 1996; Grider et al., 1998; Pan and Gershon, 2000; Grider, 2003).

Coordinated peristaltic motility requires a consecutive interplay of (ascending) contraction and (descending) relaxation of the circular muscle so that the contraction can propel the intestinal content aborally to an adjacent segment that is relaxed. The implication of CGRP in this motor pattern may explain why both excitatory (contractile) and inhibitory (relaxant) responses to CGRP have been reported in various isolated preparations of the intestine. It should not go unnoticed in this respect that experimental studies have provided consistent evidence that exogenous CGRP slows gastric emptying in rats (Lenz, 1988; Plourde et al., 1993; Julia and Buéno, 1997) and dogs (L'Heureux et al., 2000). Whether the peptide has a similar action in humans has not yet been examined. If CGRP were to inhibit gastric emptying in humans, this action might be a factor contributing to nausea which is another documented adverse effect of anti-CGRP migraine therapeutics. It is worth noting in this context that an ongoing phase 4 clinical trial investigates in which way treatment of adult migraine patients with erenumab or galcanezumab affects gastric emptying and gastrointestinal transit (ClinicalTrials.gov identifier: NCT04294147)[1].

The important clues to CGRP's role in facilitating intestinal propulsion and ion/water secretion obtained in *in vitro* investigations have been confirmed by the ability of CGRP to induce diarrhea *in vivo* as observed in mice (Kaiser et al., 2017), dogs (Reasbeck et al., 1988), and humans (Falkenberg et al., 2020). Thus, the pharmacological and physiological activities of CGRP in the intestine of laboratory animals can be extrapolated to humans. Although pretreatment of mice with CGRP antibodies and olcegepant is able to blunt CGRP-induced diarrhea (Kaiser et al., 2017), there is scarce information as to whether CGRP antagonism/neutralization in laboratory animals has *per se* an adverse effect on intestinal propulsion *in vivo*. While one study found $CGRP_{8-37}$ to delay gastrointestinal transit in mice (De Winter et al., 2009), most studies failed to see gastric corpus motility (Zittel et al., 1994), gastric emptying (De Winter et al., 2009), gastrointestinal transit (Glowka et al., 2015) and colonic transit (Zittel et al., 1998; Glowka et al., 2015) in rats and mice to be altered by $CGRP_{8-37}$, olcegepant or a CGRP antibody. In classifying these findings two aspects need be taken into account. On the one hand, peptidic antagonists such as $CGRP_{8-37}$ appear to have insufficient access to endosomal compartments that are also relevant to CLR signaling (Yarwood et al., 2017). On the other hand, in laboratory animals CGRP antibodies and antagonists have been tested only in short-term experiments whereas in humans CGRP-directed migraine therapeutics are used repeatedly for a prolonged period of time.

On the basis of the available evidence it can be concluded that CGRP plays a physiological role in stimulating intestinal propulsion, secretion and transit in humans. Blockade of this function is poised to lead to constipation as shown by the adverse effect of anti-CGRP therapeutics, a finding which at the same time confirms the involvement of CGRP in the maintenance of intestinal transit. In view of this reasoning, constipation cannot be classified as an indirect effect of CGRP-directed migraine therapeutics, as hoped by Haanes et al. (2020), but need be regarded as the result of blockade of CGRP signaling in the neural pathways underlying intestinal motor activity and secretion. In its medical implication this contention poses a major pharmacological challenge: How can the adverse effect of anti-CGRP therapeutics in the intestine be kept in check without compromising their anti-migraine action which is also thought to take place at a peripheral site outside the blood-brain barrier (Raffaelli and Reuter, 2018; Hargreaves and Olesen, 2019; Johnson et al., 2019; Messlinger and Russo, 2019; Christensen et al., 2020; Noseda et al., 2020)? At any rate, the risk of constipation associated with the use of antibodies targeting CGRP or its receptor need be accounted for in therapeutic decision making and treatment monitoring (Deligianni et al., 2021).

[1]https://clinicaltrials.gov/ct2/show/NCT04294147

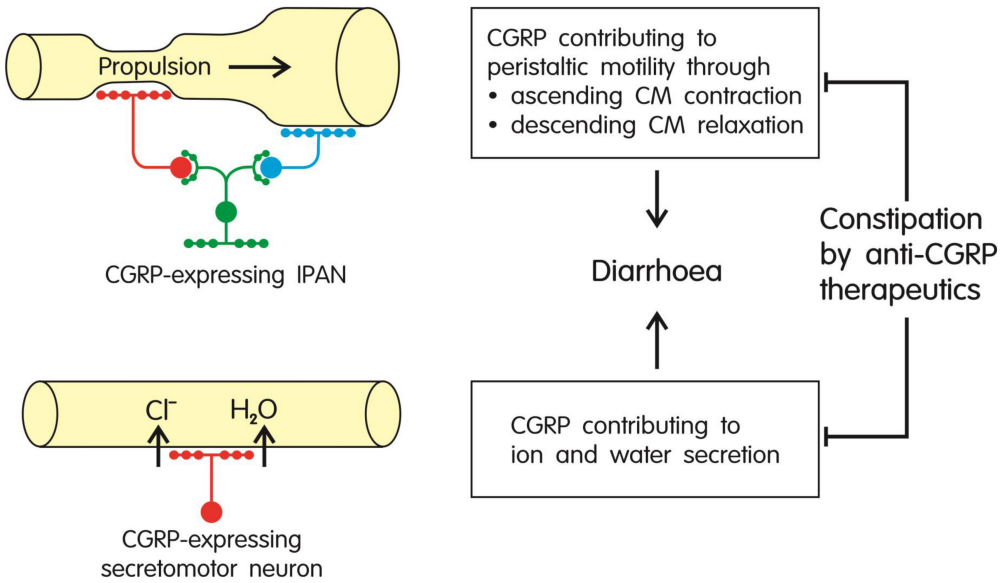

FIGURE 3 | Summary of the major effects of CGRP in the gut: stimulation of peristaltic motor activity and increased secretion of ions (primarily Cl^-) and water, both effects in combination causing diarrhea. As these effects of the peptide are likely to contribute to the maintenance of propulsive motility, ion and water homeostasis and intestinal transit, inhibition of CGRP signaling by anti-CGRP migraine therapeutics is expected to cause constipation. CM, circular muscle; IPAN, intrinsic primary afferent neuron.

Open Questions and Future Challenges

In its quest to explain whether constipation caused by anti-CGRP therapeutics can be explained by antagonism of the physiological role of CGRP in the intestine, this review was based on the disparity between the comparably low rates of constipation seen in the pre-approval RCTs of antibodies targeting CGRP or its receptor and the much higher rates reported in post-approval real-world surveys. Constipation was noted only in one RCT of atogepant (Ailani et al., 2021) and fremanezumab (Ferrari et al., 2019) but in three phase 3 trials of erenumab (Goadsby et al., 2017; Tepper et al., 2017; Takeshima et al., 2021) although in five other RCTs of erenumab (Sun et al., 2016; Dodick et al., 2018a; Reuter et al., 2018; Goadsby et al., 2020; Hirata et al., 2021) constipation went unmentioned. It was unforeseen, therefore, that substantial rates of constipation in patients treated with erenumab, fremanezumab and galcanezumab (see **Table 1**) were reported in post-approval surveys. This outcome contradicted the initial contention that the adverse effect profile of anti-CGRP migraine therapeutics is favorable and within the limits expected for this class of therapeutics. However, concerns that gastrointestinal adverse effects, particularly constipation, may be underrated and underreported were voiced before the post-approval survey data went public (Hargreaves and Olesen, 2019; Robbins, 2019; Falkenberg et al., 2020).

A straightforward explanation for the significantly higher constipation rates in migraine patients on a CGRP-targeting therapy under real world conditions as compared to RCT conditions is not yet available. Differences in the patient populations in terms of medical history and comorbidities as well as a more detailed assessment of adverse events and disturbances of life quality are likely to be contributory factors (Lambru et al., 2020; Faust et al., 2021). Important information may also be deduced from a future comparison of the real world constipation rates associated with the long-term use of gepants, on the one hand, and antibodies targeting the CGRP system, on the other hand. Differences in the constipation rates may provide clues as to whether pharmacodynamic and/or pharmacokinetic factors play a role. The two classes of anti-CGRP therapeutics differ profoundly in their pharmacokinetic profile, given that gepants have a comparatively short biological half-life (5–11 h) while the CGRP and CGRP receptor antibodies have a biological half-life of 27–31 d (for reviews see Szkutnik-Fiedler, 2020; Omaer et al., 2021). Thus it may make a difference if CGRP signaling in the intestine is continuously blocked by antibody therapeutics for a prolonged period of time as compared to a more intermittent receptor blockade caused by gepant therapy.

In addition, there may also be appreciable pharmacodynamic differences between the different classes of anti-CGRP migraine therapeutics. The CGRP receptors operating in the human intestine that are blocked by the gepant and antibody therapeutics have not yet been characterized in sufficient detail. Together with calcitonin, CGRP, amylin, adrenomedullin and intermedin form a family of peptides that can use different receptors of a related structure for their biological actions (Hay et al., 2018). CGRP is not only an agonist at the CGRP receptor (a CLR/RAMP1 heterodimer) but also at the AMY_1 receptor, a heterodimer of CTR (calcitonin receptor) and RAMP1, at which amylin is also an agonist (Hay et al., 2018). It has recently been reported that the available CGRP-directed migraine therapeutics differ in their receptor selectivity and their signaling via cAMP production (Bhakta et al., 2021). While erenumab and telcagepant antagonize not only CGRP but also adrenomedullin and intermedin at

cAMP signaling through the CGRP receptor, fremanezumab is a specific antagonist of CGRP at the CGRP receptor (Bhakta et al., 2021). In contrast, erenumab and telcagepant also bind to the AMY_1 receptor and inhibit cAMP signaling through this receptor (Bhakta et al., 2021). If different receptors such a CLR/RAMP1 and CTR/RAMP1 were to be involved in the action of CGRP and related peptides on intestinal motor activity and secretion, different classes of anti-CGRP therapeutics may also differ in their propensity to cause constipation. Indeed, the potency of $CGRP_{8-37}$ in antagonizing the prosecretory response to α-CGRP in the rat and mouse colon and in the human epithelial cell line Colony-29 is very low as compared with other effects of the peptide (Cox and Tough, 1994; Cox, 1995; Esfandyari et al., 2000; Tough et al., 2018), which has been taken to conclude that a different type of receptor mediates the $CGRP_{8-37}$-insensitive action of CGRP.

An important open question is: what medications are available to control the constipation brought about by anti-CGRP therapeutics while their anti-migraine action is maintained? Arguably this goal may be best achieved by prokinetic and laxative drugs that are suitable for long-term use and have a site of action that is restricted to the gut. In this respect it is reassuring to note that the repository of prescription medications used to treat chronic constipation has been appreciably expanded in the past 15 years (Herbert and Holzer, 2008; Bassotti et al., 2021; Wechsler and Shah, 2021). The drugs at hand include 5-HT_4 receptor agonists (e.g., tegaserod, prucalopride), osmotic laxatives (e.g., polyethylene glycols), stimulant laxatives (e.g., bisacodyl) and a number of compounds that act on newly identified gastrointestinal targets. These include lubiprostone (an activator of CIC2 chloride channels), linaclotide and plecanatide (activators of guanylate cyclase C), tenapanor (an inhibitor of the sodium-hydrogen exchanger NHE3) and elobixibat (an inhibitor of the ileal bile acid transporter). It is not possible to judge which of these medications would be particularly suitable for the control of constipation caused by CGRP blockade before any experience based on clinical studies has been gained. Acetylcholinesterase inhibitors such as neostigmine and distigmine which enhance muscle tone are used to treat gastrointestinal pseudo-obstruction disorder but not chronic constipation because they induce muscle spasms at higher dosage, inhibit rather than promote peristalsis (Fruhwald et al., 2004), affect other peripheral cholinergic systems (e.g., in urinary system, heart and lung) and may lead to cholinergic crisis. The gastroprokinetic drugs domperidone (a dopamine D_2 receptor antagonist) and metoclopramide (a dopamine D_2 and 5-HT_3 receptor antagonist and 5-HT_4 receptor agonist) are likewise contra-indicated since safety concerns led EMA to restrict these drugs to short-term use.

In a final note, the activity of antibodies targeting the CGRP system to bring about constipation may be exploited therapeutically in intestinal disorders in which endogenous CGRP contributes to diarrhea, as has been suggested by Kaiser et al. (2017). For instance, it would be an option to explore the capacity of anti-CGRP therapeutics to control the diarrhea associated with neuroendocrine tumors such MTC, carcinoid and prostatic adenocarcinoma, in which the overexpression of CGRP results in elevated circulating levels of the peptide (Morris et al., 1984; Schifter et al., 1986; Takami et al., 1990; Shulkes et al., 1991; Hanna et al., 1997). If CGRP were a causative factor, CGRP blockade could be another approach to control diarrhea, complementing established treatment modes including somatostatin analogs such as octreotide. Other pathological conditions in which anti-CGRP therapeutics could have a beneficial effect include diarrhea caused by bacterial infection, given that the intestinal water secretion and inflammation evoked by *Clostridioides difficile* toxin A in rats depends on endogenous CGRP (Keates et al., 1998; Bhargava et al., 2013). Similarly, the accelerated gastrointestinal transit evoked by ovalbumin anaphylaxis, which is blunted in mice deficient in RAMP1 (Yoshikawa et al., 2011), suggests that food allergy-associated diarrhea could be susceptible to the therapeutic use of CGRP signaling blockers. Inflammatory bowel disease and the diarrhea-predominant form of irritable bowel syndrome (IBS-D) are also pathologies characterized by enhanced intestinal secretion and transit. It is unlikely, however, that inhibition of CGRP signaling is a therapeutic option under inflammatory conditions, given that both exogenous and endogenous CGRP protects from experimentally induced colitis in laboratory animals (Evangelista and Tramontana, 1993; Reinshagen et al., 1998; Mazelin et al., 1999; Clifton et al., 2007; Thompson et al., 2008; Engel et al., 2011; de Jong et al., 2015; Kawashima-Takeda et al., 2017; Yamasaki et al., 2020). In conclusion, the capacity of antibodies targeting CGRP or its receptor to inhibit intestinal secretion and transit offers a new lead for therapeutic exploitation in select pathologies in which CGRP overactivity is a pathophysiological factor.

AUTHOR CONTRIBUTIONS

PH and UH-P wrote the manuscript and drew the figures. Both authors contributed to the article and approved the submitted version.

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REFERENCES

Aamodt, A. H., Stovner, L. J., Hagen, K., and Zwart, J. A. (2008). Comorbidity of headache and gastrointestinal complaints. The Head-HUNT Study. *Cephalalgia* 28, 1444–1451. doi: 10.1111/j.1468-2982.2007.01486.x

Ailani, J., Lipton, R. B., Goadsby, P. J., Guo, H., Miceli, R., Severt, L., et al. (2021). Atogepant for the preventive treatment of migraine. *N. Engl. J. Med.* 385, 695–706. doi: 10.1056/NEJMoa2035908

Ailani, J., Lipton, R. B., Hutchinson, S., Knievel, K., Lu, K., Butler, M., et al. (2020). Long-term safety evaluation of ubrogepant for the acute treatment of

migraine: phase 3, randomized, 52-week extension trial. *Headache* 60, 141–152. doi: 10.1111/head.13682

Akiba, Y., Nakamura, M., Nagata, H., Kaunitz, J. D., and Ishii, H. (2002). Acid-sensing pathways in rat gastrointestinal mucosa. *J. Gastroenterol.* 37(Suppl. 14), 133–138. doi: 10.1007/BF03326432

Alemi, F., Poole, D. P., Chiu, J., Schoonjans, K., Cattaruzza, F., Grider, J. R., et al. (2013). The receptor TGR5 mediates the prokinetic actions of intestinal bile acids and is required for normal defecation in mice. *Gastroenterology* 144, 145–154. doi: 10.1053/j.gastro.2012.09.055

Alex, A., Vaughn, C., and Rayhill, M. (2020). Safety and tolerability of 3 CGRP monoclonal antibodies in practice: a retrospective cohort study. *Headache* 60, 2454–2462. doi: 10.1111/head.13956

Ashina, H., Iljazi, A., Al-Khazali, H. M., Eigenbrodt, A. K., Larsen, E. L., Andersen, A. M., et al. (2020). Efficacy, tolerability, and safety of erenumab for the preventive treatment of persistent post-traumatic headache attributed to mild traumatic brain injury: an open-label study. *J. Headache Pain* 21:62. doi: 10.1186/s10194-020-01136-z

Ashina, M., Saper, J., Cady, R., Schaeffler, B. A., Biondi, D. M., Hirman, J., et al. (2020). Eptinezumab in episodic migraine: a randomized, double-blind, placebo-controlled study (PROMISE-1). *Cephalalgia* 40, 241–254. doi: 10.1177/0333102420905132

Bäck, N., Ahonen, M., Häppölä, O., Kivilaakso, E., and Kiviluoto, T. (1994). Effect of vagotomy on expression of neuropeptides and histamine in rat oxyntic mucosa. *Digest. Dis. Sci.* 39, 353–361. doi: 10.1007/BF02090208

Barthó, L., and Holzer, P. (1995). The inhibitory modulation of guinea-pig intestinal peristalsis caused by capsaicin involves calcitonin gene-related peptide and nitric oxide. *Naunyn Schmiedebergs Arch. Pharmacol.* 353, 102–109. doi: 10.1007/BF00168922

Barthó, L., Kóczán, G., Holzer, P., Maggi, C. A., and Szolcsányi, J. (1991). Antagonism of the effects of calcitonin gene-related peptide and of capsaicin on the guinea-pig isolated ileum by human alpha-calcitonin gene-related peptide(8-37). *Neurosci. Lett.* 129, 156–159. doi: 10.1016/0304-3940(91)90744-e

Bassotti, G., Usai Satta, P., and Bellini, M. (2021). Chronic idiopathic constipation in adults: a review on current guidelines and emerging treatment options. *Clin. Exp. Gastroenterol.* 14, 413–428. doi: 10.2147/CEG.S256364

Belvís, R., Irimia, P., Pozo-Rosich, P., González-Oria, C., Cano, A., Viguera, J., et al. (2021). MAB-MIG: registry of the Spanish Neurological Society of erenumab for migraine prevention. *J. Headache Pain* 22:74. doi: 10.1186/s10194-021-01267-x

Bhakta, M., Vuong, T., Taura, T., Wilson, D. S., Stratton, J. R., and Mackenzie, K. D. (2021). Migraine therapeutics differentially modulate the CGRP pathway. *Cephalalgia* 41, 499–514. doi: 10.1177/0333102420983282

Bhargava, A., Clifton, M. S., Mhaske, P., Liao, M., Pothoulakis, C., Leeman, S. E., et al. (2013). Local injection of dsRNA targeting calcitonin receptor-like receptor (CLR) ameliorates *Clostridium difficile* toxin A-induced ileitis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 731–736. doi: 10.1073/pnas.1219733110

Brehmer, A., Croner, R., Dimmler, A., Papadopoulos, T., Schrödl, F., and Neuhuber, W. (2004). Immunohistochemical characterization of putative primary afferent (sensory) myenteric neurons in human small intestine. *Auton. Neurosci.* 112, 49–59. doi: 10.1016/j.autneu.2004.03.005

Brehmer, A., Schrödl, F., and Neuhuber, W. (2002). Correlated morphological and chemical phenotyping in myenteric type V neurons of porcine ileum. *J. Comp. Neurol.* 453, 1–9. doi: 10.1002/cne.10358

Camporeale, A., Kudrow, D., Sides, R., Wang, S., Van Dycke, A., Selzler, K. J., et al. (2018). A phase 3, long-term, open-label safety study of galcanezumab in patients with migraine. *BMC Neurol.* 18:188. doi: 10.1186/s12883-018-1193-2

Chen, B. N., Olsson, C., Sharrad, D. F., and Brookes, S. J. (2016). Sensory innervation of the guinea pig colon and rectum compared using retrograde tracing and immunohistochemistry. *Neurogastroenterol. Motil.* 28, 1306–1316. doi: 10.1111/nmo.12825

Chiang, C. C., Arca, K. N., Dunn, R. B., Girardo, M. E., Quillen, J. K., Dodick, D. W., et al. (2021). Real-world efficacy, tolerability, and safety of ubrogepant. *Headache* 61, 620–627. doi: 10.1111/head.14062

Christensen, S. L., Ernstsen, C., Olesen, J., and Kristensen, D. M. (2020). No central action of CGRP antagonising drugs in the GTN mouse model of migraine. *Cephalalgia* 40, 924–934. doi: 10.1177/0333102420914913

Clifton, M. S., Hoy, J. J., Chang, J., Idumalla, P. S., Fakhruddin, H., Grady, E. F., et al. (2007). Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am. J. Physiol.* 293, G36–G44. doi: 10.1152/ajpgi.00464.2006

Cottrell, G. S., Alemi, F., Kirkland, J. G., Grady, E. F., Corvera, C. U., and Bhargava, A. (2012). Localization of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1) in human gastrointestinal tract. *Peptides* 35, 202–211. doi: 10.1016/j.peptides.2012.03.020

Cox, H. M. (1995). Receptors for calcitonin gene related peptide (CGRP) in gastrointestinal epithelia. *Can. J. Physiol. Pharmacol.* 73, 974–980. doi: 10.1139/y95-135

Cox, H. M., and Tough, I. R. (1994). Calcitonin gene-related peptide receptors in human gastrointestinal epithelia. *Br. J. Pharmacol.* 113, 1243–1248. doi: 10.1111/j.1476-5381.1994.tb17131.x

Croop, R., Goadsby, P. J., Stock, D. A., Conway, C. M., Forshaw, M., Stock, E. G., et al. (2019). Efficacy, safety, and tolerability of rimegepant orally disintegrating tablet for the acute treatment of migraine: a randomised, phase 3, double-blind, placebo-controlled trial. *Lancet* 394, 737–745. doi: 10.1016/S0140-6736(19)31606-X

de Jong, P. R., Takahashi, N., Peiris, M., Bertin, S., Lee, J., Gareau, M. G., et al. (2015). TRPM8 on mucosal sensory nerves regulates colitogenic responses by innate immune cells via CGRP. *Mucosal Immunol.* 8, 491–504. doi: 10.1038/mi.2014.82

de Vries Lentsch, S., Verhagen, I. E., van den Hoek, T. C., MaassenVanDenBrink, A., and Terwindt, G. M. (2021). Treatment with the monoclonal calcitonin gene-related peptide receptor antibody erenumab: a real-life study. *Eur. J. Neurol.* 28, 4194–4203. doi: 10.1111/ene.15075

De Winter, B. Y., Bredenoord, A. J., Van Nassauw, L., De Man, J. G., De Schepper, H. U., Timmermans, J. P., et al. (2009). Involvement of afferent neurons in the pathogenesis of endotoxin-induced ileus in mice: role of CGRP and TRPV1 receptors. *Eur. J. Pharmacol.* 615, 177–184. doi: 10.1016/j.ejphar.2009.04.055

Deen, M., Correnti, E., Kamm, K., Kelderman, T., Papetti, L., Rubio-Beltrán, E., et al. (2017). Blocking CGRP in migraine patients - a review of pros and cons. *J. Headache Pain* 18:96. doi: 10.1186/s10194-017-0807-1

Deligianni, C. I., Mitsikostas, D. D., and Ashina, M. (2021). Safety and tolerability evaluation of erenumab for the preventive treatment of migraine. *Expert Opin. Drug Saf.* 20, 867–876. doi: 10.1080/14740338.2021.1933941

Detke, H. C., Goadsby, P. J., Wang, S., Friedman, D. I., Selzler, K. J., and Aurora, S. K. (2018). Galcanezumab in chronic migraine: the randomized, double-blind, placebo-controlled REGAIN study. *Neurology* 91, e2211–e2221. doi: 10.1212/WNL.0000000000006640

Dodick, D. W., Ashina, M., Brandes, J. L., Kudrow, D., Lanteri-Minet, M., Osipova, V., et al. (2018a). A Phase 3 randomized trial of erenumab for episodic migraine. *Cephalalgia* 38, 1026–1037. doi: 10.1177/0333102418759786

Dodick, D. W., Silberstein, S. D., Bigal, M. E., Yeung, P. P., Goadsby, P. J., Blankenbiller, T., et al. (2018b). Effect of fremanezumab compared with placebo for prevention of episodic migraine: a randomized clinical trial. *JAMA* 319, 1999–2008. doi: 10.1001/jama.2018.4853

Dodick, D. W., Lipton, R. B., Ailani, J., Lu, K., Finnegan, M., Trugman, J. M., et al. (2019). Ubrogepant for the treatment of migraine. *N. Engl. J. Med.* 381, 2230–2241. doi: 10.1056/NEJMoa1813049

Dolk, A., Brodén, G., Holmström, B., Johansson, C., and Schultzberg, M. (1990). Slow transit chronic constipation (Arbuthnot Lane's disease). An immunohistochemical study of neuropeptide-containing nerves in resected specimens from the large bowel. *Int. J. Colorectal Dis.* 5, 181–187. doi: 10.1007/BF00303272

Edvinsson, L. (2019). Role of CGRP in migraine. *Handb. Exp. Pharmacol.* 255, 121–130. doi: 10.1007/164_2018_201

Eghtesadi, M., Leroux, E., and Pagé, G. (2021). Real-life response to erenumab in a therapy-resistant case series of migraine patients from the province of Quebec, Eastern Canada. *Clin. Drug Investig.* 41, 733–739. doi: 10.1007/s40261-021-01059-w

Ekblad, E., Winther, C., Ekman, R., Håkanson, R., and Sundler, F. (1987). Projections of peptide-containing neurons in rat small intestine. *Neuroscience* 20, 169-188. doi: 10.1016/0306-4522(87)90010-8

Engel, M. A., Leffler, A., Niedermirtl, F., Babes, A., Zimmermann, K., Filipović, M. R., et al. (2011). TRPA1 and substance P mediate colitis in mice. *Gastroenterology* 141, 1346–1358. doi: 10.1053/j.gastro.2011.07.002

Esfandyari, T., Macnaughton, W. K., Quirion, R., St Pierre, S., Junien, J. L., and Sharkey, K. A. (2000). A novel receptor for calcitonin gene-related peptide (CGRP) mediates secretion in the rat colon: implications for secretory function in colitis. *FASEB J.* 14, 1439–1446. doi: 10.1096/fj.14.10.1439

Evangelista, S., and Tramontana, M. (1993). Involvement of calcitonin gene-related peptide in rat experimental colitis. *J. Physiol. Paris* 87, 277–280. doi: 10.1016/0928-4257(93)90017-n

Falkenberg, K., Bjerg, H. R., and Olesen, J. (2020). Two-hour CGRP infusion causes gastrointestinal hyperactivity: possible relevance for CGRP antibody treatment. *Headache* 60, 929–937. doi: 10.1111/head.13795

Farmer, A. D., Drewes, A. M., Chiarioni, G., De Giorgio, R., O'Brien, T., Morlion, B., et al. (2019). Pathophysiology and management of opioid-induced constipation: European expert consensus statement. *United Eur. Gastroenterol. J.* 7, 7–20. doi: 10.1177/2050640618818305

Faust, E., Pivneva, I., Yang, K., Betts, K. A., Ahmed, Z., Joshi, S., et al. (2021). Real-world treatment profiles, clinical outcomes, and healthcare resource utilization of patients with migraine prescribed erenumab: a multicenter chart-review study of US Headache Centers. *Neurol. Ther.* 10, 293–306. doi: 10.1007/s40120-021-00245-4

Ferrari, M. D., Diener, H. C., Ning, X., Galic, M., Cohen, J. M., Yang, R., et al. (2019). Fremanezumab versus placebo for migraine prevention in patients with documented failure to up to four migraine preventive medication classes (FOCUS): a randomised, double-blind, placebo-controlled, phase 3b trial. *Lancet* 394, 1030–1040. doi: 10.1016/S0140-6736(19)31946-4

Forster, E. R., and Dockray, G. J. (1991). Immunoneutralization suggests that calcitonin gene related peptide regulates gastric emptying in the rat. *Neurosci. Lett.* 131, 5–7. doi: 10.1016/0304-3940(91)90324-m

Foxx-Orenstein, A. E., Kuemmerle, J. F., and Grider, J. R. (1996). Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. *Gastroenterology* 111, 1281–1290. doi: 10.1053/gast.1996.v111.pm8898642

Freeman, M. E., Cheng, G., and Hocking, M. P. (1999). Role of alpha- and beta-calcitonin gene-related peptide in postoperative small bowel ileus. *J. Gastrointest. Surg.* 3, 39–43. doi: 10.1016/s1091-255x(99)80006-7

Fruhwald, S., Herk, E., Hammer, H. F., Holzer, P., and Metzler, H. (2004). Differential reversal of drug-induced small bowel paralysis by cerulein and neostigmine. *Intensive Care Med.* 30, 1414–1420. doi: 10.1007/s00134-004-2317-2

Furness, J. B., Costa, M., Gibbins, I. L., Llewellyn-Smith, I. J., and Oliver, J. R. (1985). Neurochemically similar myenteric and submucous neurons directly traced to the mucosa of the small intestine. *Cell Tissue Res.* 241, 155–163. doi: 10.1007/BF00214637

Furness, J. B., Robbins, H. L., Xiao, J., Stebbing, M. J., and Nurgali, K. (2004). Projections and chemistry of Dogiel type II neurons in the mouse colon. *Cell Tissue Res.* 317, 1–12. doi: 10.1007/s00441-004-0895-5

Gates, T. S., Zimmerman, R. P., Mantyh, C. R., Vigna, S. R., and Mantyh, P. W. (1989). Calcitonin gene-related peptide-α receptor binding sites in the gastrointestinal tract. *Neuroscience* 31, 757–770. doi: 10.1016/0306-4522(89)90439-9

Glowka, T. R., Steinebach, A., Stein, K., Schwandt, T., Lysson, M., Holzmann, B., et al. (2015). The novel CGRP receptor antagonist BIBN4096BS alleviates a postoperative intestinal inflammation and prevents postoperative ileus. *Neurogastroenterol. Motil.* 27, 1038–1049. doi: 10.1111/nmo.12584

Goadsby, P. J., Reuter, U., Hallström, Y., Broessner, G., Bonner, J. H., Zhang, F., et al. (2017). A controlled trial of erenumab for episodic migraine. *N. Engl. J. Med.* 377, 2123–2132. doi: 10.1056/NEJMoa1705848

Goadsby, P. J., Reuter, U., Hallström, Y., Broessner, G., Bonner, J. H., Zhang, F., et al. (2020). One-year sustained efficacy of erenumab in episodic migraine: results of the STRIVE study. *Neurology* 95, e469–e479. doi: 10.1212/WNL.0000000000010019

Green, T., and Dockray, G. J. (1988). Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. *Neuroscience* 25, 181-193. doi: 10.1016/0306-4522(88)90017-6

Grider, J. R. (1994). CGRP as a transmitter in the sensory pathway mediating peristaltic reflex. *Am. J. Physiol.* 266, G1139–G1145. doi: 10.1152/ajpgi.1994.266.6.G1139

Grider, J. R. (2003). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J. Pharmacol. Exp. Ther.* 307, 460–467. doi: 10.1124/jpet.103.053512

Grider, J. R., Foxx-Orenstein, A. E., and Jin, J. G. (1998). 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* 115, 370–380. doi: 10.1016/s0016-5085(98)70203-3

Haanes, K. A., Edvinsson, L., and Sams, A. (2020). Understanding side-effects of anti-CGRP and anti-CGRP receptor antibodies. *J. Headache Pain* 21:26. doi: 10.1186/s10194-020-01097-3

Hagner, S., Stahl, U., Knoblauch, B., McGregor, G. P., and Lang, R. E. (2002). Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. *Cell Tissue Res.* 310, 41–50. doi: 10.1007/s00441-002-0616-x

Hanna, F. W., Ardill, J. E., Johnston, C. F., Cunningham, R. T., Curry, W. J., Russell, C. F., et al. (1997). Regulatory peptides and other neuroendocrine markers in medullary carcinoma of the thyroid. *J. Endocrinol.* 152, 275–281. doi: 10.1677/joe.0.1520275

Hargreaves, R., and Olesen, J. (2019). Calcitonin gene-related peptide modulators - the history and renaissance of a new migraine drug class. *Headache* 59, 951–970. doi: 10.1111/head.13510

Hay, D. L., Garelja, M. L., Poyner, D. R., and Walker, C. S. (2018). Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. *Br. J. Pharmacol.* 175, 3–17. doi: 10.1111/bph.14075

Herbert, M. K., and Holzer, P. (2008). Standardized concept for the treatment of gastrointestinal dysmotility in critically ill patients - current status and future options. *Clin. Nutr.* 27, 25–41. doi: 10.1016/j.clnu.2007.08.001

Hibberd, T. J., Travis, L., Wiklendt, L., Costa, M., Brookes, S. J. H., Hu, H., et al. (2018). Synaptic activation of putative sensory neurons by hexamethonium-sensitive nerve pathways in mouse colon. *Am. J. Physiol.* 314, G53–G64. doi: 10.1152/ajpgi.00234.2017

Hirata, K., Sakai, F., Takeshima, T., Imai, N., Matsumori, Y., Yoshida, R., et al. (2021). Efficacy and safety of erenumab in Japanese migraine patients with prior preventive treatment failure or concomitant preventive treatment: subgroup analyses of a phase 3, randomized trial. *J. Headache Pain* 22:110. doi: 10.1186/s10194-021-01313-8

Ho, T. W., Ferrari, M. D., Dodick, D. W., Galet, V., Kost, J., Fan, X., et al. (2008). Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. *Lancet* 372, 2115–2123. doi: 10.1016/S0140-6736(08)61626-8

Holzer, P. (1994). "Calcitonin gene-related peptide," in *Gut Peptides: Biochemistry and Physiology*, eds J. H. Walsh and G. J. Dockray (New York: Raven Press), 493–523.

Holzer, P. (1998). Neural emergency system in the stomach. *Gastroenterology* 114, 823–839. doi: 10.1016/s0016-5085(98)70597-9

Holzer, P. (2000). "Calcitonin gene-related peptide in gastrointestinal homeostasis," in *The CGRP Family: Calcitonin Gene-Related Peptide (CGRP), Amylin, and Adrenomedullin*, eds D. Poyner, I. Marshall, and S. D. Brain (Georgetown, TX: Landes Bioscience), 125–139.

Holzer, P., Barthó, L., Matusák, O., and Bauer, V. (1989). Calcitonin gene-related peptide action on intestinal circular muscle. *Am. J. Physiol.* 256, G546–G552. doi: 10.1152/ajpgi.1989.256.3.G546

Jakate, A., Blumenfeld, A. M., Boinpally, R., Butler, M., Borbridge, L., Contreras-De Lama, J., et al. (2021). Pharmacokinetics and safety of ubrogepant when coadministered with calcitonin gene-related peptide-targeted monoclonal antibody migraine preventives in participants with migraine: a randomized phase 1b drug-drug interaction study. *Headache* 61, 642–652. doi: 10.1111/head.14095

Johnson, K. W., Morin, S. M., Wroblewski, V. J., and Johnson, M. P. (2019). Peripheral and central nervous system distribution of the CGRP neutralizing antibody [^{125}I]galcanezumab in male rats. *Cephalalgia* 39, 1241–1248. doi: 10.1177/0333102419844711

Julia, V., and Buéno, L. (1997). Tachykininergic mediation of viscerosensitive responses to acute inflammation in rats: role of CGRP. *Am. J. Physiol.* 272, G141–G146. doi: 10.1152/ajpgi.1997.272.1.G141

Kaiser, E. A., Rea, B. J., Kuburas, A., Kovacevich, B. R., Garcia-Martinez, L. F., Recober, A., et al. (2017). Anti-CGRP antibodies block CGRP-induced diarrhea in mice. *Neuropeptides* 64, 95–99. doi: 10.1016/j.npep.2016.11.004

Kanaan, S., Hettie, G., Loder, E., and Burch, R. (2020). Real-world effectiveness and tolerability of erenumab: a retrospective cohort study. *Cephalalgia* 40, 1511–1522. doi: 10.1177/0333102420946725

Kawashima-Takeda, N., Ito, Y., Nishizawa, N., Kawashima, R., Tanaka, K., Tsujikawa, K., et al. (2017). RAMP1 suppresses mucosal injury from dextran sodium sulfate-induced colitis in mice. *J. Gastroenterol. Hepatol.* 32, 809–818. doi: 10.1111/jgh.13505

Keates, A. C., Castagliuolo, I., Qiu, B., Nikulasson, S., Sengupta, A., and Pothoulakis, C. (1998). CGRP upregulation in dorsal root ganglia and ileal mucosa during *Clostridium difficile* toxin A-induced enteritis. *Am. J. Physiol.* 274, G196–G202. doi: 10.1152/ajpgi.1998.274.1.G196

Kelman, L. (2004). The premonitory symptoms (prodrome): a tertiary care study of 893 migraineurs. *Headache* 44, 865–872. doi: 10.1111/j.1526-4610.2004.04168.x

Kojima, S., and Shimo, Y. (1995). Calcitonin gene-related peptide (CGRP)-enhanced non-adrenergic non-cholinergic contraction of guinea-pig proximal colon. *Br. J. Pharmacol.* 115, 1290–1294. doi: 10.1111/j.1476-5381.1995.tb15038.x

Kudrow, D., Cady, R. K., Allan, B., Pederson, S. M., Hirman, J., Mehta, L. R., et al. (2021). Long-term safety and tolerability of eptinezumab in patients with chronic migraine: a 2-year, open-label, phase 3 trial. *BMC Neurol.* 21:126. doi: 10.1186/s12883-021-02123-w

Lacy, B. E., Mearin, F., Chang, L., Chey, W. D., Lembo, A. J., Simrén, M., et al. (2016). Bowel disorders. *Gastroenterology* 150, 1393–1407. doi: 10.1053/j.gastro.2016.02.031

Lambru, G., Hill, B., Murphy, M., Tylova, I., and Andreou, A. P. (2020). A prospective real-world analysis of erenumab in refractory chronic migraine. *J. Headache Pain* 21:61. doi: 10.1186/s10194-020-01127-0

Lenz, J. H. (1988). Calcitonin and CGRP inhibit gastrointestinal transit via distinct neuronal pathways. *Am. J. Physiol.* 254, G920–G924. doi: 10.1152/ajpgi.1988.254.6.G920

L'Heureux, M. C., St-Pierre, S., Trudel, L., Plourde, V., Lepage, R., and Poitras, P. (2000). Digestive motor effects and vascular actions of CGRP in dog are expressed by different receptor subtypes. *Peptides* 21, 425–430. doi: 10.1016/s0196-9781(00)00160-1

Lipton, R. B., Croop, R., Stock, E. G., Stock, D. A., Morris, B. A., Frost, M., et al. (2019a). Rimegepant, an oral calcitonin gene-related peptide receptor antagonist, for migraine. *N. Engl. J. Med.* 381, 142–149. doi: 10.1056/NEJMoa1811090

Lipton, R. B., Dodick, D. W., Ailani, J., Lu, K., Finnegan, M., Szegedi, A., et al. (2019b). Effect of ubrogepant vs placebo on pain and the most bothersome associated symptom in the acute treatment of migraine: the ACHIEVE II randomized clinical trial. *JAMA* 322, 1887–1898. doi: 10.1001/jama.2019.16711

Lipton, R. B., Goadsby, P. J., Smith, J., Schaeffler, B. A., Biondi, D. M., Hirman, J., et al. (2020). Efficacy and safety of eptinezumab in patients with chronic migraine: PROMISE-2. *Neurology* 94, e1365–e1377. doi: 10.1212/WNL.0000000000009169

MaassenVanDenBrink, A., Meijer, J., Villalón, C. M., and Ferrari, M. D. (2016). Wiping out CGRP: potential cardiovascular risks. *Trends Pharmacol. Sci.* 37, 779–788. doi: 10.1016/j.tips.2016.06.002

Maggi, C. A. (1995). Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. *Prog. Neurobiol.* 45, 1–98. doi: 10.1016/0301-0082(94)e0017-b

Maggi, C. A., Giuliani, S., and Santicioli, P. (1997). CGRP potentiates excitatory transmission to the circular muscle of guinea-pig colon. *Regul. Pept.* 69, 127–136. doi: 10.1016/s0167-0115(97)00006-2

Maggi, C. A., Giuliani, S., and Zagorodnyuk, V. (1996). Calcitonin gene-related peptide (CGRP) in the circular muscle of guinea-pig colon: role as inhibitory transmitter and mechanisms of relaxation. *Regul. Pept.* 61, 27–36. doi: 10.1016/0167-0115(95)00133-6

Maggi, C. A., Patacchini, R., Santicioli, P., Giuliani, S., Turini, D., Barbanti, G., et al. (1990). Human isolated ileum: motor responses of the circular muscle to electrical field stimulation and exogenous neuropeptides. *Naunyn Schmiedebergs Arch. Pharmacol.* 341, 256–261. doi: 10.1007/BF00169740

Mazelin, L., Theodorou, V., Fioramonti, J., and Bueno, L. (1999). Vagally dependent protective action of calcitonin gene-related peptide on colitis. *Peptides* 20, 1367–1374. doi: 10.1016/s0196-9781(99)00144-8

McCulloch, C. R., and Cooke, H. J. (1989). Human α-calcitonin gene-related peptide influences colonic secretion by acting on myenteric neurons. *Regul. Pept.* 24, 87–96. doi: 10.1016/0167-0115(89)90214-0

Messlinger, K., and Russo, A. F. (2019). Current understanding of trigeminal ganglion structure and function in headache. *Cephalalgia* 39, 1661–1674. doi: 10.1177/0333102418786261

Mitsui, R. (2009). Characterisation of calcitonin gene-related peptide-immunoreactive neurons in the myenteric plexus of rat colon. *Cell Tissue Res.* 337, 37–43. doi: 10.1007/s00441-009-0798-6

Mitsui, R. (2011). Immunohistochemical analysis of substance P-containing neurons in rat small intestine. *Cell Tissue Res.* 343, 331–341. doi: 10.1007/s00441-010-1080-7

Mongardi Fantaguzzi, C., Thacker, M., Chiocchetti, R., and Furness, J. B. (2009). Identification of neuron types in the submucosal ganglia of the mouse ileum. *Cell Tissue Res.* 336, 179–189. doi: 10.1007/s00441-009-0773-2

Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I., and MacIntyre, I. (1984). Isolation and characterization of human calcitonin gene-related peptide. *Nature* 308, 746–748. doi: 10.1038/308746a0

Mulderry, P. K., Ghatei, M. A., Spokes, R. A., Jones, P. M., Pierson, A. M., Hamid, Q. A., et al. (1988). Differential expression of α-CGRP and ß-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 25, 195–205. doi: 10.1016/0306-4522(88)90018-8

Mulleners, W. M., Kim, B. K., Láinez, M. J. A., Lanteri-Minet, M., Pozo-Rosich, P., Wang, S., et al. (2020). Safety and efficacy of galcanezumab in patients for whom previous migraine preventive medication from two to four categories had failed (CONQUER): a multicentre, randomised, double-blind, placebo-controlled, phase 3b trial. *Lancet Neurol.* 19, 814–825. doi: 10.1016/S1474-4422(20)30279-9

Noseda, R., Schain, A. J., Melo-Carrillo, A., Tien, J., Stratton, J., Mai, F., et al. (2020). Fluorescently-labeled fremanezumab is distributed to sensory and autonomic ganglia and the dura but not to the brain of rats with uncompromised blood brain barrier. *Cephalalgia* 40, 229–240. doi: 10.1177/0333102419896760

Ohno, T., Hattori, Y., Komine, R., Ae, T., Mizuguchi, S., Arai, K., et al. (2008). Roles of calcitonin gene-related peptide in maintenance of gastric mucosal integrity and in enhancement of ulcer healing and angiogenesis. *Gastroenterology* 134, 215–225. doi: 10.1053/j.gastro.2007.10.001

Olesen, J., Diener, H. C., Husstedt, I. W., Goadsby, P. J., Hall, D., Meier, U., et al. (2004). Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N. Engl. J. Med.* 350, 1104–1110. doi: 10.1056/NEJMoa030505

Omaer, A., Aldosari, F. M., McGlamery, E., Wool, S., Fazel, M. T., and Alrashed, S. (2021). Calcitonin gene-related peptide (CGRP) antagonists: a comprehensive review of safety, efficacy and prescribing information. *J. Clin Pharm. Ther.* [Epub ahead of print]. doi: 10.1111/jcpt.13445

Ornello, R., Casalena, A., Frattale, I., Gabriele, A., Affaitati, G., Giamberardino, M. A., et al. (2020). Real-life data on the efficacy and safety of erenumab in the Abruzzo region, central Italy. *J. Headache Pain* 21:32. doi: 10.1186/s10194-020-01102-9

Ozdemir, F. A., Berghöfer, P., Göke, R., Göke, B., and McGregor, G. P. (1999). Specific calcitonin gene-related peptide binding sites present throughout rat intestine. *Peptides* 20, 1361–1366. doi: 10.1016/s0196-9781(99)00142-4

Palmer, J. M., Schemann, M., Tamura, K., and Wood, J. D. (1986). Calcitonin gene-related peptide excites myenteric neurons. *Eur. J. Pharmacol.* 132, 163–170. doi: 10.1016/0014-2999(86)90601-1

Pan, H., and Gershon, M. D. (2000). Activation of intrinsic afferent pathways in submucosal ganglia of the guinea pig small intestine. *J. Neurosci.* 20, 3295–3309. doi: 10.1523/JNEUROSCI.20-09-03295.2000

Peskar, B. M., Wong, H. C., Walsh, J. H., and Holzer, P. (1993). A monoclonal antibody to calcitonin gene-related peptide abolishes capsaicin-induced gastroprotection. *Eur. J. Pharmacol.* 250, 201–203. doi: 10.1016/0014-2999(93)90645-X

Plourde, V., Wong, H. C., Walsh, J. H., Raybould, H. E., and Taché, Y. (1993). CGRP antagonists and capsaicin on celiac ganglia partly prevent postoperative gastric ileus. *Peptides* 14, 1225–1229. doi: 10.1016/0196-9781(93)90180-o

Raffaelli, B., and Reuter, U. (2018). The biology of monoclonal antibodies: focus on calcitonin gene-related peptide for prophylactic migraine therapy. *Neurotherapeutics* 15, 324–335. doi: 10.1007/s13311-018-0622-7

Rask-Madsen, J., Bukhave, K., and Beubler, E. (1990). Influence on intestinal secretion of eicosanoids. *J. Intern. Med. Suppl.* 732, 137–144. doi: 10.1111/j.1365-2796.1990.tb01486.x

Rasmussen, T. N., Schmidt, P., Poulsen, S. S., and Holst, J. J. (2001). Localisation and neural control of the release of calcitonin gene-related peptide (CGRP) from the isolated perfused porcine ileum. *Regul. Pept.* 98, 137–143. doi: 10.1016/s0167-0115(00)00242-1

Raybould, H. E., Kolve, E., and Taché, Y. (1988). Central nervous system action of calcitonin gene-related peptide to inhibit gastric emptying in the conscious rat. *Peptides* 9, 735–737. doi: 10.1016/0196-9781(88)90114-3

Reasbeck, P. G., Burns, S. M., and Shulkes, A. (1988). Calcitonin gene-related peptide: enteric and cardiovascular effects in the dog. *Gastroenterology* 95, 966–971. doi: 10.1016/0016-5085(88)90170-9

Reinshagen, M., Flämig, G., Ernst, S., Geerling, I., Wong, H., Walsh, J. H., et al. (1998). Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J. Pharmacol. Exp. Ther.* 286, 657–661.

Rekik, M., Delvaux, M., Frexinos, J., and Bueno, L. (1997). The calcitonin gene-related peptide activates both cAMP and NO pathways to induce relaxation of circular smooth muscle cells of guinea-pig ileum. *Peptides* 18, 1517–1522. doi: 10.1016/s0196-9781(97)00246-5

Reuter, U., Goadsby, P. J., Lanteri-Minet, M., Wen, S., Hours-Zesiger, P., Ferrari, M. D., et al. (2018). Efficacy and tolerability of erenumab in patients with episodic migraine in whom two-to-four previous preventive treatments were unsuccessful: a randomised, double-blind, placebo-controlled, phase 3b study. *Lancet* 392, 2280–2287. doi: 10.1016/S0140-6736(18)32534-0

Robbins, L. (2019). Erenumab side effects. *Headache* 59, 1088–1089. doi: 10.1111/head.13589

Robbins, L., and Phenicie, B. (2020). CGRP monoclonal antibodies for chronic migraine prevention: evaluation of adverse effects using a checklist. *Pract. Pain Manag.* 20, 48–52.

Robblee, J., Devick, K. L., Mendez, N., Potter, J., Slonaker, J., and Starling, A. J. (2020). Real-world patient experience with erenumab for the preventive treatment of migraine. *Headache* 60, 2014–2025. doi: 10.1111/head.13951

Robinson, D. R., McNaughton, P. A., Evans, M. L., and Hicks, G. A. (2004). Characterization of the primary spinal afferent innervation of the mouse colon using retrograde labelling. *Neurogastroenterol. Motil.* 16, 113–124. doi: 10.1046/j.1365-2982.2003.00456.x

Rolston, R. K., Ghatei, M. A., Mulderry, P. K., and Bloom, S. R. (1989). Intravenous calcitonin gene-related peptide stimulates net water secretion in rat colon *in vivo*. *Digest. Dis. Sci.* 34, 612–616. doi: 10.1007/BF01536340

Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., et al. (1983). Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 304, 129–135. doi: 10.1038/304129a0

Roza, C., and Reeh, P. W. (2001). Substance P, calcitonin gene related peptide and PGE_2 co-released from the mouse colon: a new model to study nociceptive and inflammatory responses in viscera, in vitro. *Pain* 93, 213–219. doi: 10.1016/S0304-3959(01)00318-9

Russell, F. A., King, R., Smillie, S. J., Kodji, X., and Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* 94, 1099–1142. doi: 10.1152/physrev.00034.2013

Russo, A., Silvestro, M., Scotto di Clemente, F., Trojsi, F., Bisecco, A., Bonavita, S., et al. (2020). Multidimensional assessment of the effects of erenumab in chronic migraine patients with previous unsuccessful preventive treatments: a comprehensive real-world experience. *J. Headache Pain* 21:69. doi: 10.1186/s10194-020-01143-0

Sakai, F., Suzuki, N., Kim, B. K., Tatsuoka, Y., Imai, N., Ning, X., et al. (2021). Efficacy and safety of fremanezumab for episodic migraine prevention: multicenter, randomized, double-blind, placebo-controlled, parallel-group trial in Japanese and Korean patients. *Headache* 61, 1102–1111. doi: 10.1111/head.14178

Sarna, S. K. (2003). Neuronal locus and cellular signaling for stimulation of ileal giant migrating and phasic contractions. *Am. J. Physiol.* 284, G789–G797. doi: 10.1152/ajpgi.00451.2001

Scheffler, A., Messel, O., Wurthmann, S., Nsaka, M., Kleinschnitz, C., Glas, M., et al. (2020). Erenumab in highly therapy-refractory migraine patients: first German real-world evidence. *J. Headache Pain* 21:84. doi: 10.1186/s10194-020-01151-0

Scheffler, A., Schenk, H., Wurthmann, S., Nsaka, M., Kleinschnitz, C., Glas, M., et al. (2021). CGRP antibody therapy in patients with drug resistant migraine and chronic daily headache: a real-world experience. *J. Headache Pain* 22:111. doi: 10.1186/s10194-021-01323-6

Schifter, S., Williams, E. D., Craig, R. K., and Hansen, H. H. (1986). Calcitonin gene-related peptide and calcitonin in medullary thyroid carcinoma. *Clin. Endocrinol.* 25, 703–710. doi: 10.1111/j.1365-2265.1986.tb03626.x

Schütz, B., Mauer, D., Salmon, A. M., Changeux, J. P., and Zimmer, A. (2004). Analysis of the cellular expression pattern of beta-CGRP in alpha-CGRP-deficient mice. *J. Comp. Neurol.* 476, 32–43. doi: 10.1002/cne.20211

Schwörer, H., Schmidt, W. E., Katsoulis, S., and Creutzfeldt, W. (1991). Calcitonin gene-related peptide (CGRP) modulates cholinergic neurotransmission in the small intestine of man, pig and guinea-pig via presynaptic CGRP receptors. *Regul. Pept.* 36, 345–358. doi: 10.1016/0167-0115(91)90068-r

Shulkes, A., Fletcher, D. R., Rubinstein, C., Ebeling, P. R., and Martin, T. J. (1991). Production of calcitonin gene related peptide, calcitonin and PTH-related protein by a prostatic adenocarcinoma. *Clin. Endocrinol.* 34, 387–393. doi: 10.1111/j.1365-2265.1991.tb00310.x

Silberstein, S., Diamond, M., Hindiyeh, N. A., Biondi, D. M., Cady, R., Hirman, J., et al. (2020). Eptinezumab for the prevention of chronic migraine: efficacy and safety through 24 weeks of treatment in the phase 3 PROMISE-2 (Prevention of migraine via intravenous ALD403 safety and efficacy-2) study. *J. Headache Pain* 21:120. doi: 10.1186/s10194-020-01186-3

Silberstein, S. D., Dodick, D. W., Bigal, M. E., Yeung, P. P., Goadsby, P. J., Blankenbiller, T., et al. (2017). Fremanezumab for the preventive treatment of chronic migraine. *N. Engl. J. Med.* 377, 2113–2122. doi: 10.1056/NEJMoa1709038

Silvestro, M., Orologio, I., Bonavita, S., Scotto di Clemente, F., Fasano, C., Tessitore, A., et al. (2021). Effectiveness and safety of CGRP-mAbs in menstrual-related migraine: a real-world experience. *Pain Ther.* 10, 1203–1214. doi: 10.1007/s40122-021-00273-w

Sjölund, K., Fasth, S., Ekman, R., Hultén, L., Jiborn, H., Nordgren, S., et al. (1997). Neuropeptides in idiopathic chronic constipation (slow transit constipation). *Neurogastroenterol. Motil.* 9, 143–150. doi: 10.1046/j.1365-2982.1997.d01-46.x

Skljarevski, V., Matharu, M., Millen, B. A., Ossipov, M. H., Kim, B. K., and Yang, J. Y. (2018). Efficacy and safety of galcanezumab for the prevention of episodic migraine: results of the EVOLVE-2 Phase 3 randomized controlled clinical trial. *Cephalalgia* 38, 1442–1454. doi: 10.1177/0333102418779543

Smith, A. S., and Smid, S. D. (2005). Impaired capsaicin and neurokinin-evoked colonic motility in inflammatory bowel disease. *J. Gastroenterol. Hepatol.* 20, 697–704. doi: 10.1111/j.1440-1746.2005.03759.x

Smolilo, D. J., Hibberd, T. J., Costa, M., Wattchow, D. A., De Fontgalland, D., and Spencer, N. J. (2020). Intrinsic sensory neurons provide direct input to motor neurons and interneurons in mouse distal colon via varicose baskets. *J. Comp. Neurol.* 528, 2033–2043. doi: 10.1002/cne.24872

Stauffer, V. L., Dodick, D. W., Zhang, Q., Carter, J. N., Ailani, J., and Conley, R. R. (2018). Evaluation of Galcanezumab for the prevention of episodic migraine: the EVOLVE-1 randomized clinical trial. *JAMA Neurol.* 75, 1080–1088. doi: 10.1001/jamaneurol.2018.1212

Sternini, C. (1992). Enteric and visceral afferent CGRP neurons. Targets of innervation and differential expression patterns. *Ann. N.Y. Acad. Sci.* 657, 170–186. doi: 10.1111/j.1749-6632.1992.tb22766.x

Sternini, C., and Anderson, K. (1992). Calcitonin gene-related peptide-containing neurons supplying the rat digestive system: differential distribution and expression pattern. *Somatosens. Mot. Res.* 9, 45–59. doi: 10.3109/08990229209144762

Sternini, C., De Giorgio, R., and Furness, J. B. (1992). Calcitonin gene-related peptide neurons innervating the canine digestive system. *Regul. Pept.* 42, 15–26. doi: 10.1016/0167-0115(92)90020-u

Sternini, C., Reeve, J. R., and Brecha, N. (1987). Distribution and characterization of calcitonin gene-related peptide immunoreactivity in the digestive system of normal and capsaicin-treated rats. *Gastroenterology* 93, 852-862. doi: 10.1016/0016-5085(87)90450-1

Su, H. C., Bishop, A. E., Power, R. F., Hamada, Y., and Polak, J. M. (1987). Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of rat gut and pancreas. *J. Neurosci.* 7, 2674-2687. doi: 10.1523/JNEUROSCI.07-09-02674.1987

Sun, H., Dodick, D. W., Silberstein, S., Goadsby, P. J., Reuter, U., Ashina, M., et al. (2016). Safety and efficacy of AMG 334 for prevention of episodic migraine: a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* 15, 382–390. doi: 10.1016/S1474-4422(16)00019-3

Sun, Y. D., and Benishin, C. G. (1995). Effects of calcitonin gene-related peptide on cyclic AMP production and relaxation of longitudinal muscle of guinea pig ileum. *Peptides* 16, 293–297. doi: 10.1016/0196-9781(94)00189-8

Suzuki, T., Kagoshima, M., Shibata, M., Inaba, N., Onodera, S., Yamaura, T., et al. (1997). Effects of several denervation procedures on distribution of calcitonin gene-related peptide and substance P immunoreactive fibers in rat stomach. *Digest. Dis. Sci.* 42, 1242–1254. doi: 10.1023/a:1018858208532

Szkutnik-Fiedler, D. (2020). Pharmacokinetics, pharmacodynamics and drug-drug interactions of new anti-migraine drugs-lasmiditan, gepants, and calcitonin-gene-related peptide (CGRP) receptor monoclonal antibodies. *Pharmaceutics* 12:1180. doi: 10.3390/pharmaceutics12121180

Taché, Y., Raybould, H., and Wei, J. Y. (1991). Central and peripheral actions of calcitonin gene-related peptide on gastric secretory and motor function. *Adv. Exp. Med. Biol.* 298, 183–198. doi: 10.1007/978-1-4899-0744-8_17

Takami, H., Shikata, J., Kakudo, K., and Ito, K. (1990). Calcitonin gene-related peptide in patients with endocrine tumors. *J. Surg. Oncol.* 43, 28–32. doi: 10.1002/jso.2930430108

Takeshima, T., Sakai, F., Hirata, K., Imai, N., Matsumori, Y., Yoshida, R., et al. (2021). Erenumab treatment for migraine prevention in Japanese patients: efficacy and safety results from a phase 3, randomized, double-blind, placebo-controlled study. *Headache* 61, 927–935. doi: 10.1111/head

Tan, L. L., Bornstein, J. C., and Anderson, C. R. (2008). Distinct chemical classes of medium-sized transient receptor potential channel vanilloid 1-immunoreactive dorsal root ganglion neurons innervate the adult mouse jejunum and colon. *Neuroscience* 156, 334–343. doi: 10.1016/j.neuroscience.2008.06.071

Tan, L. L., Bornstein, J. C., and Anderson, C. R. (2009). Neurochemical and morphological phenotypes of vagal afferent neurons innervating the adult mouse jejunum. *Neurogastroenterol. Motil.* 21, 994–1001. doi: 10.1111/j.1365-2982.2009.01322.x

Tepper, S., Ashina, M., Reuter, U., Brandes, J. L., Doležil, D., Silberstein, S., et al. (2017). Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol.* 16, 425–434. doi: 10.1016/S1474-4422(17)30083-2

Thompson, B. J., Washington, M. K., Kurre, U., Singh, M., Rula, E. Y., and Emeson, R. B. (2008). Protective roles of alpha-calcitonin and beta-calcitonin gene-related peptide in spontaneous and experimentally induced colitis. *Dig. Dis. Sci.* 53, 229–241. doi: 10.1007/s10620-007-9848-7

Timmermans, J. P., Scheuermann, D. W., Barbiers, M., Adriaensen, D., Stach, W., Van Hee, R., et al. (1992). Calcitonin gene-related peptide-like immunoreactivity in the human small intestine. *Acta Anat.* 143, 48–53. doi: 10.1159/000147227

Torres-Ferrús, M., Gallardo, V. J., Alpuente, A., Caronna, E., Gine-Cipres, E., and Pozo-Rosich, P. (2021). The impact of anti-CGRP monoclonal antibodies in resistant migraine patients: a real-world evidence observational study. *J. Neurol.* 268, 3789–3798. doi: 10.1007/s00415-021-10523-8

Tough, I. R., Moodaley, R., and Cox, H. M. (2018). Mucosal glucagon-like peptide 1 (GLP-1) responses are mediated by calcitonin gene-related peptide (CGRP) in the mouse colon and both peptide responses are area-specific. *Neurogastroenterol. Motil.* 30:e13149. doi: 10.1111/nmo.13149

Trudel, L., Bouin, M., Tomasetto, C., Eberling, P., St-Pierre, S., Bannon, P., et al. (2003). Two new peptides to improve post-operative gastric ileus in dog. *Peptides* 24, 531–534. doi: 10.1016/s0196-9781(03)00113-x

Wechsler, E. V., and Shah, E. D. (2021). Diarrhea-predominant and constipation-predominant irritable bowel syndrome: current prescription drug treatment options. *Drugs* 81, 1953–1968. doi: 10.1007/s40265-021-01634-7

Winner, P. K., McAllister, P., Chakhava, G., Ailani, J., Ettrup, A., Krog Josiassen, M., et al. (2021). Effects of intravenous eptinezumab vs placebo on headache pain and most bothersome symptom when initiated during a migraine attack: a randomized clinical trial. *JAMA* 325, 2348–2356. doi: 10.1001/jama.2021.7665

Wolf, M., Schrödl, F., Neuhuber, W., and Brehmer, A. (2007). Calcitonin gene-related peptide: a marker for putative primary afferent neurons in the pig small intestinal myenteric plexus? *Anat. Rec.* 290, 1273–1279. doi: 10.1002/ar.20577

Yamasaki, H., Mitsuyama, K., Yoshioka, S., Kuwaki, K., Yamauchi, R., Fukunaga, S., et al. (2020). Leukocyte apheresis using a fiber filter suppresses colonic injury through calcitonin gene-related peptide induction. *Inflamm. Bowel Dis.* 26, 709–719. doi: 10.1093/ibd/izz303

Yarwood, R. E., Imlach, W. L., Lieu, T., Veldhuis, N. A., Jensen, D. D., Klein Herenbrink, C., et al. (2017). Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc. Natl. Acad. Sci. U.S.A.* 114, 12309–12314. doi: 10.1073/pnas.1706656114

Yoshikawa, R., Mikami, N., Otani, I., Kishimoto, T., Nishioka, S., Hashimoto, N., et al. (2011). Suppression of ovalbumin-induced allergic diarrhea by diminished intestinal peristalsis in RAMP1-deficient mice. *Biochem. Biophys. Res. Commun.* 410, 389–393. doi: 10.1016/j.bbrc.2011.05.141

Zittel, T. T., Lloyd, K. C., Rothenhöfer, I., Wong, H., Walsh, J. H., and Raybould, H. E. (1998). Calcitonin gene-related peptide and spinal afferents partly mediate postoperative colonic ileus in the rat. *Surgery* 123, 518–527. doi: 10.1067/msy.1998.88090

Zittel, T. T., Reddy, S. N., Plourde, V., and Raybould, H. E. (1994). Role of spinal afferents and calcitonin gene-related peptide in the postoperative gastric ileus in anesthetized rats. *Ann. Surg.* 219, 79–87. doi: 10.1097/00000658-199401000-00013

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REVIEW
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Neuropeptides and the Nodes of Ranvier in Cranial Headaches

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The trigeminovascular system (TGV) comprise of the trigeminal ganglion with neurons and satellite glial cells, with sensory unmyelinated C-fibers and myelinated Aδ-fibers picking up information from different parts of the head and sending signals to the brainstem and the central nervous system. In this review we discuss aspects of signaling at the distal parts of the sensory fibers, the extrasynaptic signaling between C-fibers and Aδ-fibers, and the contact between the trigeminal fibers at the nerve root entry zone where they transit into the CNS. We also address the possible role of the neuropeptides calcitonin gene-related peptide (CGRP), the neurokinin family and pituitary adenylyl cyclase-activating polypeptide 38 (PACAP-38), all found in the TGV system together with their respective receptors. Elucidation of the expression and localization of neuropeptides and their receptors in the TGV system may provide novel ways to understand their roles in migraine pathophysiology and suggest novel ways for treatment of migraine patients.

Keywords: trigeminal ganglion, nodes of Ranvier, Remak bundles, vascular neuroeffector site, nerve entry zone, Redlich-Obersteiner zone

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INTRODUCTION

There is strong evidence for a role of calcitonin gene-related peptide (CGRP) and the calcitonin receptor-like receptor/receptor activity-modifying protein 1 (CLR/RAMP1, the CGRP receptor elements) in migraine pathophysiology (Edvinsson et al., 2018). CGRP is expressed in small to medium sized neurons in the trigeminal ganglion (TG) that project sensory, afferent non-myelinated C-fibers to both intra- and extracranial structures and centrally to the trigeminal nucleus caudalis (TNC; Edvinsson and Uddman, 2005; Eftekhari et al., 2010, 2013). The TG also harbors larger neurons and satellite glial cells (SGCs) which express the CGRP receptor. These neurons project thinly myelinated afferent sensory Aδ-fibers often in close relation to the C-fibers to the same regions as the C-fibers (Eftekhari et al., 2010, 2013). Early work demonstrated that CGRP is released from the trigeminovascular (TGV) system while more recent work has revealed CGRP release in attacks of primary headaches from sites such as the TG with neuronal bodies (soma), the trigeminal fibers (soma-free parts) and the dura mater and cranial vasculature which represent the most distal parts of the sensory nerve fibers (Edvinsson et al., 2018). Since neither CGRP nor CGRP receptor blocking drugs can pass the blood-brain barrier (BBB) freely, the likely site of anti-migraine action of the novel gepants and monoclonal antibodies (mAbs) is within the TGV system which lacks a BBB (Lundblad et al., 2015).

The aim of this review is to discuss in some detail the close relation between the C- and Aδ-fibers, occurring when the boutons align with or come close to proximal nodes of Ranvier, proposing the hypothesis that this might be a site of action of headache medications. Current data point toward the intimate location here between CGRP containing C-fiber boutons in proximity to the nodes of Ranvier (**Figure 1** and **Supplementary Video**) where recent work has identified location of CGRP receptors on the Aδ-fibers (Edvinsson et al., 2019). Substance P (neurokinin family) (Edvinsson et al., 2021) and the pituitary adenylyl cyclase-activating polypeptide - 38 (PACAP-38) and their receptors in relation to the CGRP system (Edvinsson J. C. A. et al., 2020), has also been investigated in light of the node of Ranvier, and discussed below.

EXPRESSION AND RELEASE OF CALCITONIN GENE-RELATED PEPTIDE, SUBSTANCE P, AND PITUITARY ADENYLYL CYCLASE-ACTIVATING POLYPEPTIDE FROM THE TRIGEMINOVASCULAR SYSTEM

Studies have examined the release of CGRP, substance P, and PACAP from neuron rich regions the TG, neuron soma poor regions of the TG and the dura mater with its vasculature (Edvinsson J. C. A. et al., 2020; Edvinsson et al., 2021). Along the unmyelinated C-fibers from the TG in both central and peripheral direction the thin C-fibers often appear as a pearl necklace with the "pearls" being the so-called "boutons," sometimes described also as a pattern "en-passant" in the fibers with accumulations of neurotransmitters. Immunohistochemistry revealed that the C-fibers and TG neurons contain a rich supply of CGRP, while about 15% of these contain substance P (Edvinsson et al., 2021) and PACAP (Edvinsson J. C. A. et al., 2020); even fewer contain neurokinins A and B. Our analysis revealed a minor content of substance P in the CGRP containing C-fiber boutons while there was no observable presence of PACAP. Detailed analysis of other peptides of the CGRP family such as amylin and adrenomedullin revealed a minor contribution, mainly localized in neuronal somas which co-store CGRP (Edvinsson L. et al., 2020). PACAP-38 is expressed in the TG of rat and man, and shown to co-localized with CGRP specifically in the neurons in TG (Frederiksen et al., 2018). In the walls of cerebral arteries there was a rich distribution of CGRP, but some perivascular fibers also contain substance P and PACAP-38 (Edvinsson and Uddman, 2005; Frederiksen et al., 2019). The dura mater with the middle meningeal artery (MMA) and its branches showed CGRP positive fibers but few containing substance P and essentially none with PACAP-38 (Edvinsson J. C. A. et al., 2020). In studies of the C-fiber distribution within the trigeminal nerve, there were C-fiber boutons being enriched with CGRP (Edvinsson et al., 2019); these also expressed some vesicular substance P but no PACAP-38 (Edvinsson J. C. A. et al., 2020). Based on these findings, co-release experiments were performed in different parts of the TGV system.

When stimulating the tissue preparations, it was observed that 60 mM K^+ (depolarization) resulted in minor release of substance P from the dura mater but no release from the soma-poor or soma-rich samples (Edvinsson et al., 2021). However, there was abundant release from all three preparations of CGRP as measured in parallel. When adding the TRPV1 agonist capsaicin there was no observed release of substance P from the dura mater samples or soma-rich parts of the TG but a low release was detected from the soma-poor TG. In contrast, all three samples showed high and consistent release of CGRP following capsaicin treatment (Edvinsson et al., 2021). This pattern of release agrees well with the immunohistochemical expression of substance P and CGRP.

The PACAP-38 release data demonstrated that CGRP was released from all three parts of the TGV system; however, PACAP-38 was only released from the soma-rich part of the TG (with the neurons) and showed no differences if it was stimulated with 60 mM K^+ or capsaicin (Edvinsson J. C. A. et al., 2020). This finding agrees well with the immunohistochemistry revealing that PACAP is only expressed in a subpopulation of CGRP positive neurons in the TG (Eftekhari et al., 2013; Frederiksen et al., 2018; Edvinsson J. C. A. et al., 2020). Taken together the data strongly suggest that the C-fiber boutons may function as local release sites.

Immunohistochemistry of the major blood vessels, cerebral and MMA, revealed some structural differences. Previous studies revealed a rich distribution of CGRP and substance P in cerebral vessel walls while the perivascular network of these fibers was comparatively sparse in MMA and in dura mater (Edvinsson et al., 1981, 1987). Only few PACAP-38 immunoreactive fibers were observed. All these fibers were observed in the adventitia, sometimes near the outermost part of the medial layer with vascular smooth muscle (VSM) cells. This would represent the far end of the TGV system.

RECEPTORS FOR CALCITONIN GENE-RELATED PEPTIDE, SUBSTANCE P, AND PITUITARY ADENYLYL CYCLASE-ACTIVATING POLYPEPTIDE IN THE TRIGEMINOVASCULAR SYSTEM AND IN INTRACRANIAL VESSEL WALLS

Numerous experiments on various types of vascular preparations, *in vivo* as well as *in vitro*, have been performed (Edvinsson and Uddman, 2005). Since migraine is a human disorder this section will focus mainly on human material when possible. CGRP, amylin, and adrenomedullin relax human MMA/branches with CGRP being strongest and the most potent of these agonists. Human cerebral arteries respond even more potently to CGRP than the MMA (Sams et al., 2000; Edvinsson et al., 2007). In perfused middle cerebral artery (MCA) from rat neither CGRP, amylin nor adrenomedullin pass cerebral artery endothelium when given luminally due to the presence of the BBB. However, when given abluminal these peptides elicited concentration-dependent

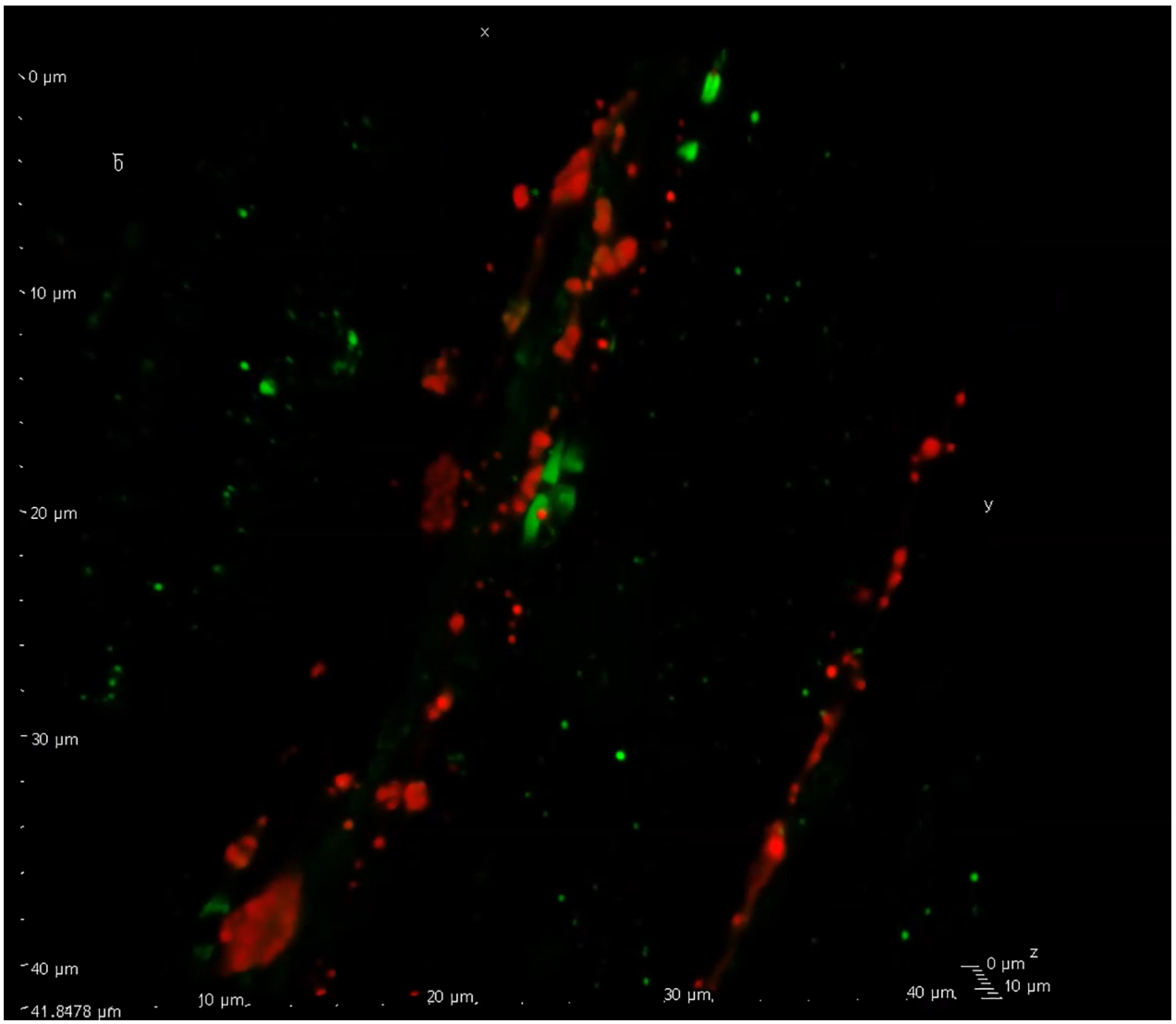

FIGURE 1 | Confocal visualization of the node of Ranvier. Confocal microscopy displaying calcitonin gene-related peptide (CGRP) (red) immunreactive C-fiber boutons flanked by two Aδ-fibers with their paranodal regions marked with contactin associated protein 1 (CASPR, green). This close relationship between the two fiber types display a point of interaction for CGRP signaling within the trigeminovascular (TGV) system (see **Supplementary Video**). For experimental details on antibodies etc., see Edvinsson et al. (2019).

dilatation that could be blocked by gepants or mAbs (Edvinsson et al., 2007).

The parasympathetic peptides: vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), and PACAP-38, are members of the secretin/glucagon superfamily of peptides, and have been shown to relax in a concentration-dependent manner, human temporal, meningeal, and cerebral arteries (Jansen et al., 1992; Grande et al., 2014). In a study on the MMA (Chan et al., 2011) it was reported that VIP (pEC_{50} = 7.4) was more potent than PACAP (<6.9); in addition, neither $VPAC_1$ nor PAC_1 receptor blockers antagonized the vasodilator responses to PACAP-38 in the MMA. This is probably due to low blocking capacity in the concentrations used, because both cerebral and MMAs contain mRNA for all three receptors (Knutsson and Edvinsson, 2002; Chan et al., 2011). In this work 30 mM K^+ was used for pre-contraction and substance P to evaluate vasodilator capacity and endothelial function (Chan et al., 2011). Substance P is a strong vasodilator that acts *via* endothelial receptors to induce formation of nitric oxide (NO) which in turn activates guanylyl cyclase in the vascular smooth muscle cells (VSMCs). While several studies with the "infusion model" used NO or an NO donor (Lassen et al., 2003) to elicit migraine-like headache, there is no study on the effects of substance P in this paradigm (Ashina et al., 2017).

The pharmacological approach, has shown that VIP is a more potent vasodilator compared to PACAP-38 in man, both *in vivo* (Rahmann et al., 2008; Schytz et al., 2009) and *in vitro* on human MMA (Chan et al., 2011). Subsequent work on the dichotomy

of VIP/PACAP infusions with magnetic resonance angiography showed dilatation of the extracranial superficial temporal artery but no effect on the diameter of the intracranial arteries, MMA, or cerebral arteries (Amin et al., 2014). Based on these findings, Tfelt-Hansen suggested that PACAP-38 might have an effect on sites within the CNS. We would as an alternative propose an effect on the sensory afferent nerves since there are receptors for both peptides available (Knutsson and Edvinsson, 2002; Chan et al., 2011). More precisely, the PAC_1 receptor is present in the TG satellite cells but not on the Aδ-fibers which explains why the clinical studies with a specific mAb toward the PAC_1 receptor was negative in prevention of migraine (Ashina et al., 2021a). The role of PACAP-38 in the TGV is still under scrutiny and the recently developed mAbs toward PACAP-38 will give more final answers on the involvement of PACAP in migraine pathophysiology (Moldovan Loomis et al., 2019).

THE NODES OF RANVIER – A PLACE FOR INTERACTION BETWEEN C- AND Aδ-FIBERS

Nodes of Ranvier are essential for the regeneration of diminishing action potentials in the nervous system (saltatory conduction). Although they have traditionally been regarded as passive contribution to action potential propagation, more recent work has suggest that they may have an active role in regulating neuronal excitability (Edvinsson et al., 2019). The nodal propagation has been suggested to be plastic and hence could excitingly be explaining the sensitization process which is a key element in migraine symptomatology. With the use of different antibodies toward elements of the nodal region we have shown with clarity their dense expression in the trigeminal nerve (**Figure 2**), both within the ganglion and in the nerve fibers going in central and peripheral directions (Edvinsson et al., 2019).

Neurons of the TG project with either thin, unmyelinated C-fibers or larger, myelinated A-fibers. While C-fibers are classically categorized, based on conduction velocity, as a single type, the A-fibers are of three subtypes: Aα, Aβ, and Aδ. Aα-fibers have the largest diameter (13–20 μm) with a conduction velocity of 80–120 m/s, followed by Aβ-fibers (6–12 μm; 30–70 m/s) and last the Aδ-fibers (1–5 μm; 3–30 m/s), while the C-fibers are slow conductors (0.2–1.5 μm; 0.5–2 m/s). This categorization may vary slightly between different species which makes it difficult to morphologically determine or differentiate a small Aβ or a larger Aδ-fiber. C-fibers and Aδ-fibers make up most axons within the trigeminal system and are related to nociceptive signaling. C-fibers are responsible for slow, dull burning pain while Aδ-fibers signal a quicker, sharper pain. This makes them important biological structures to consider when studying headache, pain, or allodynia related to the trigeminal system. Electrophysiology at different parts of the TGV system, mainly the neurons, shows two types of signaling velocities, one is the C-fibers and the other often categorized as Aδ-fiber neurons.

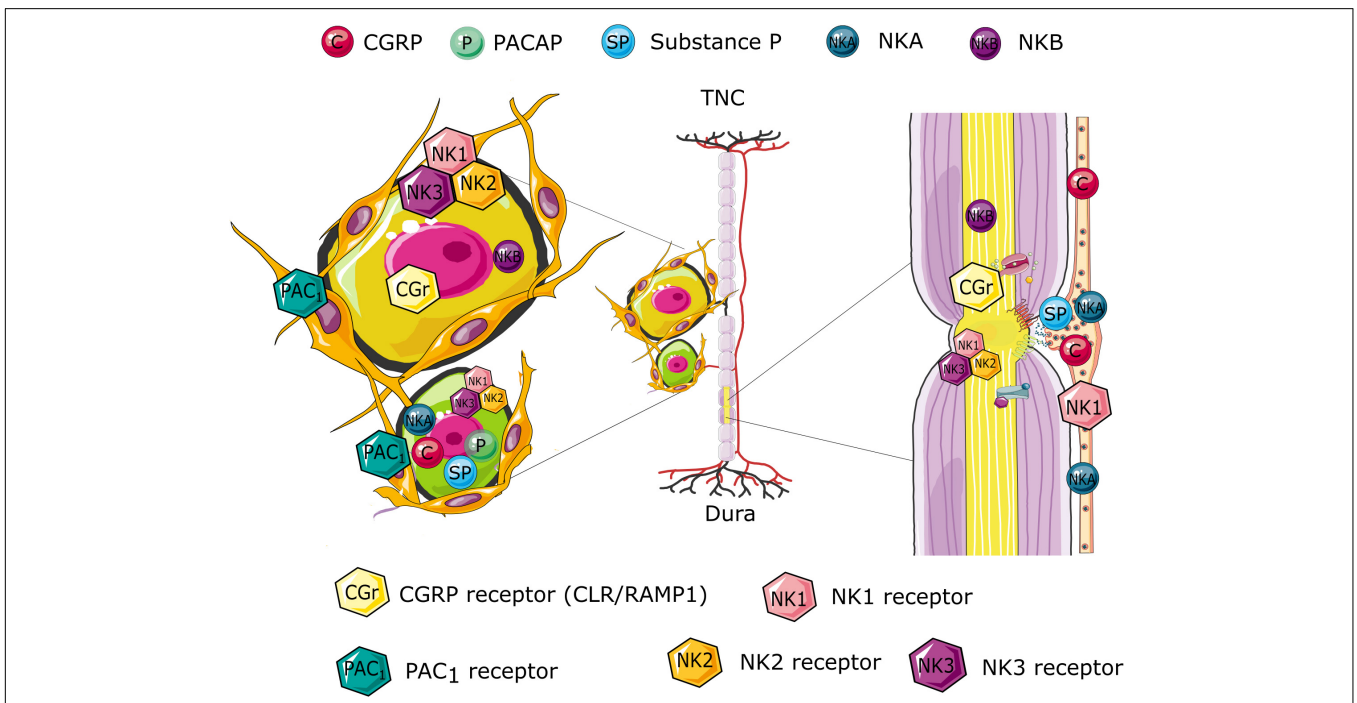

FIGURE 2 | Schematic illustration of the differential distribution of the CGRP, pituitary adenylyl cyclase-activating polypeptide (PACAP), and Neurokinin signaling compounds in the trigeminal system. The illustration suggests possible site of origin if the various peptides and their associated release. Further, the receptor expression highlights possible receptor targets, following the peptide release. The expression is based on immunology findings from the following studies (Edvinsson et al., 2019, 2021; Edvinsson J. C. A. et al., 2020; Edvinsson L. et al., 2020).

Stimulation of these nerve fibers at TG regions lacking neuronal somas results in the release of CGRP, which supports release from the C-fibers. In relation to the nodes of Ranvier in the trigeminal system we have shown that the C-fiber boutons contain large amounts of CGRP (Edvinsson et al., 2019) and smaller amounts of substance P (Edvinsson et al., 2021) but no PACAP (Edvinsson J. C. A. et al., 2020). This would favor the view that the first two neuropeptides may have a role albeit perhaps at different degrees.

The release from extra synaptic regions such as the varicosities, also referred to as "en-passant boutons," are characteristic of presynaptic release sites in peripheral nerves (Smolen, 1988). We have postulated that CGRP and substance P could be released from these varicosities, although these areas are not classically understood to be synaptic. There are increasing evidence that neuropeptide release does not typically occur at defined synapses (Torrealba and Carrasco, 2004). Neuropeptides, such as CGRP, are typically stored in large dense-core vesicles, which are typically stored away from the presynaptic membrane, which for example has been shown for orexin (Torrealba et al., 2003). Other exocytosis sites have been suggested also due to the location of receptors for signaling molecules outside of typical synaptic regions (Trueta and De-Miguel, 2012). Although many of the mechanisms are common mechanisms, there are other aspects that are more similar to those of exocytosis from excitable endocrine cells. One such example is related to substance P, where exocytosis from the somata of dorsal root ganglion neurons has been demonstrated (Huang and Neher, 1996). This highlights the possible extra synaptic release of neuropeptides, and we suggest that the varicosities at the nodes of Ranvier provides such a possible release site (Edvinsson et al., 2019).

On the adjacent nodes of Ranvier where the myelin sheath is absent, and the Aδ-fiber is exposed we have demonstrated the presence of the CGRP receptor (Edvinsson et al., 2019) and recently also the neurokinin 1 receptor (NK1R; Edvinsson et al., 2021). This opens for diffusion of the two peptides to reach targets on the Aδ-fiber. The presence of adenylyl cyclase and protein kinase A further add to the activity of this pathway in the Aδ-fiber presumably modifying the activity of potassium K^+ and sodium Na^+ channels and the saltatory activity within the Aδ-fiber and the pain signaling. The Nav channels have received recent attention in light of their strong link to pain transmission (Hameed, 2019). In the nodes of Ranvier both Nav1.6, Nav1.7, and Nav1.8 have been shown to be expressed specifically in the nodal structure (Caldwell et al., 2000; Devaux and Scherer, 2005; Black et al., 2012), although Nav1.8 typically only do so in disease model (Devaux and Scherer, 2005). For Nav1.6 the dependency of PKA is present but does not have a major influence (Chen et al., 2008). However, Nav1.7 and Nav1.8, when expressed in Xenopus oocytes, are differentially regulated by PKA and PKC. Focusing on the cAMP pathway, PKA activation resulted in a dose-dependent potentiation of Nav1.8 currents and an attenuation of Nav1.7 currents (Vijayaragavan et al., 2004). This work is in its infancy but could potentially add novel understanding to processes of sensitization and as a drug target for headache disorders.

HUMAN DATA IN LIGHT OF THE NODE OF RANVIER

Although the infusion model (Ashina et al., 2017) as a basis of triggering migraine-pain appears to be based primarily on the vascular theory of migraine more specifically on vasodilatation of cranial arteries, we suggest that some of the data can be extrapolated to the nodes of Ranvier. We have previously proposed that some of these data, e.g., when applying a K^+ channel opener, could lead to long lasting hyperpolarization that activates Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) Channels (Haanes and Edvinsson, 2020). The fact that both cAMP and cGMP can activate these channels (Momin et al., 2008) and lead to increases in the open-probability and augmented neuronal excitability and firing of the neurons (Momin et al., 2008) supports this notion. Furthermore, injection of CGRP or other migraine triggering molecules, also share the common cAMP pathway (Ashina et al., 2021b). We hypothesize that the key regulators of the node of Ranvier are the cyclic nucleotides, and indeed we have localized the downstream PKA to the nodes (Edvinsson et al., 2019). It cannot be ruled out that local potassium or other secondary messengers following dilation also activate the C-fibers (Al-Karagholi et al., 2021) but we propose nodal or neuronal activation could be the key to understanding the infusion model. It is further worth pointing out that only passive vasodilation of cranial arteries is not the likely inducer of CGRP release, as it was shown that the TGV reflex demonstrated that vasoconstriction of cortex pial arterioles results in activation of the TGV system and release of the stored vasoactive peptides (McCulloch et al., 1986; Edvinsson et al., 1990).

THE REMAK BUNDLES ON C-FIBERS

Schwann cells can either be "myelinating" or "non-myelinating" meaning that they are either specialized in insulating a portion of an A-fiber with myelin, or mainly providing structural stability for a bundle of C-fibers (called Remak bundles) (Aguayo et al., 1976). The myelin sheath provides isolation making the transmission of an electrical signal move faster through the myelinated segments of the axon. Without the nodes of Ranvier, a signal traveling too far would lose energy and fade out or be diminished before reaching its intended terminal, making the signal unable to proliferate. This process is an important prerequisite for adequate function of nerves both in the CNS and in the PNS. As an alternative to the myelin coverings of the Aδ-fibers in the TGV system the C-fibers have been suggested to be partly covered by so-called non-myelinating Schwann cells. The main population of axons surrounded by non-myelinating Schwann cells are the small nociceptive C-type of axons. This also applies for postganglionic sympathetic axons, and some of the preganglionic sympathetic and parasympathetic fibers (Griffin and Thompson, 2008). Remak non-myelinating Schwann cells envelop sections of the axons within troughs on their surface. To our knowledge, no specific antibody exists for differentiating between myelinating and non-myelinating Schwann cells. During

our immunohistochemical studies we have therefore, so far, not been able to identify these structures. If Remak bundles are abundant in the TGV system it could limit some of the C-fibers from signaling to adjacent fibers (Edvinsson et al., 2019). However, in our experimental studies on soma-poor trigeminal nerves we observed strong release of CGRP which indicates that peptides located in C-fibers can diffuse outside the Remak bundles and act on the CGRP receptors located on the Aδ-fibers at the nodes of Ranvier. The released CGRP can also be measured in cranial vein plasma as shown in several species including man. With existing information from literature, the Remak bundles usually refer to groups of C-fibers and not, as we often see them, as single C-fibers in the TGV system (Faroni et al., 2014; Miyamoto et al., 2017).

Calcitonin gene-related peptide has been shown to lead to increases in iNOS expression (Vause and Durham, 2009). And although not in the scope of the current review, there is also extensive research showing that nitric oxide can modulate action potentials. Relating to the node of Ranvier, iNOS immunoreactivity has been shown to be expressed in Schwann cells of peripheral nerves and was particularly enriched at the paranodal regions of the nodes of Ranvier (Chen et al., 2010). In addition, activation of cGMP might link to membrane channel activities such as the HCN channel (Momin et al., 2008), which deserves further research.

THE NERVE ROOT ENTRY ZONE – THE REDLICH-OBERSTEINER ZONE

Ever since its discovery 150 years ago much attention has been directed to the Redlich-Obersteiner zone (Savvaidou and Triarhou, 2016). This region exists for all cranial nerves and hence is postulated to be involved in many cranial nerve disorders such as trigeminal neuralgia (Nomura et al., 2019) and other clinical applications (Xenellis and Linthicum, 2003). It represents the" junction zone" of glia and Schwann sheath of the cranial nerves. In this zone, the Schwann cells are abruptly replaced by the more numerous oligodendrocytes and a clearly visible zone between the PNS and the CNS appear. This area is believed to be linked to trigeminal neuralgia caused by compression of the nerve root (Jannetta, 1967). However, many of the functional details remains to be understood; how the PNS and CNS communicates, how the functionality of the BBB is upheld in this region and what role the change of glial cells may have on axonal signaling. Possible cross-talk among fibers in this region may be crucial in understanding parts of the migraine pathology.

CONCLUSION

Here we have discussed the possibility that the nodes of Ranvier, expressed in most parts of the CNS and the PNS may be involved as an important mechanism to modify signaling in the trigeminal system and perhaps even have a role in the sensitization process. CGRP and its receptor represent the most expressed system while substance P is less densely supplied but still may be involved in the headache processes. PACAP has been suggested as a putative novel molecule in headache disorders but future work remains to unravel its role in migraine. While there was no PACAP in the trigeminal nerves we observed some expression in trigeminal ganglion cells and the PAC_1 receptor in satellite glial cells in the ganglion. There remain numerous questions to address also for the other members of the CGRP family of peptides and their receptors (Edvinsson L. et al., 2020).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.820037/full#supplementary-material

REFERENCES

Aguayo, A. J., Charron, L., and Bray, G. M. (1976). Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. *J. Neurocytol.* 5, 565–573. doi: 10.1007/BF01175570

Al-Karagholi, M. A., Ghanizada, H., Nielsen, C. A. W., Hougaard, A., and Ashina, M. (2021). Opening of ATP sensitive potassium channels causes migraine attacks with aura. *Brain* 144, 2322–2332. doi: 10.1093/brain/awab136

Amin, F. M., Hougaard, A., Schytz, H. W., Asghar, M. S., Lundholm, E., Parvaiz, A. I., et al. (2014). Investigation of the pathophysiological mechanisms of migraine attacks induced by pituitary adenylate cyclase-activating polypeptide-38. *Brain* 137, 779–794. doi: 10.1093/brain/awt369

Ashina, M., Dolezil, D., Bonner, J. H., Zhou, L., Klatt, J., Picard, H., et al. (2021a). A phase 2, randomized, double-blind, placebo-controlled trial of AMG 301, a pituitary adenylate cyclase-activating polypeptide PAC1 receptor monoclonal antibody for migraine prevention. *Cephalalgia* 41, 33–44. doi: 10.1177/0333102420970889

Ashina, M., Terwindt, G. M., Al-Karagholi, M. A., De Boer, I., Lee, M. J., Hay, D. L., et al. (2021b). Migraine: disease characterisation, biomarkers, and precision medicine. *Lancet* 397, 1496–1504. doi: 10.1016/S0140-6736(20)32162-0

Ashina, M., Hansen, J. M., Bo, A. D., and Olesen, J. (2017). Human models of migraine - short-term pain for long-term gain. *Nat. Rev. Neurol.* 13, 713–724. doi: 10.1038/nrneurol.2017.137

Black, J. A., Frezel, N., Dib-Hajj, S. D., and Waxman, S. G. (2012). Expression of Nav1.7 in DRG neurons extends from peripheral terminals in the skin to central preterminal branches and terminals in the dorsal horn. *Mol. Pain* 8:82. doi: 10.1186/1744-8069-8-82

Caldwell, J. H., Schaller, K. L., Lasher, R. S., Peles, E., and Levinson, S. R. (2000). Sodium channel Na(v)1.6 is localized at nodes of ranvier, dendrites, and synapses. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5616–5620. doi: 10.1073/pnas.090034797

Chan, K. Y., Baun, M., De Vries, R., Van Den Bogaerdt, A. J., Dirven, C. M., Danser, A. H., et al. (2011). Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery. *Cephalalgia* 31, 181–189. doi: 10.1177/0333102410375624

Chen, K., Northington, F. J., and Martin, L. J. (2010). Inducible nitric oxide synthase is present in motor neuron mitochondria and Schwann cells and contributes to disease mechanisms in ALS mice. *Brain Struct. Funct.* 214, 219–234. doi: 10.1007/s00429-009-0226-4

Chen, Y., Yu, F. H., Sharp, E. M., Beacham, D., Scheuer, T., and Catterall, W. A. (2008). Functional properties and differential neuromodulation of Na(v)1.6 channels. *Mol. Cell Neurosci.* 38, 607–615. doi: 10.1016/j.mcn.2008.05.009

Devaux, J. J., and Scherer, S. S. (2005). Altered ion channels in an animal model of Charcot-Marie-Tooth disease type IA. *J. Neurosci.* 25, 1470–1480. doi: 10.1523/JNEUROSCI.3328-04.2005

Edvinsson, J. C., Reducha, P., Sheykhzade, M., Warfvinge, K., Haanes, K. A., and Edvinsson, L. (2021). Neurokinins and their receptors in the rat trigeminal system: differential localization and release with implications for migraine pain. *Mol. Pain* 17:17448069211059400. doi: 10.1177/17448069211059400

Edvinsson, J. C. A., Grell, A. S., Warfvinge, K., Sheykhzade, M., Edvinsson, L., and Haanes, K. A. (2020). Differences in pituitary adenylate cyclase-activating peptide and calcitonin gene-related peptide release in the trigeminovascular system. *Cephalalgia* 40, 1296–1309. doi: 10.1177/0333102420929026

Edvinsson, L., Grell, A. S., and Warfvinge, K. (2020). Expression of the CGRP family of neuropeptides and their receptors in the trigeminal ganglion. *J. Mol. Neurosci.* 70, 930–944. doi: 10.1007/s12031-020-01493-z

Edvinsson, J. C. A., Warfvinge, K., Krause, D. N., Blixt, F. W., Sheykhzade, M., Edvinsson, L., et al. (2019). C-fibers may modulate adjacent Aδ-fibers through axon-axon CGRP signaling at nodes of Ranvier in the trigeminal system. *J. Headache Pain* 20:105. doi: 10.1186/s10194-019-1055-3

Edvinsson, L., Ekman, R., Jansen, I., Mcculloch, J., and Uddman, R. (1987). Calcitonin gene-related peptide and cerebral blood vessels: distribution and vasomotor effects. *J. Cereb. Blood Flow Metab.* 7, 720–728. doi: 10.1038/jcbfm.1987.126

Edvinsson, L., Haanes, K. A., Warfvinge, K., and Krause, D. N. (2018). CGRP as the target of new migraine therapies - successful translation from bench to clinic. *Nat. Rev. Neurol.* 14, 338–350. doi: 10.1038/s41582-018-0003-1

Edvinsson, L., Jansen, I., Kingman, T. A., and Mcculloch, J. (1990). Cerebrovascular responses to capsaicin in vitro and in situ. *Br. J. Pharmacol.* 100, 312–318. doi: 10.1111/j.1476-5381.1990.tb15801.x

Edvinsson, L., Mcculloch, J., and Uddman, R. (1981). Substance P: immunohistochemical localization and effect upon cat pial arteries in vitro and in situ. *J. Physiol.* 318, 251–258. doi: 10.1113/jphysiol.1981.sp013862

Edvinsson, L., Nilsson, E., and Jansen-Olesen, I. (2007). Inhibitory effect of BIBN4096BS, CGRP(8-37), a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery. *Br. J. Pharmacol.* 150, 633–640. doi: 10.1038/sj.bjp.0707134

Edvinsson, L., and Uddman, R. (2005). Neurobiology in primary headaches. *Brain Res. Brain Res. Rev.* 48, 438–456. doi: 10.1016/j.brainresrev.2004.09.007

Eftekhari, S., Salvatore, C. A., Calamari, A., Kane, S. A., Tajti, J., and Edvinsson, L. (2010). Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. *Neuroscience* 169, 683–696. doi: 10.1016/j.neuroscience.2010.05.016

Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *J. Pain* 14, 1289–1303. doi: 10.1016/j.jpain.2013.03.010

Faroni, A., Castelnovo, L. F., Procacci, P., Caffino, L., Fumagalli, F., Melfi, S., et al. (2014). Deletion of GABA-B receptor in Schwann cells regulates remak bundles and small nociceptive C-fibers. *Glia* 62, 548–565. doi: 10.1002/glia.22625

Frederiksen, S. D., Haanes, K. A., Warfvinge, K., and Edvinsson, L. (2019). Perivascular neurotransmitters: regulation of cerebral blood flow and role in primary headaches. *J. Cereb. Blood Flow Metab.* 39, 610–632. doi: 10.1177/0271678X17747188

Frederiksen, S. D., Warfvinge, K., Ohlsson, L., and Edvinsson, L. (2018). Expression of pituitary adenylate cyclase-activating peptide, calcitonin gene-related peptide and headache targets in the trigeminal ganglia of rats and humans. *Neuroscience* 393, 319–332. doi: 10.1016/j.neuroscience.2018.10.004

Grande, G., Labruijere, S., Haanes, K. A., Maassenvandenbrink, A., and Edvinsson, L. (2014). Comparison of the vasodilator responses of isolated human and rat middle meningeal arteries to migraine related compounds. *J. Headache Pain* 15:22. doi: 10.1186/1129-2377-15-22

Griffin, J. W., and Thompson, W. J. (2008). Biology and pathology of nonmyelinating Schwann cells. *Glia* 56, 1518–1531. doi: 10.1002/glia.20778

Haanes, K. A., and Edvinsson, L. (2020). Hyperpolarization through ATP-sensitive potassium channels; relevance to migraine pathology. *Brain* 143:e13.

Hameed, S. (2019). Nav1.7 and Nav1.8: role in the pathophysiology of pain. *Mol. Pain* 15:1744806919858801.

Huang, L. Y., and Neher, E. (1996). Ca(2+)-dependent exocytosis in the somata of dorsal root ganglion neurons. *Neuron* 17, 135–145. doi: 10.1016/s0896-6273(00)80287-1

Jannetta, P. J. (1967). Arterial compression of the trigeminal nerve at the pons in patients with trigeminal neuralgia. *J. Neurosurg.* 26, 159–162. doi: 10.3171/jns.1967.26.1part2.0159

Jansen, I., Uddman, R., Ekman, R., Olesen, J., Ottosson, A., and Edvinsson, L. (1992). Distribution and effects of neuropeptide Y, vasoactive intestinal peptide, substance P, and calcitonin gene-related peptide in human middle meningeal arteries: comparison with cerebral and temporal arteries. *Peptides* 13, 527–536. doi: 10.1016/0196-9781(92)90084-g

Knutsson, M., and Edvinsson, L. (2002). Distribution of mRNA for VIP and PACAP receptors in human cerebral arteries and cranial ganglia. *Neuroreport* 13, 507–509. doi: 10.1097/00001756-200203250-00030

Lassen, L. H., Christiansen, I., Iversen, H. K., Jansen-Olesen, I., and Olesen, J. (2003). The effect of nitric oxide synthase inhibition on histamine induced headache and arterial dilatation in migraineurs. *Cephalalgia* 23, 877–886. doi: 10.1046/j.1468-2982.2003.00586.x

Lundblad, C., Haanes, K. A., Grande, G., and Edvinsson, L. (2015). Experimental inflammation following dural application of complete Freund's adjuvant or inflammatory soup does not alter brain and trigeminal microvascular passage. *J. Headache Pain* 16:91. doi: 10.1186/s10194-015-0575-8

McCulloch, J., Uddman, R., Kingman, T. A., and Edvinsson, L. (1986). Calcitonin gene-related peptide: functional role in cerebrovascular regulation. *Proc. Natl. Acad. Sci. U. S. A.* 83, 5731–5735. doi: 10.1073/pnas.83.15.5731

Miyamoto, Y., Torii, T., Kawahara, K., Inoue, M., Morimoto, T., Yamamoto, M., et al. (2017). Data on the effect of in vivo knockdown using artificial ErbB3 miRNA on Remak bundle structure. *Data Brief* 12, 313–319. doi: 10.1016/j.dib.2017.04.014

Moldovan Loomis, C., Dutzar, B., Ojala, E. W., Hendrix, L., Karasek, C., Scalley-Kim, M., et al. (2019). Pharmacologic characterization of ALD1910, a potent humanized monoclonal antibody against the pituitary adenylate cyclase-activating peptide. *J. Pharmacol. Exp. Ther.* 369, 26–36. doi: 10.1124/jpet.118.253443

Momin, A., Cadiou, H., Mason, A., and Mcnaughton, P. A. (2008). Role of the hyperpolarization-activated current Ih in somatosensory neurons. *J. Physiol.* 586, 5911–5929. doi: 10.1113/jphysiol.2008.163154

Nomura, K., Ryu, H., Ohno, K., and Sato, K. (2019). Varying length of central myelin along the trigeminal nerve might contribute to trigeminal neuralgia. *Clin. Anat.* 32, 541–545. doi: 10.1002/ca.23348

Rahmann, A., Wienecke, T., Hansen, J. M., Fahrenkrug, J., Olesen, J., and Ashina, M. (2008). Vasoactive intestinal peptide causes marked cephalic vasodilation, but does not induce migraine. *Cephalalgia* 28, 226–236. doi: 10.1111/j.1468-2982.2007.01497.x

Sams, A., Knyihar-Csillik, E., Engberg, J., Szok, D., Tajti, J., Bodi, I., et al. (2000). CGRP and adrenomedullin receptor populations in human cerebral arteries: in vitro pharmacological and molecular investigations in different artery sizes. *Eur. J. Pharmacol.* 408, 183–193. doi: 10.1016/s0014-2999(00)00781-0

Savvaidou, N. E., and Triarhou, L. C. (2016). Sesquicentennial tribute to Emil Redlich (1866-1930), The 'embodied conscience of neurology'. *Eur. Neurol.* 76, 267–277. doi: 10.1159/000452244

Schytz, H. W., Birk, S., Wienecke, T., Kruuse, C., Olesen, J., and Ashina, M. (2009). PACAP38 induces migraine-like attacks in patients with migraine without aura. *Brain* 132, 16–25. doi: 10.1093/brain/awn307

Smolen, A. J. (1988). Morphology of synapses in the autonomic nervous system. *J. Electron Microsc. Tech.* 10, 187–204. doi: 10.1002/jemt.1060100205

Torrealba, F., and Carrasco, M. A. (2004). A review on electron microscopy and neurotransmitter systems. *Brain Res. Brain Res. Rev.* 47, 5–17. doi: 10.1016/j.brainresrev.2004.06.004

Torrealba, F., Yanagisawa, M., and Saper, C. B. (2003). Colocalization of orexin a and glutamate immunoreactivity in axon terminals in the tuberomammillary nucleus in rats. *Neuroscience* 119, 1033–1044. doi: 10.1016/s0306-4522(03)00238-0

Trueta, C., and De-Miguel, F. F. (2012). Extrasynaptic exocytosis and its mechanisms: a source of molecules mediating volume transmission in the nervous system. *Front. Physiol.* 3:319. doi: 10.3389/fphys.2012.00319

Vause, C. V., and Durham, P. L. (2009). CGRP stimulation of iNOS and NO release from trigeminal ganglion glial cells involves mitogen-activated protein kinase pathways. *J Neurochem.* 110, 811–821. doi: 10.1111/j.1471-4159.2009.06154.x

Vijayaragavan, K., Boutjdir, M., and Chahine, M. (2004). Modulation of Nav1.7 and Nav1.8 peripheral nerve sodium channels by protein kinase A and protein kinase C. *J. Neurophysiol.* 91, 1556–1569. doi: 10.1152/jn.00676.2003

Xenellis, J. E., and Linthicum, F. H. Jr. (2003). On the myth of the glial/schwann junction (Obersteiner-Redlich zone): origin of vestibular nerve schwannomas. *Otol. Neurotol.* 24:1. doi: 10.1097/00129492-200301000-00001

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REVIEW
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Alpha-Calcitonin Gene Related Peptide: New Therapeutic Strategies for the Treatment and Prevention of Cardiovascular Disease and Migraine

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Alpha-calcitonin gene-related peptide (α-CGRP) is a vasodilator neuropeptide of the calcitonin gene family. Pharmacological and gene knock-out studies have established a significant role of α-CGRP in normal and pathophysiological states, particularly in cardiovascular disease and migraines. α-CGRP knock-out mice with transverse aortic constriction (TAC)-induced pressure-overload heart failure have higher mortality rates and exhibit higher levels of cardiac fibrosis, inflammation, oxidative stress, and cell death compared to the wild-type TAC-mice. However, administration of α-CGRP, either in its native- or modified-form, improves cardiac function at the pathophysiological level, and significantly protects the heart from the adverse effects of heart failure and hypertension. Similar cardioprotective effects of the peptide were demonstrated in pressure-overload heart failure mice when α-CGRP was delivered using an alginate microcapsules-based drug delivery system. In contrast to cardiovascular disease, an elevated level of α-CGRP causes migraine-related headaches, thus the use of α-CGRP antagonists that block the interaction of the peptide to its receptor are beneficial in reducing chronic and episodic migraine headaches. Currently, several α-CGRP antagonists are being used as migraine treatments or in clinical trials for migraine pain management. Overall, agonists and antagonists of α-CGRP are clinically relevant to treat and prevent cardiovascular disease and migraine pain, respectively. This review focuses on the pharmacological and therapeutic significance of α-CGRP-agonists and -antagonists in various diseases, particularly in cardiac diseases and migraine pain.

Keywords: alpha-calcitonin gene-related peptide (α-CGRP), cardiovascular diseases, CGRP-agonist, CGRP-antagonist, heart failure, hypertension, migraine, neuropeptide

Abbreviations: Ang-II, Angiotensin-II, α-CGRP, Alpha-calcitonin gene-related peptide, DRG, Dorsal root ganglia, I/R injury, Ischemia/reperfusion injury, KO, Knock-out, NO, Nitric oxide, NOS, Nitric oxide synthase, RAAS, Renin angiotensin aldosterone system, RAMP, Receptor activity modifying protein, ROS, Reactive oxygen species, SP, Substance P, TAC, Transverse aortic constriction, WT, Wild-type.

INTRODUCTION

Alpha-calcitonin gene-related peptide (α-CGRP) is a regulatory neuropeptide, a potent vasodilator, and belongs to calcitonin gene family (Brain et al., 1985; Russell et al., 2014). It is predominantly produced in the central and peripheral nervous systems, specifically in the dorsal root ganglia (DRG) of Aδ and C sensory neurons. Another form of CGRP also exists, named β-CGRP, which is primarily synthesized in the enteric nervous system, pituitary gland, and immune cells (Steenbergh et al., 1985; Mulderry et al., 1988). On chromosome 11, α-CGRP is produced from the CALC-I gene while β-CGRP is produced from the CALC-II gene. Although, α- and β-CGRP are 94% homologous, α-CGRP is predominantly involved in hemodynamic actions, while β-CGRPs exert gastric effects in humans (Steenbergh et al., 1986). The present review is mainly focused on the pathophysiological function of α-CGRP in normal and disease conditions, particularly cardiovascular disease and migraine, and recent advances in developing CGRP-agonists and -antagonists.

ALPHA-CALCITONIN GENE RELATED PEPTIDE AND ITS RECEPTOR

α-CGRP is a peptide of 37 amino acids that contains a disulfide bond at amino acids 2 and 7, and a phenylalanine amide group at the carboxy-terminal end (Kumar et al., 2019a; **Figure 1**). The first seven amino acids at the N-terminus end form a ring-like structure (Maggi et al., 1990; Breeze et al., 1991). Deletion of disulfide bond at amino acids 2 and 7 prevents α-CGRP from exerting its physiological actions. A CGRP fragment devoid of first seven amino acids, a.k.a. $CGRP_{8-37}$, can bind to the CGRP receptor, although its binding affinity for the receptor is 10-fold less than α-CGRP, and does not stimulate any physiologic response. $CGRP_{8-37}$ acts as a competitive antagonist of the CGRP receptor (Chiba et al., 1989). Thus, the first seven amino acids play an essential role in α-CGRP's high affinity binding for the CGRP receptor and are responsible for activating this receptor. Amino acid residues from 8 to 18 form an α-helix, while amino acids spanning from 19 to 27 form a β- or γ-twist (Rovero et al., 1992; Howitt et al., 2003). The C-terminal end of α-CGRP containing amino acids 28–37 interacts with N-terminus region of the CGRP receptor (Carpenter et al., 2001; Banerjee et al., 2006). Several biological and mutational studies have established that the N-terminal region of α-CGRP from amino acids 1–7 is responsible for receptor activation, while amino acids 8–37 are needed for binding affinity to the CGRP receptor (Watkins et al., 2013; **Figure 1**).

The CGRP receptor is a heterotrimer of three protein subunits: (1)- Calcitonin receptor-like receptor (CLR), (2)- receptor activity modifying protein 1 (RAMP1), and (3)- receptor component protein (RCP) (Walker et al., 2010; **Figure 2**). All three protein components are essential to exhibit cellular functions of CGRP receptor. CLR is a member of the class B "secretin-like" family of the G-protein-coupled receptors (GPCR), which also contains the receptors for calcitonin, parathyroid hormone, vasoactive intestinal polypeptide, and pituitary adenylate cyclase activating polypeptide. The ligand-binding CLR is a 461 amino acid protein that contains seven transmembrane-spanning domains, an extracellular N-terminus, and a cytosolic C-terminus (Aiyar et al., 1996). To initiate the signaling cascade, α-CGRP binds to the extracellular N-terminus of CLR. RAMP1 is a member of the RAMP protein family, which also includes RAMP2 and RAMP3 (Hay and Pioszak, 2016; Serafin et al., 2020). The RAMP family shares a similar structural motif even though their amino acid sequences are <30% homologous with one another. RAMP1 is specific to the CGRP receptor, while RAMP2 and RAMP3 are part of the adrenomedullin (AM) receptors (McLatchie et al., 1998; **Figure 2**). RAMP1 consists of one transmembrane-spanning domain with a long extracellular N-terminal domain (∼100 amino acids) and a short intracellular C-terminus (∼10 amino acids) (Steiner et al., 2002; Udawela et al., 2006; Qi and Hay, 2010). In order for CLR to be transported from the endoplasmic reticulum to the plasma membrane, it must be heterodimerized with RAMP1 as a mature glycoprotein (Hilairet et al., 2001). The heterodimeric complex of CLR and RAMP1 is stabilized via non-covalent interactions between the two proteins. Production of RAMP1 in the absence of CLR inhibits RAMP1 transport to the plasma membrane, resulting in RAMP1 accumulation in the Golgi apparatus as a disulfide-linked homodimer. In RAMP1-KO mice, an increase in BP (but no change in heart rate) was observed suggesting that deletion of RAMP1 disrupted CGRP-mediated cellular signaling (Tsujikawa et al., 2007). Receptor component protein (RCP) is a small (∼17 kDa) cytosolic protein that ionically binds to CLR and is required for G protein coupling (Evans et al., 2000; Egea and Dickerson, 2012). Experiments using RCP-depleted NIH3T3 cells concluded that although loss of RCP levels had no effect between the binding affinity of α-CGRP and CLR, these cells were unable to initiate the CGRP signaling cascade to create a physiologic response due to decreased intracellular cyclic adenosine monophosphate (cAMP) levels (Evans et al., 2000). Therefore, RCP serves as the connection between CLR and the intracellular G protein-mediated signaling pathway, which triggers production of cAMP (Routledge et al., 2020).

The expression of CGRP receptor is widely reported within the central and peripheral nervous system, and cardiovascular system. Molecular methods like radioligand binding assay, immunohistochemistry, and Northern blot analysis were employed to determine the localization of CGRP receptor in different tissues and organs. Using radiolabelled ^{125}I-CGRP in radioligand binding assays, the specific binding site for the CGRP ligand has been shown in the rat cardiovascular system, human and rat brain, central and peripheral tissues including bronchial and pulmonary blood vessels of human and guinea pig, and the smooth muscle layer of arteries and arterioles of the human urinary bladder (Inagaki et al., 1986; Sexton et al., 1986; Mak and Barnes, 1988; Wimalawansa and MacIntyre, 1988; van Rossum et al., 1997; Burcher et al., 2000). In the cardiovascular system and visceral organs, CGRP-binding sites were detected in the right atrium, and within the intima and media layers of blood vessels, such as the mesenteric artery, aorta, carotid arteries, renal arteries, pulmonary arteries and veins, spleen, lung, and

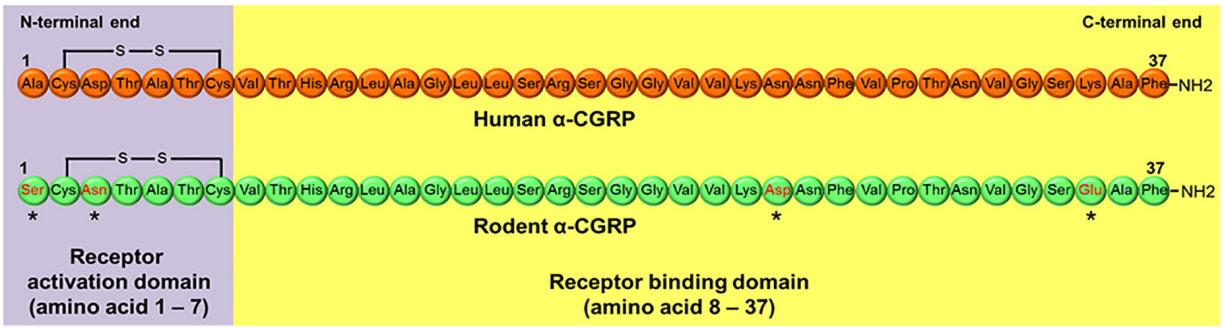

FIGURE 1 | Peptide sequence of human and rodent α-CGRP. Rodent α-CGRP differs at four amino acids (*marked) with human α-CGRP. Amino acids 1–7 at N-terminal end of the peptide forms receptor activation domain, while amino acid region 8–37 consists of receptor binding domain.

liver (Nakamuta et al., 1986; Wimalawansa and MacIntyre, 1988; McCormack et al., 1989). The mRNA transcripts of CLR (by RT-PCR assay) and RAMP1 (by Northern blot analysis) were detected in myocyte and non-myocyte cells prepared from cardiac ventricles of 1-day-old rat pups (Tomoda et al., 2001). In addition, using immunohistochemistry the expression of CGRP receptor components (CLR and RAMP1) in arterial blood vessels and nerve fibers present in the rodent cranial dura mater, human trigeminal ganglion, myelinated A-fiber axons, and rat cerebellar Purkinje cells were detected (Lennerz et al., 2008; Eftekhari et al., 2010, 2013; Edvinsson et al., 2011). In the rat brain, the immunoreactivity for CLR1 and RAMP1 was detected in the neuronal processes of the cerebral cortex, hippocampus, cerebellum, thalamic and hypothalamic nuclei, and brainstem nuclei (Warfvinge and Edvinsson, 2019). Using human subcutaneous arteries, Edvinsson et al. (2014), detected the immunoreactivity of CLR and RAMP1 in the endothelial and smooth muscle cells, and to a minor extent in the vascular adventitia (Edvinsson et al., 2014). Eftekhari and Edvinsson (2011) performed immunohistochemistry for CGRP, CLR and RAMP1 and compared their expression in the human and rat spinal trigeminal nucleus (STN) and the C1 region of the spinal cord (Eftekhari and Edvinsson, 2011). Immunohistochemistry data demonstrated that CLR and RAMP1 positive fibers were present in the human spinal trigeminal tract region. CLR and RAMP1 were co-localized in this region, but no co-localization of CGRP and CLR or CGRP and RAMP1 was observed. In rat STN, CLR and RAMP1 (but not CGRP and CLR or CGRP and RAMP1) were co-localized in the spinal trigeminal tract region, in both fiber bundles and fibers spanning from the spinal trigeminal tract. In human C1, CLR and RAMP1 signals were detected within laminae I and II, but no co-localization of these receptor components was observed. However, in rat C1, CLR and RAMP1 were co-localized in the laminae I and II region, but no co-localization of CGRP and CLR or CGRP and RAMP1 was observed. In contrast to Eftekhari and Edvinsson (2011) study, one study reported partial co-localization of CGRP and its receptor component CLR and RAMP1 in the rat superficial laminae (Lennerz et al., 2008). The discrepancy in the detection of CGRP, CLR, and RAMP1 in both studies might be due the difference in the recognition sites of the antibodies used and change in methods of the tissue processing. Miller et al. (2016), raised antibodies against a fusion protein of the extracellular domains of RAMP1 and CLR that comprise the CGRP binding site of the CGRP receptor. Using these antibodies, researchers performed immunohistochemistry and confirmed the expression of CGRP receptor in human vascular smooth muscle cells of dural meningeal arteries and neurons in the trigeminal ganglion (Miller et al., 2016). Immunohistology method further confirmed expression of CLR and RAMP1 in human cerebral vasculature particularly in middle meningeal, middle cerebral, superficial temporal, and pial vessels (Oliver et al., 2002). These studies suggested that although immunohistochemistry is a powerful tool to detect regional distribution of proteins in tissue, it has several limitations as well. A variety of antibodies raised against CGRP and CGRP receptor components are commercially available, however, considerations such as consistency in tissue processing, use of validated antibodies specific for the histochemistry, and species specificity of these antibodies are needed while performing immunohistochemistry to detect CGRP and its receptor in the tissue.

SYNTHESIS AND RELEASE OF ALPHA-CALCITONIN GENE RELATED PEPTIDE

The α-CGRP peptide is created via tissue-specific alternative splicing of the pre-mRNA transcript of the calcitonin gene CALC-I on chromosome 11 (Amara et al., 1982). Thyroid C cells produce calcitonin by splicing out exons 5 and 6 from the CALC-I gene transcript, while sensory neurons form α-CGRP via removal of exon 4 from the CALC-I gene pre-mRNA product (**Figure 3**). To produce mature α-CGRP, it is first translated as a 121 amino acid pro-hormone and then cleaved into a final 37 amino acid polypeptide (Rosenfeld et al., 1983). α-CGRP is primarily stored and released from Aδ- and C-fiber sensory neurons of the afferent nervous system, though it is also distributed in smooth muscle of blood vessels and in regions of the central nervous system (Gibson et al., 1984; Uddman et al., 1986; Kee et al., 2018). α-CGRP synthesis occurs almost exclusively in the dorsal root ganglia (DRG) of sensory neurons, however, endothelial cells

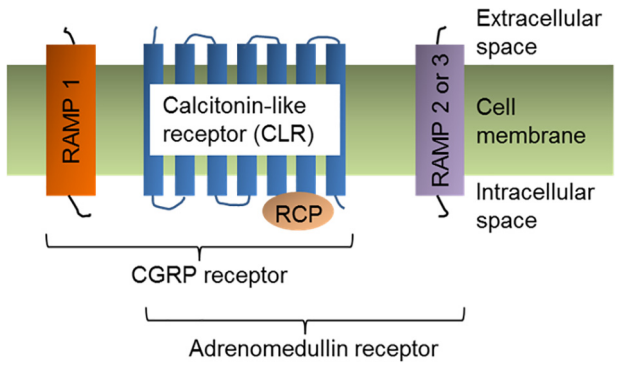

FIGURE 2 | α-CGRP receptor structure. Cell-membrane bound α-CGRP receptor is a heterotrimer of three protein subunits: Calcitonin-like receptor (CLR), RAMP1, and RCP. Coupling of RAMP2 or RAMP3 with CLR and RCP subunits form adrenomedullin receptor.

and certain immune cells, such as lymphocytes and monocytes, also have the ability to produce the peptide (Doi et al., 2001; Bracci-Laudiero et al., 2002; Wang et al., 2002; Linscheid et al., 2004). Additionally, the trigeminal ganglion releases α-CGRP to regulate cerebral vascular tone, which contributes to the pathology of certain migraines (McCulloch et al., 1986). Aδ- and C-fiber sensory neurons release α-CGRP when stimulated by mechanical, thermal, and chemical stimuli, but because of their efferent function, they can also excrete α-CGRP without any stimulation. It is hypothesized that the efferent release of α-CGRP from these neurons plays an important role in blood flow regulation, as circulating α-CGRP levels are attributable to leakage of the peptide from the synaptic cleft after its release. Once α-CGRP is generated, it is stored in neuronal vesicles with substance P (SP) in the axon terminals until axonal depolarization and calcium-dependent exocytosis trigger its release (Gibbins et al., 1985; Matteoli et al., 1988; **Figure 4**). In motor neurons, α-CGRP is stored and released with acetylcholine (Ach), which led some to hypothesize that α-CGRP plays a role in Ach receptor synthesis (New and Mudge, 1986; Csillik et al., 1993).

α-CGRP is released in response to high levels of angiotensin-II (Ang-II) and norepinephrine to combat the potent vasoconstrictive actions of these compounds, which is why α-CGRP agonists are likely beneficial in hypertensive patients (Supowit et al., 1995b). Capsaicin, nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), low pH, bradykinins, prostaglandins, tissue inflammation, and the renin angiotensin aldosterone system (RAAS) also promote α-CGRP excretion from DRG sensory neurons (Lindsay et al., 1989; Donnerer and Stein, 1992; Andreeva and Rang, 1993; Strecker et al., 2006; Salio et al., 2007; Supowit et al., 2011). Additionally, α-CGRP synthesis increases in the presence of neuronal damage (Donnerer et al., 1992). It is thought that macrophages and keratinocytes excrete NGF in a paracrine-like manner when exposed to inflammation in order to stimulate nearby sensory neurons to release α-CGRP. One study demonstrated that addition of NGF to adult DRG neurons promotes α-CGRP expression through activation of the Ras/Raf/MEK-1/p42/p44 MAPK pathway (Freeland et al., 2000). Another study illustrated that NGF intraperitoneal injections in spontaneously hypertensive rats (SHRs) induced α-CGRP production and release in DRG sensory neurons (Supowit et al., 2001). Stimulation of the transient receptor potential vanilloid 1 (TRPV1) receptor, a type of non-selective cation channel in the TRP family found on sensory nerve axon terminals, prompts α-CGRP release when exposed to noxious heat, capsaicin, and acidic environments (Kichko and Reeh, 2009; Meng et al., 2009; Dux et al., 2020). It has been observed that phenotypically TRPV1-KO mice are not hypertensive, however, these KO mice, after undergoing TAC procedure, exhibit increased LV hypertrophy and fibrosis compared to their wild-type counterparts (Marshall et al., 2013; Zhong et al., 2018). Transient receptor potential ankyrin 1 (TRPA1), another member of the TRP family, induces α-CGRP excretion when exposed to noxious cold, reactive oxygen species (ROS), and cellular stress (Pozsgai et al., 2012; Wang et al., 2019; Gebhardt et al., 2020).

A CGRP-KO mouse strain, where both CGRP and calcitonin genes were deleted, showed an increase in basal BP (Gangula et al., 2000), while no difference in heart rate and BP was observed in another CGRP-KO mouse strain (Lu et al., 1999). Moreover, compared with their WT-counterparts, α-CGRP mice exhibit a higher extent of cardiac fibrosis and inflammation when undergoing TAC-induced pressure overload, and their renal and cardiovascular systems are more susceptible to hypertension-induced end-organ damage (Supowit et al., 2005; Li et al., 2013).

THERAPEUTIC BENEFITS OF ALPHA-CALCITONIN GENE RELATED PEPTIDE AND ITS ANALOGS IN CARDIOVASCULAR DISEASE

The effects of α-CGRP on the cardiovascular system are well-documented: it induces positive chronotropic, ionotropic, and hypertrophic effects on the heart, it mediates blood flow, and it is the most potent vasodilator known to date (Fisher et al., 1983; Brain et al., 1985; Franco-Cereceda and Lundberg, 1985; Struthers et al., 1986; Gennari et al., 1990; Bell et al., 1995; Al-Rubaiee et al., 2013). The positive ionotropic and chronotropic actions of α-CGRP are attributed to reflexive sympathetic activity and direct stimulation of α-CGRP on cardiomyocytes to oppose the hypotensive effects from vasodilation. Specifically, α-CGRP stimulation of cardiomyocytes triggers positive ionotropic effects via the cAMP/PKA or the PKC intracellular messenger pathways. An *in vitro* study of guinea pig atria found that exposure to capsaicin, a TRPV1 agonist, triggered α-CGRP release and produced positive ionotropic and chronotropic effects in the cardiac cells, but these results were diminished when hearts were infused with capsaicin to desensitize the nerves and induce tachyphylaxis (Lundberg et al., 1984). The vasodilatory capabilities of α-CGRP are ∼1,000 times more potent than acetylcholine, substance P, and 5-hydroxytriptamine, and 10 times more potent than the most powerful vasodilatory

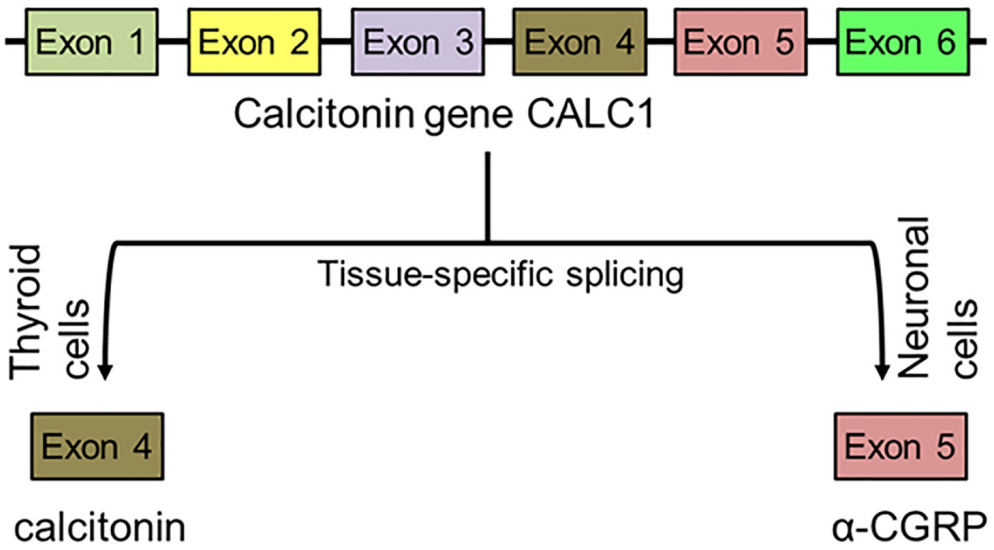

FIGURE 3 | Synthesis of peptide α-CGRP. After tissue-specific alternative splicing of calcitonin gene CALC1, α-CGRP is synthesized in the neuronal cells while calcitonin is formed in the thyroid cells.

prostaglandins (Brain et al., 1985). Additionally, α-CGRP-induced vasodilation persists longer than other vasodilators. Injection of 15 pmol of α-CGRP intradermally in humans augments local blood flow and causes erythema formation of the skin that lasts 5–6 h (Brain et al., 1985). One study carried out by DiPette et al. (1989) demonstrated that a bolus intravenous injection of α-CGRP at doses of 22, 65, 220, and 2,200 pmol in conscious rats reduced mean blood pressure in a dose dependent manner (DiPette et al., 1989). Because of its potency and sustainability, α-CGRP stimulates vasodilation of various vascular beds, including the coronary, cerebral, and renal vessels, and systemic infusion reduces blood pressure in normotensive and hypertensive species (DiPette et al., 1989; Dubois-Rande et al., 1992; Gulbenkian et al., 1993). The vasodilatory actions of α-CGRP are most prominent in small peripheral arteries, as opposed to large vessels, respectively. It has been shown in the rat isolated mesenteric resistance arteries that α-CGRP is used to combat the vasoconstrictive effects of endothelin via interaction with the CGRP receptor and a $G_{\beta\gamma}$ -coupled protein (Meens et al., 2011, 2012).

α-CGRP can stimulate vasodilation of peripheral arteries using either the nitric oxide (NO)-/endothelium-independent pathway or the NO-/endothelium-dependent pathway. However, in most blood vessels, vasodilatation occurs via the NO-/endothelium-independent route (Russell et al., 2014; Kumar et al., 2019b). To initiate the NO-/endothelium-independent pathway, α-CGRP first binds to the CGRP receptor on a vascular smooth muscle cell, which stimulates $G\alpha_s$ to activate adenylate cyclase. Once adenylate cyclase is stimulated, it will synthesize cAMP followed by activation of protein kinase A (PKA). The activated PKA subsequentially induces K^+-ATP channels to open, causing smooth muscle relaxation and vasodilation. Administration of glibenclamide, a K^+-ATP channel inhibitor, blocks α-CGRP-induced hyperpolarization, and therefore vasodilation, of vascular smooth muscle cells (Edvinsson et al., 1985; Nelson et al., 1990).

There are several reports showing that vasodilation of the rat aorta and pulmonary arteries, and human internal mammary arteries are regulated via the NO-/endothelium-dependent pathway. One study found that delivery of NO synthase (NOS) inhibitors in the rat aorta weakened the vasodilatory capability of α-CGRP (Gray and Marshall, 1992b). Another study demonstrated that treatment of rat aortas with human α-CGRP augmented intracellular cAMP and cGMP concentrations, and induced vasodilation. This occurred only when the endothelium was intact, further supporting the idea that these vessels are NO-/endothelium-dependent (Gray and Marshall, 1992a).

Several studies have highlighted the importance of α-CGRP as a cardioprotective molecule. Specifically, α-CGRP prevents pathologies such as hypertension, myocardial infarctions, heart failure, and ischemia from damaging cardiac cells through vasodilation and inhibition of oxidative stress, inflammation, and apoptosis. The role of α-CGRP in normal and pathophysiological states of these diseases is disused below.

Hypertension

Plasma levels of α-CGRP are elevated in hypertensive patients with primary aldosteronism or on a high-salt diet, but are decreased in patients with secondary hypertension who have undergone an adrenalectomy, suggesting that α-CGRP might act as a compensatory mechanism: first the peptide is released to oppose the effects of high blood pressure, but later CGRP synthesis and/or release may become inhibited as the disorder advances (Masuda et al., 1992). Several models of hypertension have revealed that expression of α-CGRP and its receptor increased in these pathological states (Kawasaki et al., 1990; Supowit et al., 1995a; Li and Wang, 2005). In a number of experimental hypertension models such as deoxycorticosterone

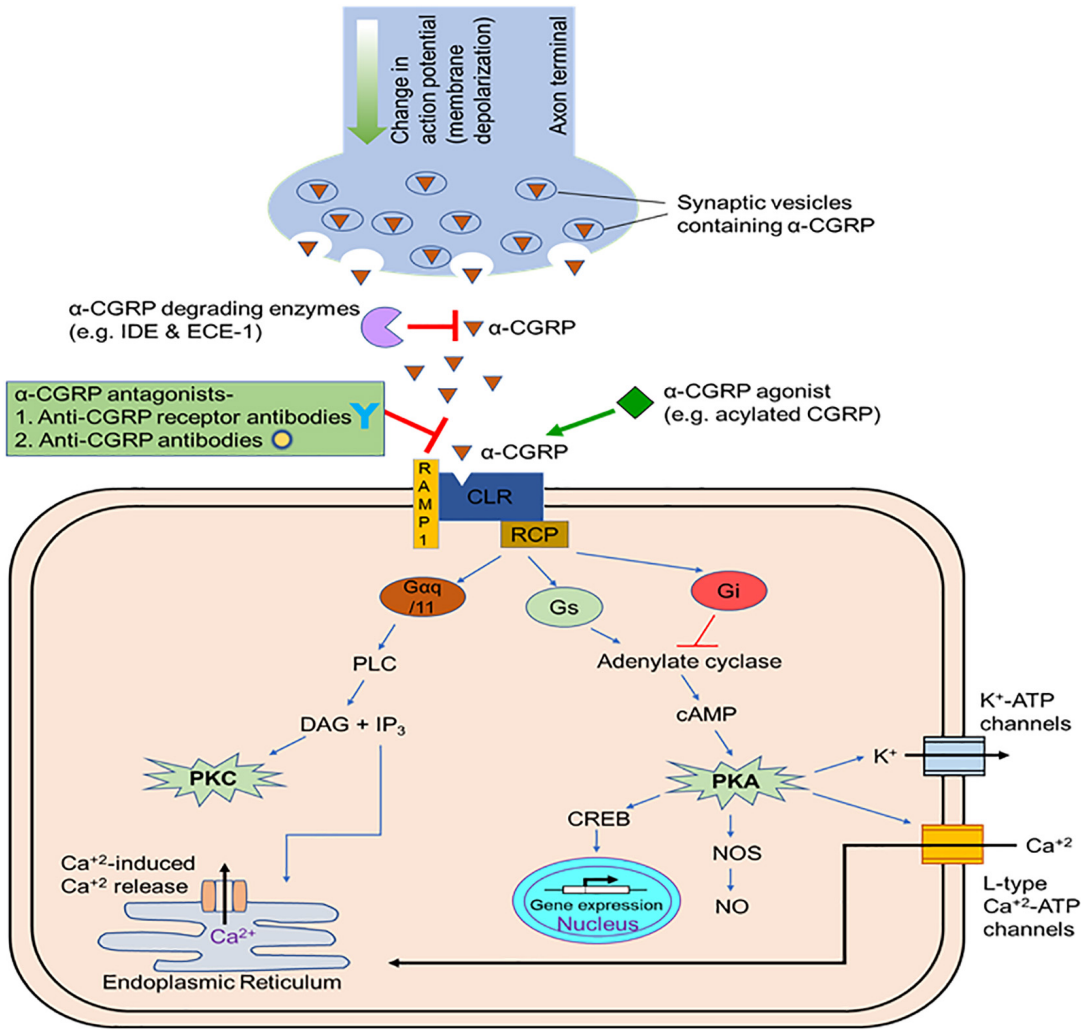

FIGURE 4 | Release and mode of action of α-CGRP. After synthesis, α-CGRP remains stored in vehicles at the terminal ends of the nerves. As nerve depolarize, α-CGRP is released form the nerve terminals through the exocytosis process and interacts to its receptor and elicits its cellular effects mainly via the generation of intracellular cAMP. In the circulation, endopeptidases (insulin degrading enzyme, IDE, and endothelin converting enzyme-1, ECE-1) degrades α-CGRP. CGRP-agonists with increased self-life and bioactivity prolong the cellular activity of α-CGRP and might be beneficial to use as therapeutic agents in cardiac diseases. Several CGRP-antagonists that block interaction of α-CGRP to its receptor and, thus, inhibit downstream signaling pathways are employed to treat migraine pain.

(DOC)-salt, subtotal nephrectomy (SN)-salt, the two-kidney one-clip (2K1C), N-nitro-L-arginine methyl ester (L-NAME)-induced hypertension during pregnancy, and chronic hypoxic pulmonary hypertension, α-CGRP blunts blood pressure elevations as a compensatory mechanism (Tjen et al., 1992; Gangula et al., 1997; Supowit et al., 1997, 1998). Depending on the model of hypertension, neuronal levels of α-CGRP are regulated differently. For example, in hypertensive rats induced by either the DOC-salt or the 2K1C model, levels of immunoreactive CGRP in the spinal cord and α-CGRP mRNA in DRG were elevated in order to stimulate vasodilation and combat the developing hypertension (Supowit et al., 1995a, 1997, 1998). Administration of capsaicin or the CGRP receptor antagonist, $CGRP_{8-37}$, in these rats increased the mean arterial pressure (MAP), further supporting the idea that the high α-CGRP levels attenuated the hypertension. Delivery of $CGRP_{8-37}$ in SN-induced hypertensive rats and L-NAME-induced hypertension during pregnancy also augmented the hypertensive levels (Gangula et al., 1997). In contrast to the DOC-salt model, immunoreactive CGRP concentrations in laminae I and II of the spinal cord and α-CGRP mRNA levels in the DRG were reduced in spontaneously hypertensive rats (SHRs) (Supowit et al., 1993). Further, pre-treatment administration of capsaicin or $CGRP_{8-37}$ had no effect on blood pressure, indicating that the lack of α-CGRP contributes to the development of high blood pressure due to loss of vasodilatory control.

One study demonstrated that Ang-II-induced hypertensive mice displayed higher amounts of ROS, inflammation, and apoptotic cell death in their hearts and kidneys. Additionally, they showed reduced cardiac function due to Ang-II upregulation

of oxidative stress response proteins and downregulation of the anti-oxidative enzyme glutathione peroxidase-1, resulting in disruption of endothelial cell function and vascular hypertrophy (Aubdool et al., 2017). Furthermore, Ang-II induces RAMP1 expression in the cardiovascular system. Delivery of acylated α-CGRP, an α-CGRP agonist, in mice significantly reduced the adverse effects created by Ang-II. A similar study using α-CGRP KO mice with hypertension via Ang-II administration, led to aortic hypertrophy and reduced eNOS expression (Smillie et al., 2014). Therefore, α-CGRP appears to delay the onset and development of hypertension through cardioprotective mechanisms.

A study comparing α-CGRP KO mice to WT mice in a DOC-salt model revealed that the KO mice developed greater amounts of cellular damage and oxidative stress in the cardiovascular and renal systems and had elevated concentrations of pro-inflammatory cytokines and chemokines than the DOC-salt WT mice (Supowit et al., 2005). Baseline blood pressure of α-CGRP KO mice was significantly elevated compared to WT counterparts, indicating that α-CGRP plays a role in preventing hypertension. Therefore, lack of α-CGRP increases the likelihood of hypertension-induced damage to the heart and kidneys. Another basis for α-CGRP KO mice to develop high blood pressure is that they have greater activity of the RAAS, which is a well-known mechanism involved in the development of hypertension (Li et al., 2004). α-CGRP opposes the hypertensive effects of RAAS through vasodilation and by inhibiting Ang-II induced aldosterone secretion. Electric field stimulation of the mesenteric arteries in SHRs triggers a vasoconstrictive response, but the vasodilatory response to α-CGRP was augmented by aldosterone, suggesting that α-CGRP controls blood pressure homeostasis by interacting with the RAAS (Balfagon et al., 2004). Delivery of sub-depressor doses of α-CGRP for 6 days in hypertensive rats induced by Ang-II or norepinephrine decreased blood pressure significantly (Fujioka et al., 1991). Much like α-CGRP KO mice, RAMP1 KO mice develop high blood pressure (Tsujikawa et al., 2007). However, even with exogenous α-CGRP administration, RAMP1 KO mice are hypertensive because α-CGRP is unable to activate its signaling cascade and therefore, vasodilation is unachievable. On the other hand, upregulation of RAMP1 in Ang-II-induced hypertensive mice enhanced the ability of α-CGRP to reduce blood pressure (Sabharwal et al., 2010). Additionally, because the baroreflex sensitivity of RAMP1 transgenic mice was augmented, resulting in a lower blood pressure, suggesting that α-CGRP modulates the baroreflex response to help reduce blood pressure.

α-CGRP is also protective against pulmonary hypertension, likely as the result of the high expression in the lungs and dilatation of pulmonary arteries (Bivalacqua et al., 2002). α-CGRP specifically acts to blunt the effects of hypoxia-induced tissue remodeling that occurs in pulmonary hypertension. Rats affected by pulmonary hypertension have reduced plasma levels of α-CGRP, and these effects are exacerbated by $CGRP_{8-37}$ infusion (Tjen et al., 1992). Administration of α-CGRP to these rats attenuated the effects of pulmonary hypertension. One study carried out using left-to-right shunt-induced pulmonary hypertensive rats demonstrated that intravenous injection of endothelial progenitor cells modified to secrete α-CGRP reduced the severity of the disease and prevented adverse vascular remodeling (Zhao et al., 2007). These studies demonstrated that α-CGRP is a potent vasopressor and is a potential therapeutic peptide to treat patients suffering from high blood pressure.

Ischemia/Reperfusion Injury

The protective function of α-CGRP against ischemia/reperfusion (I/R) injury has been reported in human and a numerous animal models. α-CGRP KO mice subjected to 30-min ischemic episodes followed by reperfusion to stimulate I/R injury experienced significant deterioration in cardiac function compared to WT mice who underwent the same procedure (Huang et al., 2008). Sensory Aδ- and C-fibers excrete α-CGRP as a cardioprotective mechanism after a cardiac I/R injury. For example, levels of circulating α-CGRP increase in humans following an acute myocardial infarction and isolated hearts of guinea pigs and rats release more α-CGRP after experiencing myocardial ischemia (Franco-Cereceda, 1988; Mair et al., 1990; Lechleitner et al., 1992). During these pathological events, stimuli such as bradykinin, prostaglandins, and low pH activate TRPV1 receptors to induce α-CGRP release. An increased CGRP-immunoreactivity has been reported in principal ganglionic neurons and perineuronal nets in the human stellate ganglia following acute myocardial infarction (Roudenok et al., 2001). Other investigations have revealed that α-CGRP also plays a role in cardiac and remote preconditioning, in which CGRP infusion reduces the cardiac damage caused by an ischemic event in a rat model of I/R injury (Wolfrum et al., 2005). When Langendorff-perfused rat hearts were administered exogenous α-CGRP, the peptide imitated the beneficial preconditioning effects induced by transient ischemia and resulted in increased endogenous α-CGRP release (Wu et al., 2001). Conversely, infusion of the CGRP receptor antagonist BIBN4096BS inhibited all cardioprotective effects of α-CGRP and preconditioning, suggesting that α-CGRP is involved in the physiologic response to cardiac I/R injuries. BIBN4096BS or olcegepant (developed by Boehringer Ingelheim Pharmaceuticals) is a small molecule non-peptide antagonist that exhibits greater antagonism to the human CGRP receptor when compared to rodent CGRP-receptor, and does not show affinity for adrenomedullin receptors (AM1 or AM2 receptor) (Doods et al., 2000; Hay et al., 2003; Arulmani et al., 2004). In addition to the CGRP-receptor, BIBN4096BS is reported to block the binding of CGRP to another distinct CGRP-responsive receptor, the amylin subtype 1 receptor known as the AMY1 receptor, however, BIBN4096BS has higher binding affinity to the CGRP receptor than to the AMY1 receptor and it is a relatively selective antagonist to the CGRP receptor (Hay et al., 2006; Walker et al., 2018). A series of *in vitro* receptor assays carried out in Cos7 cells transfected with CGRP receptor or AMY1 receptor and in cultured rat trigeminal ganglia neurons demonstrated that BIBN4096BS treatment significantly inhibited α-CGRP-stimulated phosphorylation of cAMP response element-binding protein (CREB) and the accumulation of cAMP in these cells (Walker et al., 2018). A comparison study in the transfected Cos7 cells showed that BIBN4096BS was ∼132-fold more potent

at the CGRP receptor than the AMY1 receptor in blocking α-CGRP-stimulated cAMP accumulation and ∼26-fold more potent at the CGRP receptor than the AMY1 receptor in blocking α-CGRP-stimulated CREB phosphorylation (Walker et al., 2018). Moreover, the antagonist selectivity of this molecule has been shown to be pathway-dependent as BIBN4096BS inhibited α-CGRP-stimulated CREB phosphorylation to a greater extent compared to cAMP accumulation in rat trigeminal ganglia neurons and AMY1 receptor transfected Cos7 cells (but not in the CGRP receptor transfected Cos7 cells) (Walker et al., 2018). The AMY1 receptor is a complex of the calcitonin receptor (CTR) and RAMP1 subunit (CTR-RAMP1), and it is responsive to both CGRP and amylin peptides (Muff et al., 1999; Walker et al., 2015; Hay and Walker, 2017). As RAMP1 is a common component in the CGRP receptor and AMY1 receptor, it is thought that binding of BIBN4096BS to RAMP1 might be involved in the antagonism of BIBN4096BS (Mallee et al., 2002). Due to poor oral bioavailability, BIBN4096BS is not considered a viable choice as a therapeutic molecule where inhibition of CGRP-receptor activation is needed, e.g., migraine headache. Adenoviral transfection of cardiac tissue with α-CGRP, in diabetic rodents, increased the ischemic preconditioning response, which is typically reduced in these models (Zheng et al., 2012). Zheng et al. (2012) showed that streptozotocin-induced diabetic mice who underwent ischemia (30 min) and reperfusion (24 h) had increased myocardial infarct size and higher plasma level of lactate dehydrogenase (LDH) compared to their non-diabetic counterparts. However, intramyocardial injection of adenovirus encoding the CGRP gene reduced myocardial infarct size and plasma LDH level in both non-diabetic and diabetic mice (Zheng et al., 2012). α-CGRP has been shown to prevent I/R damage in other tissues such as the intestines, kidneys, and brain (Song et al., 2009; Liu et al., 2011; Lu et al., 2017). When central organs, like the heart and liver, are exposed to ischemia, α-CGRP release may serve to both prevent damage to these organs and protect other organ systems, such as the intestines, from subsequent injury via reflexive neuronal release. In one study, Luo et al. (2016) demonstrated that following the intestinal ischemia or intestinal reperfusion phase in rats, the level of caspase-3 protein (an apoptotic cell death marker) increased. CGRP pretreatment reduced iNOS and caspase-3 levels, and protected against intestinal I/R injury (Luo et al., 2016).

Finally, administration of α-CGRP following a myocardial I/R injury can significantly reverse the developing cardiac dysfunction. In patients with stable angina pectoris, exogenous α-CGRP postponed the onset of myocardial ischemia (Mair et al., 1990). In total, these findings demonstrate that depletion of α-CGRP increases the risk of developing an I/R injury, augments cardiac damage, and delays the recovery process following myocardial ischemia. These results demonstrated that α-CGRP is protective against ischemia-reperfusion injury.

Heart Failure

α-CGRP-producing sensory nerves are found in the perivascular layer of coronary vessels, in the cardiac conduction system, and in myocardium of the ventricles (Gulbenkian et al., 1993). When blood pressure rises or there is a potential of damage to the heart, such as ischemia or oxidative stress, these neurons release α-CGRP to maintain cardiovascular homeostasis. Exogenous administration of α-CGRP enhances cardiac function in humans by stimulating catecholamine release to induce positive ionotropic effects and by increasing stroke volume through reducing afterload via vasodilation (Tortorella et al., 2001). Additionally, α-CGRP infusion in patients with heart disease has been shown to enhance blood flow through vasodilation (Gennari et al., 1990; Shekhar et al., 1991). For these reasons, α-CGRP protects the heart against adverse myocardial remodeling and dysfunction from hypertension and heart failure.

Although patients with heart failure have higher circulating levels of α-CGRP during the initial and middle stages, these levels rapidly fall during the later stages of heart failure as functionality declines (Dubois-Rande et al., 1992). Rats induced with pressure-overload heart failure via constriction of their ascending aorta expressed higher levels of RAMP1 mRNA and its subsequent protein in the cardiomyocytes of their atria and ventricles, indicating that more CGRP receptors were available (Cueille et al., 2002). Another study using rats with isoprenaline-induced heart failure found that delivery of rutaecarpine, a TRPV1 agonist that stimulates α-CGRP release, attenuated cardiac hypertrophy and apoptosis of cardiomyocytes (Li et al., 2010). Pre-treatment capsaicin infusion to these rats diminished the results, further indicating that α-CGRP is cardioprotective against heart failure. Our laboratory previously demonstrated that when α-CGRP KO mice underwent transverse aortic constriction (TAC) to develop pressure-overload heart failure, their survival rates were drastically decreased compared to the TAC-WT mice (Li et al., 2013). Additionally, the TAC-KO mice displayed a greater extent of dysfunctional cardiac remodeling and left ventricular hypertrophy, and they had reduced cardiac functions compared to the TAC-WT mice. Further, cardiomyocytes of the TAC-KO mice exhibited greater amounts of fibrosis, inflammation, and apoptosis, and had lower rates of angiogenesis as opposed to the TAC-WT mice. Our lab also showed that when we deliver native α-CGRP through osmotic mini-pumps for 28 days to the TAC-WT mice, CGRP administration is cardio-protective against pressure-overload induced heart failure (Kumar et al., 2019b). Our data showed that α-CGRP delivery preserved cardiac functions and inhibited left ventricular apoptosis, fibrosis, and cardiac hypertrophy in the TAC mice, and thus protecting hearts from pressure-induced heart failure. Based on our observations in this study, we proposed that TAC-induced pressure overload increased the nuclear level of sirt1 via the direct and/or indirect activation of AMPK and thus impaired mitochondrial function. As a result of mitochondrial dysfunction, there was a significantly higher number of apoptotic cells in the TAC-hearts due to larger amount of ROS produced. Administration of α-CGRP attenuated TAC-induced increased activation of sirt1 and AMPK, and inhibited oxidative stress and apoptotic cell death in TAC mice who received α-CGRP. Together, these events inhibited cardiac hypertrophy and protected hearts at the pathophysiological levels. These results suggest that α-CGRP might be a therapeutic agent to treat and prevent cardiac diseases. However, non-applicability of osmotic mini-pumps in humans and short half-life of α-CGRP in circulation (∼5.5 min in human plasma)

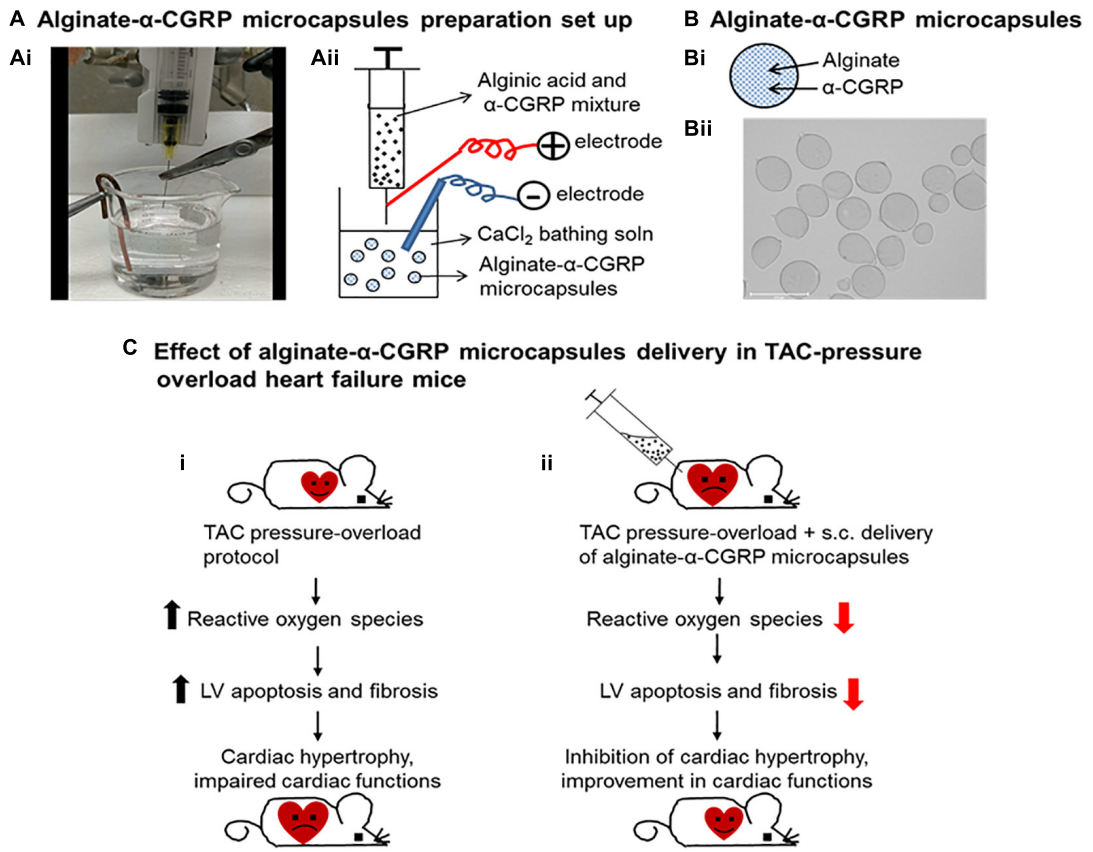

FIGURE 5 | Alginate-α-CGRP microcapsules as a peptide delivery system in heart failure mice. Laboratory set up **(Ai)** and schematic presentation **(Aii)** showing encapsulation of α-CGRP in alginate microcapsules using an electrospray method. **(B)** Schematic picture showing α-CGRP encapsulated alginate microcapsule **(Bi)**. Using an electrospray method, alginate-α-CGRP microcapsules were prepared and imaged **(Bii)**. **(C)** Schematic representation of cardio-protective effect of alginate-α-CGRP microcapsules against heart failure in mice. Transverse aortic constriction (TAC) procedure was performed in wild-type mice. TAC-induced pressure overload stimulated excessive reactive oxygen species generation and induced apoptotic cell death and fibrosis in mice left ventricles (LV). As a result, hearts got enlarged and cardiac functions impaired in the TAC-mice **(Ci)**. Subcutaneous administration of alginate-α-CGRP microcapsules in TAC-mice attenuated increased levels of TAC-induced LV oxidative stress, apoptosis, and fibrosis that, in turn, inhibited cardiac hypertrophy and improved cardiac functions in the TAC-mice **(Cii)**.

limits the use of peptide (as the case with other peptides) in long-term treatment regime. To increase the bioavailability of peptide, we utilized an FDA approved alginate biomaterial as a drug carrier and developed an α-CGRP delivery system (Kumar et al., 2020). Alginate is an immunologically inactive natural biopolymer isolated from the seaweeds and has been used as a delivery vehicle for a number of biological materials, including cells, DNA, proteins, and peptides (Zhang et al., 2011; Moore et al., 2014; Annamalai et al., 2018; Gheorghita Puscaselu et al., 2020). α-CGRP was encapsulated into alginate polymer using an electrospray method and alginate-α-CGRP microcapsules of 200 μm diameter were prepared by passing a mixture of alginate and α-CGRP through a positively charged 30-G blunt end syringe needle at a constant flow rate (60 mm/h) under high voltage current (6 KV) into the 150 mM $CaCl_2$ gelling solution. The distance between syringe needle and bathing soln was kept 7 mm (Kumar et al., 2020; **Figure 5**). α-CGRP-filled alginate microcapsules released peptide, most likely through diffusion, for an extended period (up to 6 days) where higher concentration was released in first few hours known as a "burst" and then constant but lower concentration for up to 6 days. Alginate-α-CGRP microcapsules were non-toxic to cardiac cells tested (rat H9C2 cardiac myoblast cell line and mouse HL-1 cardiac muscle cell line) in *in vitro* assays. The efficacy of these microcapsules was tested in the TAC-pressure overload mice. Alginate-α-CGRP microcapsules administered on day-2 or day-15 post-TAC attenuated cardiac hypertrophy and improved cardiac functions in the TAC-mice. Delivery of alginate-α-CGRP microcapsules lowered TAC-pressure induced LV apoptotic cell death, oxidative stress, and fibrosis in the TAC-alginate-CGRP group of mice. Alginate-α-CGRP microcapsules can be dehydrated and reconstituted in an buffered saline solution of distilled water. These capsules can be put in pluronic gels or collagen gels or injected as we have done either IP or sub cutaneious. Although α-CGRP release profile was performed in *in vitro* assays, the pharmacology and pharmacokinetics of released peptide in different organs and tissues under *in vivo* conditions are being investigated. The use of alginate is

further enhanced due to it being an immunologically inactive polymer hence does not exhibit adverse effects *in vivo*. The cardioprotective effects of CGRP have been demonstrated in TAC-mice after administering CGRP encapsulated microcapsules via subcutaneous route. However, the oral route is a preferred method for delivery of most therapeutic agents, therefore the cardioprotective effects of alginate-α-CGRP microcapsules delivered through the oral route is of keen interest the lab. Furthermore, numerous additional benefits for using alginate microcapsules are: these microcapsules can be freeze-thawed without losing structural integrity; they can be stored at very low temperature, and they can be lyophilized. These studies indicate that our alginate-based delivery system is a potential approach to increase the bioavailability of CGRP in circulation and this therapeutic modality can be translated to treat cardiac diseases during long term treatment.

Several efforts are also being made in search of more stable bioactive α-CGRP-agonists to address peptide's low bioavailability in human plasma (Nilsson et al., 2016). Aubdool et al., 2017, tested an acylated form of α-CGRP (half-life ∼7 h) in rodent models of Ang-II-induced hypertension and abdominal aortic constriction (AAC)-induced heart failure (Aubdool et al., 2017). Researchers demonstrated that subcutaneous administration of acylated-α-CGRP significantly reduced cardiac hypertrophy, fibrosis, and inflammation, and improved cardiac function in these disease rodent models. Recently, the cardioprotective effect of a metabolically stable CGRP analog, SAX, has been tested in a constant left anterior descending (LAD) occlusion rat model of acute myocardial infarction (Bentsen et al., 2021). Researchers reported that intraperitoneal administration of SAX (100 nmole/kg bwt per rat) improved myocardial recovery after myocardial infarction. These studies collectively suggest that utilization of α-CGRP (native or its analogs) and their delivery systems can be a potential strategy in preventing harmful cardiac remodeling and dysfunction present in cardiac diseases. As α-CGRP is a potent vasodilator and its higher level in plasma induces migraine, monitoring of blood pressure and migraine-like headache in humans is recommended while administering high doses of the peptide.

THERAPEUTIC VALUE OF ALPHA-CALCITONIN GENE RELATED PEPTIDE-ANTAGONISTS IN MIGRAINE PAIN

Migraine is a chronic neurological disorder. Patients suffering from migraines show an increased level of CGRP in their saliva and plasma suggesting that CGRP plays an important role in the pathophysiology of migraine headache (Gallai et al., 1995; Cady et al., 2009; Hansen et al., 2010; Al-Hassany and Van Den Brink, 2020; Wattiez et al., 2020). It has been shown that the trigeminovascular system (containing the trigeminal ganglion and trigeminal nerves) becomes activated during a migraine episode that leads to the release of CGRP in the external jugular vein resulting in a migraine-like headache (Goadsby et al., 1988, 1990; Edvinsson, 2017). Recently developed CGRP-antagonists, which consist of humanized monoclonal antibodies that either bind to CGRP or its receptor, have been used to relieve the pain in migraine patients (Urits et al., 2019). These well tolerated and FDA-approved antibodies are erenumab, fremanezumab, galcanezumab, and eptinezumab, and are discussed below (**Table 1**).

Erenumab (Aimovig)

Erenumab (commercial name Aimovig) is an FDA-approved fully monoclonal IgG2 antibody that selectively blocks the CGRP receptor, and subsequently downstream molecular signaling, to prevent the onset of migraines (Shi et al., 2016; Markham, 2018). It is administered once per month in patients to prevent the onset of migraines. Erenumab's ability to attenuate migraine symptoms is primarily attributable to its ability to block CGRP receptors on the trigeminal ganglion and its branches. It is also thought that erenumab's inhibitory actions on cerebral and meningeal blood vessel vasodilation plays a role in migraine treatment and prevention. Additionally, erenumab may block satellite cells from releasing inflammatory modulators and nitric oxide (NO) in response to α-CGRP exposure (Edvinsson et al., 2018).

A randomized, double-blind, and placebo-controlled phase II clinical trial showed that administration of 70 or 140 mg doses of erenumab to 667 chronic migraine patients reduced migraine days per month (Tepper et al., 2017). In a 6 month, randomized, double-blind, placebo-controlled phase III clinical trial (STRIVE trial), erenumab was injected subcutaneously monthly in doses of either 70 mg or 140 mg in 955 patients with episodic migraines to assess its safety and efficacy (Goadsby et al., 2017). Erenumab reduced the average number of migraine days per month by 43.3% (3.2 days) in patients given 70 mg doses and 50% (3.7 days) in patients injected with 140 mg doses, compared to a 26.6% reduction (1.8 days) in the placebo group.

An open-label, 5-year treatment phase followed 383 episodic migraine patients who had enrolled in a 12-week, double-blind, placebo-controlled clinical trial of erenumab (Ashina et al., 2021). These patients began the clinical trial receiving 70 mg doses of erenumab, but 250 patients were later switched to 140 mg doses. This open-label study tested the long-term safety and efficacy of erenumab by investigating changes in baseline monthly migraine days, monthly acute migraine-specific medication days, and health-related quality of life. By year 5 of erenumab use, these patients experienced an average reduction of 62.3% of monthly migraine days (−5.3 days per month), a −4.4 days reduction in acute migraine-specific days per month. This study also found that long-term erenumab use is not associated with increased frequency of adverse side effects. Therefore, the study concluded that erenumab is a safe and effective drug for migraine prevention.

Fremanezumab (Ajovy)

Fremanezumab (TEV-48125) is a fully monoclonal IgG2 antibody that blocks CGRP signaling by binding to α- and β-forms of CGRP to inhibit these ligands from interacting with the CGRP receptor (Hoy, 2018). Approved by the FDA and developed by Teva Pharmaceuticals, fremanezumab (commercial

TABLE 1 | FDA approved anti-CGRP and anti-CGRP receptor antibodies for the treatment of migraine.

CGRP-antagonists	Manufacturer	Mode of action	Dose regime	Response in migraine patients	Potential side effects
Erenumab (Aimovig)	Amgen/ Novartis	Blocks CGRP receptor	s.c., once monthly	Reduced monthly migraine days in patients with chronic and episodic migraines	Pain and redness at injection site, and constipation
Fremanezumab (Ajovy)	Teva Pharmaceuticals	Binds to α- and β-forms of CGRP	s.c., once monthly/quarterly	Reduced migraine days by an average of 5 days a month in patients with chronic migraine and reduced migraine days by an average of 3.5 days a month in patients with episodic migraine	Injection site reactions. May cause allergic reactions, such as itching, rash, and hives
Galcanezumab (Emgality)	Eli Lilly	Binds to α- and β-forms of CGRP	s.c., once monthly	Cut the number of monthly migraine days by 50% or more for some people	Injection site reactions. May cause allergic reactions, such as itching, rash, hives, and trouble breathing
Eptinezumab (Vyepti)	Lundbeck Seattle BioPharmaceuticals	Binds to α- and β-forms of CGRP	Intravenously (i.v.), once quarterly	Reduced monthly migraine days in patients with chronic and episodic migraines	Nasopharyngitis and hypersensitivity

name Ajovy) is administered monthly or quarterly via a subcutaneous injection to prevent migraine attacks (Friedman and Cohen, 2020). *In vitro* studies found that fremanezumab inhibits CGRP-induced vasodilation of intracranial and abdominal arteries. Fremanezumab is given in doses of 225, 675, or 900 mg, and its maximum concentration in plasma is apparent 5–7 days after administration, which indicates that it is absorbed slowly into the circulation. Fremanezumab has a plasma half-life of ∼31 days (Fiedler-Kelly et al., 2019).

A randomized, double-blind, placebo-controlled, parallel-group clinical trial tested the effectiveness of fremanezumab by administering 225 mg monthly or 675 mg quarterly to episodic migraine patients (Dodick et al., 2018). Patients in the 225 mg group experienced a reduction in mean migraine days per month from 8.9 to 4.9 days. The number of mean migraine days per month in subjects injected with 675 mg of fremanezumab quarterly dropped from 9.2 to 5.3 days, while those in the placebo group reported a reduction of 9.1 migraine days per month to 6.5 days.

Another randomized, double-blind, placebo-controlled, parallel-group study administered fremanezumab monthly or quarterly in chronic migraine patients (Silberstein et al., 2017). Patients who received fremanezumab monthly experienced a 4.6 days reduction of mean number of headache days per month, while those in the quarterly group reported a 4.3 days reduction. Both groups compared to the placebo were significant, as the reduction in number of headache days per month in the placebo group was 2.5 days. Additionally, 41% of patients in the monthly fremanezumab group and 38% in the quarterly group reported a reduction of ≥50% of headache days per month compared to 18% in the placebo group.

A 12-week, phase III clinical trial determined the efficacy of fremanezumab in patients with difficult-to-treat chronic or episodic migraines (Ferrari et al., 2019). Patients with difficult-to-treat migraines were defined by failure to respond to 2–4 of the following migraine preventative medications: beta-blockers, anticonvulsants, tricyclic antidepressants, calcium channel blockers, angiotensin II receptor antagonists, or onabotulinumtoxin A. In this randomized, double-blind, parallel-group study, patients received monthly or quarterly subcutaneous injections of either fremanezumab or a placebo. At the end of the 12 weeks, the study found that patients in both fremanezumab groups experienced a significant reduction in migraine days per month compared to the placebo. The monthly fremanezumab group reported an average of 4.1 fewer migraine days per month, while the quarterly fremanezumab patients experienced a 3.7-day reduction. Fremanezumab also reduced the frequency of monthly headache days of at least moderate severity in both groups (–4.2 days in monthly group; –3.9 days in quarterly group; –0.6 days in placebo group).

The HALO long-term study (LTS), a double-blind, randomized, parallel-group, 12-month, phase III clinical trial, investigated the long-term efficacy of fremanezumab in 1,890 patients with chronic or episodic migraines (Goadsby et al., 2020). At the end of the study, over 50% of patients with chronic migraine and ∼66% of patients with episodic migraine reported a ≥50% reduction in migraine days per month from their baseline levels. This investigation also found that as the study progressed, more patients reported reductions in monthly migraine days, indicating that fremanezumab became more effective with longer treatment duration.

Galcanezumab (Emgality)

Developed by Eli Lilly & Co., galcanezumab (commercial name Emgality) is an FDA-approved humanized monoclonal antibody used for migraine and cluster headache prevention (Lamb, 2018). Galcanezumab has an apparent half-life of ∼27 days in serum and binds to the CGRP ligand to prevent interaction with the CGRP receptor, thus abolishing the biological activity of α-CGRP (Dodick et al., 2014b). Healthy volunteers injected with a single dose of 75–600 mg of galcanezumab experienced significantly more inhibition of capsaicin-induced dermal blood flow compared to a placebo group at all post-dose time intervals (days 3, 14, 28, and 42). Galcanezumab is administered

monthly via subcutaneous injection, and it is slowly absorbed into circulation, as it reaches maximum plasma concentration ∼5 days post-injection.

In a clinical trial (EVOLVE), the safety and efficacy of galcanezumab was evaluated in episodic migraine patients treated with 120 mg or 240 mg galcanezumab or a placebo group (Stauffer et al., 2018). By the end of the 6-month period, galcanezumab reduced the number of monthly migraine days by 4.7 and 4.6 days at doses 120 and 240 mg, respectively. Similarly, 65% of patients taking galcanezumab reported a > 50% reduction of mean headache days per month compared to 42% in the placebo group.

A randomized, double-blind, placebo-controlled phase III clinical trial (REGAIN) investigated the efficacy of galcanezumab by injecting monthly doses of 120 mg (with an initial 240 mg loading dose) or 240 mg of galcanezumab or a placebo in chronic migraine patients (Detke et al., 2018). Patients in the 120 mg galcanezumab group experienced a mean reduction in number of headache days per month of –4.8 days. Similarly, the 240 mg galcanezumab group reported a –4.6 day reduction, while patients in the placebo group experienced a –2.7 day reduction in number of headache days per month.

In addition, an 8-week, randomized, double-blind, placebo-controlled phase III clinical trial injected 300 mg doses of galcanezumab once per month in 106 adults with episodic cluster headaches (Goadsby et al., 2019). During weeks 1–3, galcanezumab decreased the number of weekly cluster headaches by 8.7, as opposed to 5.2 in the placebo group. Additionally, by week 3, 71% of patients receiving galcanezumab reported a ≥50% decrease in the incidence of weekly cluster headaches, compared to 53% in the placebo group.

Eptinezumab (Vyepti)

Eptinezumab is a humanized monoclonal antibody that is used to prevent the onset of migraine attacks (Dodick et al., 2014a; Baker et al., 2020). Developed by Lundbeck Seattle BioPharmaceuticals under the commercial name Vyepti, eptinezumab binds to α- and β-forms of CGRP to prevent ligand-receptor interaction, and therefore blocks CGRP signaling. Eptinezumab has a half-life of ∼27 days, and it achieves steady state serum concentrations following its initial administration and subsequentially after each infusion once every 3 months.

PROMISE-1, a randomized, double-blind, multicenter, placebo-controlled phase III clinical trial assessed the efficacy and tolerability of eptinezumab by intravenously administering 30, 100, or 300 mg doses of eptinezumab or a placebo every 3 months for 12 months in 888 adults with episodic migraines (Ashina et al., 2020; Smith et al., 2020). Patients who received 100 mg doses of eptinezumab reported a 3.9 day reduction in monthly migraine days during weeks 1–12, compared to 3.2 days in the placebo group. Similarly, patients who were given 300 mg of eptinezumab experienced a 4.3 day decrease in monthly migraine days during weeks 1–12. Because the 30 mg dose of eptinezumab failed to be statistically significant on the primary efficacy end point, this dosing regimen was not approved by the FDA. Throughout weeks 1–4, the 100 mg and 300 mg eptinezumab groups had significantly higher rates of ≥75% reductions in monthly migraine days from baseline compared to the placebo (31% in 100 mg group, 32% in 300 mg group, and 20% in placebo). During weeks 1–12, significantly more patients who received 300 mg doses of eptinezumab reported ≥50% decrease in monthly migraine days vs. placebo (56 vs. 37%). Additionally, 30% of 300 mg eptinezumab patients reported a ≥75% reduction in monthly migraine days during weeks 1–12, compared to 16% in the placebo group. All eptinezumab dosing regimens decreased the frequency of monthly migraine days compared to the placebo through week 48, and the beneficial effects of eptinezumab persisted throughout the entire study. The number of patients who experienced a ≥50% or a ≥75% reduction in monthly migraine days were significantly higher in those who received 100 or 300 mg doses of eptinezumab compared to the placebo from weeks 1 to 48. By week 48, the average reduction in monthly migraine days was –4.5 and –5.3 days in the 100 mg and 300 mg eptinezumab groups, compared to –4.0 days in the placebo group. Of all the patients treated with eptinezumab, ∼70% experienced a ≥50% migraine response during at least 6 months of the study and >20% reported ≥50% migraine response during all 12 months of the study.

Moreover, a large randomized, placebo-controlled, double-blind, multicenter, phase III clinical trial (PROMISE-2) investigated the safety and efficacy of eptinezumab in 1,072 patients with chronic migraines (Lipton et al., 2020). Patients were randomized to an eptinezumab or a placebo group and received either 100 or 300 mg intravenous doses every 3 months for 6 months. The effects of eptinezumab were apparent after the first day of dosing, as 28.6% of patients who received 100 mg of eptinezumab and 27.8% of patients administered 300 mg of eptinezumab reported having a migraine after the first day of dosing compared to 42.3% of patients in the placebo group. Eptinezumab significantly reduced the frequency of monthly migraine days during weeks 1–12 in both the 100 mg (–7.7 days) and 300 mg (–8.2 days) groups compared to the placebo (–5.6 days). During weeks 1–12, 26.7% of patients who received 100 mg doses of eptinezumab and 33.1% of the 300 mg eptinezumab group reported ≥75% reduction rates in monthly migraine days, compared to 15% in the placebo group. The most common side effect of eptinezumab use was nasopharyngitis, which occurred in 9.4% of patients who received 300 mg of eptinezumab, compared to 6.0% of patients in the placebo group. Less than 1% of patients (7 from eptinezumab group and 3 from placebo group) experienced a serious treatment emergent adverse effect (TEAE), including nervous system disorders, injury, poisoning, and procedural complications. Based on these results, eptinezumab was determined to be a well-tolerated and effective medication for migraine prevention.

CONCLUSION

Numerous biological, pharmacological, and genetic studies carried out in a variety of animal models have established the role of α-CGRP in normal and disease states, particularly in cardiovascular disease and migraine pain. In recent years

the development of CGRP-antagonists and their successful use in humans to treat/prevent migraine-like headache is remarkable. Growing evidence suggest that, in addition to classical CGRP-receptor, CGRP can interact with other CGRP-responsive receptors (such as AMY1 receptor) present on the cell surface, hence studying the pharmacological effects of developing CGRP-agonists/-antagonists on these receptor subtypes might be valuable for the successful development of CGRP-based therapy for diseases. Moreover, the ongoing efforts to enhance the bioactivity of CGRP in circulation via CGRP-analogs and delivery systems are promising therapeutic agents to treat patients suffering from cardiovascular diseases. The alginate-based delivery system for α-CGRP is showing promising results to increase the bioavailability of the peptide and protect against pressure-induced heart failure. Further testing of this delivery system is needed in other rodent models of cardiovascular diseases, including hypertension, myocardial infarction, ischemia-reperfusion injury, and also other modes of alginate-CGRP microcapsules delivery is needed to verify in these cardiac diseases. These endeavors are a hopeful avenue for CGRP-based cardiovascular therapeutics in the coming years. Another aspect that is needed to consider in CGRP biology is the accurate measurement of peptide in the biological samples. α-CGRP is a very short-lived peptide making it difficult to measure accurately in samples. Although radio-immunoassay and enzyme-immunoassay are employed, more sensitive detection methods such as high-performance liquid chromatography (HPLC) and mass spectroscopy should be considered to include in the CGRP measurement assays. It is also worthy to mention here that as α-CGRP has an important role in regulating regional organ blood flow in normal and pathophysiological conditions, it might be possible that migraine patients treated with CGRP-antagonists, that block CGRP-signaling, develop adverse side effects on heart and blood vessels. Hence long-term follow-up of these patients in terms of cardiovascular safety is advisable.

AUTHOR CONTRIBUTIONS

JP was responsible for the design and analysis of the data and construction and editing of the manuscript. DD was responsible for the design, analysis and construction, and editing of the manuscript. AK responsible for the generation of TAC animals and data presented in the manuscript, as well as contributed to the writing of the manuscript. MW was responsible for the generation of data presented in the manuscript, in addition to editing the manuscript. AH was responsible for the generation of data presented in the manuscript, in addition to editing the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aiyar, N., Rand, K., Elshourbagy, N. A., Zeng, Z., Adamou, J. E., Bergsma, D. J., et al. (1996). A cDNA encoding the calcitonin gene-related peptide type 1 receptor. *J. Biol. Chem.* 271, 11325–11329. doi: 10.1074/jbc.271.19.11325

Al-Hassany, L., and Van Den Brink, A. M. (2020). Targeting CGRP in migraine: a matter of choice and dose. *Lancet Neurol.* 19, 712–713. doi: 10.1016/S1474-4422(20)30282-9

Al-Rubaiee, M., Gangula, P. R., Millis, R. M., Walker, R. K., Umoh, N. A., Cousins, V. M., et al. (2013). Inotropic and lusitropic effects of calcitonin gene-related peptide in the heart. *Am. J. Physiol. Heart Circ. Physiol.* 304, H1525–H1537. doi: 10.1152/ajpheart.00874.2012

Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 298, 240–244. doi: 10.1038/298240a0

Andreeva, L., and Rang, H. P. (1993). Effect of bradykinin and prostaglandins on the release of calcitonin gene-related peptide-like immunoreactivity from the rat spinal cord in vitro. *Br. J. Pharmacol.* 108, 185–190. doi: 10.1111/j.1476-5381.1993.tb13460.x

Annamalai, B., Parsons, N., Belhaj, M., Brandon, C., Potts, J., and Rohrer, B. (2018). Encapsulated cell technology-based delivery of a complement inhibitor reduces choroidal neovascularization in a mouse model. *Transl. Vis. Sci. Technol.* 7:3. doi: 10.1167/tvst.7.2.3

Arulmani, U., Schuijt, M. P., Heiligers, J. P., Willems, E. W., Villalon, C. M., and Saxena, P. R. (2004). Effects of the calcitonin gene-related peptide (CGRP) receptor antagonist BIBN4096BS on alpha-CGRP-induced regional haemodynamic changes in anaesthetised rats. *Basic Clin. Pharmacol. Toxicol.* 94, 291–297. doi: 10.1111/j.1742-7843.2004.pto940606.x

Ashina, M., Goadsby, P. J., Reuter, U., Silberstein, S., Dodick, D. W., Xue, F., et al. (2021). Long-term efficacy and safety of erenumab in migraine prevention: results from a 5-year, open-label treatment phase of a randomized clinical trial. *Eur. J. Neurol.* 28, 1716–1725. doi: 10.1111/ene.14715

Ashina, M., Saper, J., Cady, R., Schaeffler, B. A., Biondi, D. M., Hirman, J., et al. (2020). Eptinezumab in episodic migraine: a randomized, double-blind, placebo-controlled study (PROMISE-1). *Cephalalgia* 40, 241–254. doi: 10.1177/0333102420905132

Aubdool, A. A., Thakore, P., Argunhan, F., Smillie, S. J., Schnelle, M., Srivastava, S., et al. (2017). A novel alpha-calcitonin gene-related peptide analogue protects against end-organ damage in experimental hypertension, cardiac hypertrophy, and heart failure. *Circulation* 136, 367–383. doi: 10.1161/circulationaha.117.028388

Baker, B., Schaeffler, B., Beliveau, M., Rubets, I., Pederson, S., Trinh, M., et al. (2020). Population pharmacokinetic and exposure-response analysis of eptinezumab in the treatment of episodic and chronic migraine. *Pharmacol. Res. Perspect.* 8:e00567. doi: 10.1002/prp2.567

Balfagon, G., Marquez-Rodas, I., Alvarez, Y., Alonso, M. J., Cachofeiro, V., Salaices, M., et al. (2004). Aldosterone modulates neural vasomotor response in hypertension: role of calcitonin gene-related peptide. *Regul. Pept.* 120, 253–260. doi: 10.1016/j.regpep.2004.03.016

Banerjee, S., Evanson, J., Harris, E., Lowe, S. L., Thomasson, K. A., and Porter, J. E. (2006). Identification of specific calcitonin-like receptor residues important for calcitonin gene-related peptide high affinity binding. *BMC Pharmacol.* 6:9. doi: 10.1186/1471-2210-6-9

Bell, D., Schluter, K. D., Zhou, X. J., Mcdermott, B. J., and Piper, H. M. (1995). Hypertrophic effects of calcitonin gene-related peptide (CGRP) and amylin on adult mammalian ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* 27, 2433–2443. doi: 10.1006/jmcc.1995.0231

Bentsen, S., Sams, A., Hasbak, P., Edvinsson, L., Kjaer, A., and Ripa, R. S. (2021). Myocardial perfusion recovery induced by an alpha-calcitonin gene-related peptide analogue. *J. Nucl. Cardiol.* doi: 10.1007/s12350-021-02678-8

Bivalacqua, T. J., Hyman, A. L., Kadowitz, P. J., Paolocci, N., Kass, D. A., and Champion, H. C. (2002). Role of calcitonin gene-related peptide (CGRP) in chronic hypoxia-induced pulmonary hypertension in the mouse. Influence of gene transfer in vivo. *Regul. Pept.* 108, 129–133. doi: 10.1016/s0167-0115(02)00100-3

Bracci-Laudiero, L., Aloe, L., Buanne, P., Finn, A., Stenfors, C., Vigneti, E., et al. (2002). NGF modulates CGRP synthesis in human B-lymphocytes: a possible anti-inflammatory action of NGF? *J. Neuroimmunol.* 123, 58–65. doi: 10.1016/s0165-5728(01)00475-1

Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R., and Macintyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313, 54–56. doi: 10.1038/313054a0

Breeze, A. L., Harvey, T. S., Bazzo, R., and Campbell, I. D. (1991). Solution structure of human calcitonin gene-related peptide by 1H NMR and distance geometry with restrained molecular dynamics. *Biochemistry* 30, 575–582. doi: 10.1021/bi00216a036

Burcher, E., Zeng, X. P., Strigas, J., Shang, F., Millard, R. J., and Moore, K. H. (2000). Autoradiographic localization of tachykinin and calcitonin gene-related peptide receptors in adult urinary bladder. *J. Urol.* 163, 331–337. doi: 10.1016/s0022-5347(05)68047-0

Cady, R. K., Vause, C. V., Ho, T. W., Bigal, M. E., and Durham, P. L. (2009). Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. *Headache* 49, 1258–1266. doi: 10.1111/j.1526-4610.2009.01523.x

Carpenter, K. A., Schmidt, R., von Mentzer, B., Haglund, U., Roberts, E., and Walpole, C. (2001). Turn structures in CGRP C-terminal analogues promote stable arrangements of key residue side chains. *Biochemistry* 40, 8317–8325. doi: 10.1021/bi0102860

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., et al. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am. J. Physiol.* 256, E331–E335. doi: 10.1152/ajpendo.1989.256.2.E331

Csillik, B., Tajti, L., Kovacs, T., Kukla, E., Rakic, P., and Knyihar-Csillik, E. (1993). Distribution of calcitonin gene-related peptide in vertebrate neuromuscular junctions: relationship to the acetylcholine receptor. *J. Histochem. Cytochem.* 41, 1547–1555. doi: 10.1177/41.10.8245413

Cueille, C., Pidoux, E., de Vernejoul, M. C., Ventura-Clapier, R., and Garel, J. M. (2002). Increased myocardial expression of RAMP1 and RAMP3 in rats with chronic heart failure. *Biochem. Biophys. Res. Commun.* 294, 340–346. doi: 10.1016/S0006-291X(02)00487-4

Detke, H. C., Goadsby, P. J., Wang, S., Friedman, D. I., Selzler, K. J., and Aurora, S. K. (2018). Galcanezumab in chronic migraine: the randomized, double-blind, placebo-controlled REGAIN study. *Neurology* 91, e2211–e2221. doi: 10.1212/WNL.0000000000006640

DiPette, D. J., Schwarzenberger, K., Kerr, N., and Holland, O. B. (1989). Dose-dependent systemic and regional hemodynamic effects of calcitonin gene-related peptide. *Am. J. Med. Sci.* 297, 65–70. doi: 10.1097/00000441-198902000-00001

Dodick, D. W., Goadsby, P. J., Spierings, E. L., Scherer, J. C., Sweeney, S. P., and Grayzel, D. S. (2014b). Safety and efficacy of LY2951742, a monoclonal antibody to calcitonin gene-related peptide, for the prevention of migraine: a phase 2, randomised, double-blind, placebo-controlled study. *Lancet Neurol.* 13, 885–892. doi: 10.1016/S1474-4422(14)70128-0

Dodick, D. W., Goadsby, P. J., Silberstein, S. D., Lipton, R. B., Olesen, J., Ashina, M., et al. (2014a). Safety and efficacy of ALD403, an antibody to calcitonin gene-related peptide, for the prevention of frequent episodic migraine: a randomised, double-blind, placebo-controlled, exploratory phase 2 trial. *Lancet Neurol.* 13, 1100–1107. doi: 10.1016/S1474-4422(14)70209-1

Dodick, D. W., Silberstein, S. D., Bigal, M. E., Yeung, P. P., Goadsby, P. J., Blankenbiller, T., et al. (2018). Effect of fremanezumab compared with placebo for prevention of episodic migraine: a randomized clinical trial. *JAMA* 319, 1999–2008. doi: 10.1001/jama.2018.4853

Doi, Y., Kudo, H., Nishino, T., Kayashima, K., Kiyonaga, H., Nagata, T., et al. (2001). Synthesis of calcitonin gene-related peptide (CGRP) by rat arterial endothelial cells. *Histol. Histopathol.* 16, 1073–1079. doi: 10.14670/HH-16.1073

Donnerer, J., Schuligoi, R., and Stein, C. (1992). Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. *Neuroscience* 49, 693–698. doi: 10.1016/0306-4522(92)90237-v

Donnerer, J., and Stein, C. (1992). Evidence for an increase in the release of CGRP from sensory nerves during inflammation. *Ann. N. Y. Acad. Sci.* 657, 505–506. doi: 10.1111/j.1749-6632.1992.tb22814.x

Doods, H., Hallermayer, G., Wu, D., Entzeroth, M., Rudolf, K., Engel, W., et al. (2000). Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. *Br. J. Pharmacol.* 129, 420–423. doi: 10.1038/sj.bjp.0703110

Dubois-Rande, J. L., Merlet, P., Benvenuti, C., Sediame, S., Macquin-Mavier, I., Chabrier, E., et al. (1992). Effects of calcitonin gene-related peptide on cardiac contractility, coronary hemodynamics and myocardial energetics in idiopathic dilated cardiomyopathy. *Am. J. Cardiol.* 70, 906–912. doi: 10.1016/0002-9149(92)90736-i

Dux, M., Rosta, J., and Messlinger, K. (2020). TRP channels in the focus of trigeminal nociceptor sensitization contributing to primary headaches. *Int. J. Mol. Sci.* 21:342. doi: 10.3390/ijms21010342

Edvinsson, L. (2017). The trigeminovascular pathway: role of CGRP and CGRP receptors in migraine. *Headache* 57(Suppl. 2), 47–55. doi: 10.1111/head.13081

Edvinsson, L., Ahnstedt, H., Larsen, R., and Sheykhzade, M. (2014). Differential localization and characterization of functional calcitonin gene-related peptide receptors in human subcutaneous arteries. *Acta. Physiol.* 210, 811–822. doi: 10.1111/apha.12213

Edvinsson, L., Eftekhari, S., Salvatore, C. A., and Warfvinge, K. (2011). Cerebellar distribution of calcitonin gene-related peptide (CGRP) and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in rat. *Mol. Cell. Neurosci.* 46, 333–339. doi: 10.1016/j.mcn.2010.10.005

Edvinsson, L., Fredholm, B. B., Hamel, E., Jansen, I., and Verrecchia, C. (1985). Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci. Lett.* 58, 213–217. doi: 10.1016/0304-3940(85)90166-1

Edvinsson, L., Haanes, K. A., Warfvinge, K., and Krause, D. N. (2018). CGRP as the target of new migraine therapies – successful translation from bench to clinic. *Nat. Rev. Neurol.* 14, 338–350. doi: 10.1038/s41582-018-0003-1

Eftekhari, S., and Edvinsson, L. (2011). Calcitonin gene-related peptide (CGRP) and its receptor components in human and rat spinal trigeminal nucleus and spinal cord at C1-level. *BMC Neurosci.* 12:112. doi: 10.1186/1471-2202-12-112

Eftekhari, S., Salvatore, C. A., Calamari, A., Kane, S. A., Tajti, J., and Edvinsson, L. (2010). Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. *Neuroscience* 169, 683–696. doi: 10.1016/j.neuroscience.2010.05.016

Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *J. Pain* 14, 1289–1303.

Egea, S. C., and Dickerson, I. M. (2012). Direct interactions between calcitonin-like receptor (CLR) and CGRP-receptor component protein (RCP) regulate CGRP receptor signaling. *Endocrinology* 153, 1850–1860. doi: 10.1210/en.2011-1459

Evans, B. N., Rosenblatt, M. I., Mnayer, L. O., Oliver, K. R., and Dickerson, I. M. (2000). CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J. Biol. Chem.* 275, 31438–31443. doi: 10.1074/jbc.M005604200

Ferrari, M. D., Diener, H. C., Ning, X., Galic, M., Cohen, J. M., Yang, R., et al. (2019). Fremanezumab versus placebo for migraine prevention in patients with documented failure to up to four migraine preventive medication classes (FOCUS): a randomised, double-blind, placebo-controlled, phase 3b trial. *Lancet* 394, 1030–1040. doi: 10.1016/S0140-6736(19)31946-4

Fiedler-Kelly, J. B., Cohen-Barak, O., Morris, D. N., Ludwig, E., Rasamoelisolo, M., Shen, H., et al. (2019). Population pharmacokinetic modelling and simulation of fremanezumab in healthy subjects and patients with migraine. *Br. J. Clin. Pharmacol.* 85, 2721–2733. doi: 10.1111/bcp.14096

Fisher, L. A., Kikkawa, D. O., Rivier, J. E., Amara, S. G., Evans, R. M., Rosenfeld, M. G., et al. (1983). Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. *Nature* 305, 534–536. doi: 10.1038/305534a0

Franco-Cereceda, A. (1988). Calcitonin gene-related peptide and tachykinins in relation to local sensory control of cardiac contractility and coronary vascular tone. *Acta Physiol. Scand. Suppl.* 569, 1–63. doi: 10.1016/0301-0082(94)e0017-b

Franco-Cereceda, A., and Lundberg, J. M. (1985). Calcitonin gene-related peptide (CGRP) and capsaicin-induced stimulation of heart contractile rate and force. *Naunyn Schmiedebergs Arch. Pharmacol.* 331, 146–151. doi: 10.1007/BF00634231

Freeland, K., Liu, Y. Z., and Latchman, D. S. (2000). Distinct signalling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor. *Biochem. J.* 345(Pt 2), 233–238.

Friedman, D. I., and Cohen, J. M. (2020). Fremanezumab: a disease-specific option for the preventive treatment of migraine, including difficult-to-treat migraine. *Emerg. Top. Life Sci.* 4, 179–190. doi: 10.1042/ETLS20200018

Fujioka, S., Sasakawa, O., Kishimoto, H., Tsumura, K., and Morii, H. (1991). The antihypertensive effect of calcitonin gene-related peptide in rats with norepinephrine- and angiotensin II-induced hypertension. *J. Hypertens.* 9, 175–179. doi: 10.1097/00004872-199102000-00013

Gallai, V., Sarchielli, P., Floridi, A., Franceschini, M., Codini, M., Glioti, G., et al. (1995). Vasoactive peptide levels in the plasma of young migraine patients with and without aura assessed both interictally and ictally. *Cephalalgia* 15, 384–390. doi: 10.1046/j.1468-2982.1995.1505384.x

Gangula, P. R., Supowit, S. C., Wimalawansa, S. J., Zhao, H., Hallman, D. M., Dipette, D. J., et al. (1997). Calcitonin gene-related peptide is a depressor in NG-nitro-L-arginine methyl ester-induced hypertension during pregnancy. *Hypertension* 29, 248–253. doi: 10.1161/01.hyp.29.1.248

Gangula, P. R., Zhao, H., Supowit, S. C., Wimalawansa, S. J., Dipette, D. J., Westlund, K. N., et al. (2000). Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. *Hypertension* 35, 470–475. doi: 10.1161/01.hyp.35.1.470

Gebhardt, L. A., Kichko, T. I., Fischer, M. J. M., and Reeh, P. W. (2020). TRPA1-dependent calcium transients and CGRP release in DRG neurons require extracellular calcium. *J. Cell Biol.* 219:e201702151.

Gennari, C., Nami, R., Agnusdei, D., and Fischer, J. A. (1990). Improved cardiac performance with human calcitonin gene related peptide in patients with congestive heart failure. *Cardiovasc. Res.* 24, 239–241. doi: 10.1093/cvr/24.3.239

Gheorghita Puscaselu, R., Lobiuc, A., Dimian, M., and Covasa, M. (2020). Alginate: from food industry to biomedical applications and management of metabolic disorders. *Polymers* 12:2417. doi: 10.3390/polym12102417

Gibbins, I. L., Furness, J. B., Costa, M., Macintyre, I., Hillyard, C. J., and Girgis, S. (1985). Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci. Lett.* 57, 125–130. doi: 10.1016/0304-3940(85)90050-3

Gibson, S. J., Polak, J. M., Bloom, S. R., Sabate, I. M., Mulderry, P. M., Ghatei, M. A., et al. (1984). Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J. Neurosci.* 4, 3101–3111. doi: 10.1523/JNEUROSCI.04-12-03101.1984

Goadsby, P. J., Dodick, D. W., Leone, M., Bardos, J. N., Oakes, T. M., Millen, B. A., et al. (2019). Trial of Galcanezumab in prevention of episodic cluster headache. *N. Engl. J. Med.* 381, 132–141. doi: 10.1056/NEJMoa1813440

Goadsby, P. J., Edvinsson, L., and Ekman, R. (1988). Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. *Ann. Neurol.* 23, 193–196. doi: 10.1002/ana.410230214

Goadsby, P. J., Edvinsson, L., and Ekman, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann. Neurol.* 28, 183–187. doi: 10.1002/ana.410280213

Goadsby, P. J., Reuter, U., Hallstrom, Y., Broessner, G., Bonner, J. H., Zhang, F., et al. (2017). A controlled trial of erenumab for episodic migraine. *N. Engl. J. Med.* 377, 2123–2132. doi: 10.1056/nejmoa1705848

Goadsby, P. J., Silberstein, S. D., Yeung, P. P., Cohen, J. M., Ning, X., Yang, R., et al. (2020). Long-term safety, tolerability, and efficacy of fremanezumab in migraine: a randomized study. *Neurology* 95, e2487–e2499. doi: 10.1212/WNL.0000000000010600

Gray, D. W., and Marshall, I. (1992b). Nitric oxide synthesis inhibitors attenuate calcitonin gene-related peptide endothelium-dependent vasorelaxation in rat aorta. *Eur. J. Pharmacol.* 212, 37–42. doi: 10.1016/0014-2999(92)90069-g

Gray, D. W., and Marshall, I. (1992a). Human alpha-calcitonin gene-related peptide stimulates adenylate cyclase and guanylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide. *Br. J. Pharmacol.* 107, 691–696. doi: 10.1111/j.1476-5381.1992.tb14508.x

Gulbenkian, S., Saetrum Opgaard, O., Ekman, R., Costa Andrade, N., Wharton, J., Polak, J. M., et al. (1993). Peptidergic innervation of human epicardial coronary arteries. *Circ. Res.* 73, 579–588. doi: 10.1161/01.res.73.3.579

Hansen, J. M., Hauge, A. W., Olesen, J., and Ashina, M. (2010). Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. *Cephalalgia* 30, 1179–1186. doi: 10.1177/0333102410368444

Hay, D. L., and Walker, C. S. (2017). CGRP and its receptors. *Headache* 57, 625–636. doi: 10.1111/head.13064

Hay, D. L., Christopoulos, G., Christopoulos, A., and Sexton, P. M. (2006). Determinants of 1-piperidinecarboxamide, N-[2-[[5-amino-l-[[4-(4-pyridinyl)-l-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl) (BIBN4096BS) affinity for calcitonin gene-related peptide and amylin receptors–the role of receptor activity modifying protein 1. *Mol. Pharmacol.* 70, 1984–1991. doi: 10.1124/mol.106.027953

Hay, D. L., Howitt, S. G., Conner, A. C., Schindler, M., Smith, D. M., and Poyner, D. R. (2003). CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct adrenomedullin receptors: a comparison of effects of adrenomedullin22-52, CGRP8-37 and BIBN4096BS. *Br. J. Pharmacol.* 140, 477–486. doi: 10.1038/sj.bjp.0705472

Hay, D. L., and Pioszak, A. A. (2016). Receptor activity-modifying proteins (RAMPs): new insights and roles. *Annu. Rev. Pharmacol. Toxicol.* 56, 469–487. doi: 10.1146/annurev-pharmtox-010715-103120

Hilairet, S., Foord, S. M., Marshall, F. H., and Bouvier, M. (2001). Protein-protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. *J. Biol. Chem.* 276, 29575–29581. doi: 10.1074/jbc.M102722200

Howitt, S. G., Kilk, K., Wang, Y., Smith, D. M., Langel, U., and Poyner, D. R. (2003). The role of the 8-18 helix of CGRP8-37 in mediating high affinity binding to CGRP receptors; coulombic and steric interactions. *Br. J. Pharmacol.* 138, 325–332. doi: 10.1038/sj.bjp.0705040

Hoy, S. M. (2018). Fremanezumab: first global approval. *Drugs* 78, 1829–1834.

Huang, R., Karve, A., Shah, I., Bowers, M. C., Dipette, D. J., Supowit, S. C., et al. (2008). Deletion of the mouse alpha-calcitonin gene-related peptide gene increases the vulnerability of the heart to ischemia-reperfusion injury. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1291–H1297. doi: 10.1152/ajpheart.00749.2007

Inagaki, S., Kito, S., Kubota, Y., Girgis, S., Hillyard, C. J., and Macintyre, I. (1986). Autoradiographic localization of calcitonin gene-related peptide binding sites in human and rat brains. *Brain Res.* 374, 287–298. doi: 10.1016/0006-8993(86)90423-3

Kawasaki, H., Nuki, C., Saito, A., and Takasaki, K. (1990). Role of calcitonin gene-related peptide-containing nerves in the vascular adrenergic neurotransmission. *J. Pharmacol. Exp. Ther.* 252, 403–409.

Kee, Z., Kodji, X., and Brain, S. D. (2018). The role of calcitonin gene related peptide (CGRP) in neurogenic vasodilation and its cardioprotective effects. *Front. Physiol.* 9:1249. doi: 10.3389/fphys.2018.01249

Kichko, T. I., and Reeh, P. W. (2009). TRPV1 controls acid- and heat-induced calcitonin gene-related peptide release and sensitization by bradykinin in the isolated mouse trachea. *Eur. J. Neurosci.* 29, 1896–1904. doi: 10.1111/j.1460-9568.2009.06747.x

Kumar, A., Belhaj, M., Dipette, D. J., and Potts, J. D. (2020). A novel alginate-based delivery system for the prevention and treatment of pressure-overload induced heart failure. *Front. Pharmacol.* 11:602952. doi: 10.3389/fphar.2020.602952

Kumar, A., Potts, J. D., and Dipette, D. J. (2019a). Protective role of alpha-calcitonin gene-related peptide in cardiovascular diseases. *Front. Physiol.* 10:821. doi: 10.3389/fphys.2019.00821

Kumar, A., Supowit, S., Potts, J. D., and Dipette, D. J. (2019b). Alpha-calcitonin gene-related peptide prevents pressure-overload induced heart failure: role of apoptosis and oxidative stress. *Physiol. Rep.* 7:e14269. doi: 10.14814/phy2.14269

Lamb, Y. N. (2018). Galcanezumab: first global approval. *Drugs* 78, 1769–1775. doi: 10.1007/s40265-018-1002-7

Lechleitner, P., Genser, N., Mair, J., Dienstl, A., Haring, C., Wiedermann, C. J., et al. (1992). Calcitonin gene-related peptide in patients with and without early reperfusion after acute myocardial infarction. *Am. Heart J.* 124, 1433–1439. doi: 10.1016/0002-8703(92)90054-y

Lennerz, J. K., Ruhle, V., Ceppa, E. P., Neuhuber, W. L., Bunnett, N. W., Grady, E. F., et al. (2008). Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. *J. Comp. Neurol.* 507, 1277–1299. doi: 10.1002/cne.21607

Li, J., Levick, S. P., Dipette, D. J., Janicki, J. S., and Supowit, S. C. (2013). Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure. *Regul. Pept.* 185, 20–28. doi: 10.1016/j.regpep.2013.06.008

Li, J., and Wang, D. H. (2005). Development of angiotensin II-induced hypertension: role of CGRP and its receptor. *J. Hypertens.* 23, 113–118. doi: 10.1097/00004872-200501000-00020

Li, J., Zhao, H., Supowit, S. C., Dipette, D. J., and Wang, D. H. (2004). Activation of the renin-angiotensin system in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. *J. Hypertens.* 22, 1345–1349. doi: 10.1097/01.hjh.0000125409.50839.f1

Li, J. Z., Peng, J., Xiao, L., Zhang, Y. S., Liao, M. C., Li, X. H., et al. (2010). Reversal of isoprenaline-induced cardiac remodeling by rutaecarpine via stimulation of calcitonin gene-related peptide production. *Can. J. Physiol. Pharmacol.* 88, 949–959. doi: 10.1139/y10-067

Lindsay, R. M., Lockett, C., Sternberg, J., and Winter, J. (1989). Neuropeptide expression in cultures of adult sensory neurons: modulation of substance P and calcitonin gene-related peptide levels by nerve growth factor. *Neuroscience* 33, 53–65. doi: 10.1016/0306-4522(89)90310-2

Linscheid, P., Seboek, D., Schaer, D. J., Zulewski, H., Keller, U., and Muller, B. (2004). Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes. *Crit. Care Med.* 32, 1715–1721. doi: 10.1097/01.ccm.0000134404.63292.71

Lipton, R. B., Goadsby, P. J., Smith, J., Schaeffler, B. A., Biondi, D. M., Hirman, J., et al. (2020). Efficacy and safety of eptinezumab in patients with chronic migraine: PROMISE-2. *Neurology* 94, e1365–e1377.

Liu, Z., Liu, Q., Cai, H., Xu, C., Liu, G., and Li, Z. (2011). Calcitonin gene-related peptide prevents blood-brain barrier injury and brain edema induced by focal cerebral ischemia reperfusion. *Regul. Pept.* 171, 19–25. doi: 10.1016/j.regpep.2011.05.014

Lu, C. X., Qiu, T., Liu, Z. F., Su, L., and Cheng, B. (2017). Calcitonin gene-related peptide has protective effect on brain injury induced by heat stroke in rats. *Exp. Ther. Med.* 14, 4935–4941. doi: 10.3892/etm.2017.5126

Lu, J. T., Son, Y. J., Lee, J., Jetton, T. L., Shiota, M., Moscoso, L., et al. (1999). Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. *Mol. Cell. Neurosci.* 14, 99–120. doi: 10.1006/mcne.1999.0767

Lundberg, J. M., Hua, Y., and Fredholm, B. B. (1984). Capsaicin-induced stimulation of the guinea-pig atrium. Involvement of a novel sensory transmitter or a direct action on myocytes? *Naunyn Schmiedebergs Arch. Pharmacol.* 325, 176–182. doi: 10.1007/BF00506198

Luo, C. C., Huang, C. S., Ming, Y. C., Chu, S. M., and Chao, H. C. (2016). Calcitonin gene-related peptide downregulates expression of inducible nitride oxide synthase and caspase-3 after intestinal ischemia-reperfusion injury in rats. *Pediatr. Neonatol.* 57, 474–479. doi: 10.1016/j.pedneo.2015.10.012

Maggi, C. A., Rovero, P., Giuliani, S., Evangelista, S., Regoli, D., and Meli, A. (1990). Biological activity of N-terminal fragments of calcitonin gene-related peptide. *Eur. J. Pharmacol.* 179, 217–219.

Mair, J., Lechleitner, P., Langle, T., Wiedermann, C., Dienstl, F., and Saria, A. (1990). Plasma CGRP in acute myocardial infarction. *Lancet* 335:168. doi: 10.1016/0140-6736(90)90040-c

Mak, J. C., and Barnes, P. J. (1988). Autoradiographic localization of calcitonin gene-related peptide (CGRP) binding sites in human and guinea pig lung. *Peptides* 9, 957–963. doi: 10.1016/0196-9781(88)90073-3

Mallee, J. J., Salvatore, C. A., Lebourdelles, B., Oliver, K. R., Longmore, J., Koblan, K. S., et al. (2002). Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. *J. Biol. Chem.* 277, 14294–14298. doi: 10.1074/jbc.M109661200

Markham, A. (2018). Erenumab: first global approval. *Drugs* 78, 1157–1161. doi: 10.1007/s40265-018-0944-0

Marshall, N. J., Liang, L., Bodkin, J., Dessapt-Baradez, C., Nandi, M., Collot-Teixeira, S., et al. (2013). A role for TRPV1 in influencing the onset of cardiovascular disease in obesity. *Hypertension* 61, 246–252. doi: 10.1161/HYPERTENSIONAHA.112.201434

Masuda, A., Shimamoto, K., Mori, Y., Nakagawa, M., Ura, N., and Iimura, O. (1992). Plasma calcitonin gene-related peptide levels in patients with various hypertensive diseases. *J. Hypertens.* 10, 1499–1504. doi: 10.1097/00004872-199210120-00010

Matteoli, M., Haimann, C., Torri-Tarelli, F., Polak, J. M., Ceccarelli, B., and De Camilli, P. (1988). Differential effect of alpha-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7366–7370. doi: 10.1073/pnas.85.19.7366

McCormack, D. G., Mak, J. C., Coupe, M. O., and Barnes, P. J. (1989). Calcitonin gene-related peptide vasodilation of human pulmonary vessels. *J. Appl. Physiol.* 67, 1265–1270. doi: 10.1152/jappl.1989.67.3.1265

McCulloch, J., Uddman, R., Kingman, T. A., and Edvinsson, L. (1986). Calcitonin gene-related peptide: functional role in cerebrovascular regulation. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5731–5735. doi: 10.1073/pnas.83.15.5731

McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., et al. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393, 333–339. doi: 10.1038/30666

Meens, M. J., Mattheij, N. J., Nelissen, J., Lemkens, P., Compeer, M. G., Janssen, B. J., et al. (2011). Calcitonin gene-related peptide terminates long-lasting vasopressor responses to endothelin 1 in vivo. *Hypertension* 58, 99–106. doi: 10.1161/HYPERTENSIONAHA.110.169128

Meens, M. J., Mattheij, N. J., van Loenen, P. B., Spijkers, L. J., Lemkens, P., Nelissen, J., et al. (2012). G-protein betagamma subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. *Br. J. Pharmacol.* 166, 297–308. doi: 10.1111/j.1476-5381.2011.01774.x

Meng, J., Ovsepian, S. V., Wang, J., Pickering, M., Sasse, A., Aoki, K. R., et al. (2009). Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. *J. Neurosci.* 29, 4981–4992. doi: 10.1523/JNEUROSCI.5490-08.2009

Miller, S., Liu, H., Warfvinge, K., Shi, L., Dovlatyan, M., Xu, C., et al. (2016). Immunohistochemical localization of the calcitonin gene-related peptide binding site in the primate trigeminovascular system using functional antagonist antibodies. *Neuroscience* 328, 165–183. doi: 10.1016/j.neuroscience.2016.04.046

Moore, K., Ghatnekar, G., Gourdie, R. G., and Potts, J. D. (2014). Impact of the controlled release of a connexin 43 peptide on corneal wound closure in an STZ model of type I diabetes. *PLoS One* 9:e86570. doi: 10.1371/journal.pone.0086570

Muff, R., Buhlmann, N., Fischer, J. A., and Born, W. (1999). An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. *Endocrinology* 140, 2924–2927. doi: 10.1210/endo.140.6.6930

Mulderry, P. K., Ghatei, M. A., Spokes, R. A., Jones, P. M., Pierson, A. M., Hamid, Q. A., et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 25, 195–205. doi: 10.1016/0306-4522(88)90018-8

Nakamuta, H., Fukuda, Y., Koida, M., Fujii, N., Otaka, A., Funakoshi, S., et al. (1986). Binding sites of calcitonin gene-related peptide (CGRP): abundant occurrence in visceral organs. *Jpn. J. Pharmacol.* 42, 175–180. doi: 10.1254/jjp.42.175

Nelson, M. T., Huang, Y., Brayden, J. E., Hescheler, J., and Standen, N. B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K+ channels. *Nature* 344, 770–773. doi: 10.1038/344770a0

New, H. V., and Mudge, A. W. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* 323, 809–811. doi: 10.1038/323809a0

Nilsson, C., Hansen, T. K., Rosenquist, C., Hartmann, B., Kodra, J. T., Lau, J. F., et al. (2016). Long acting analogue of the calcitonin gene-related peptide induces positive metabolic effects and secretion of the glucagon-like peptide-1. *Eur. J. Pharmacol.* 773, 24–31. doi: 10.1016/j.ejphar.2016.01.003

Oliver, K. R., Wainwright, A., Edvinsson, L., Pickard, J. D., and Hill, R. G. (2002). Immunohistochemical localization of calcitonin receptor-like receptor and receptor activity-modifying proteins in the human cerebral vasculature. *J. Cereb. Blood Flow Metab.* 22, 620–629. doi: 10.1097/00004647-200205000-00014

Pozsgai, G., Hajna, Z., Bagoly, T., Boros, M., Kemeny, A., Materazzi, S., et al. (2012). The role of transient receptor potential ankyrin 1 (TRPA1) receptor activation in hydrogen-sulphide-induced CGRP-release and vasodilation. *Eur. J. Pharmacol.* 689, 56–64. doi: 10.1016/j.ejphar.2012.05.053

Qi, T., and Hay, D. L. (2010). Structure-function relationships of the N-terminus of receptor activity-modifying proteins. *Br. J. Pharmacol.* 159, 1059–1068.

Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., et al. (1983). Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 304, 129–135. doi: 10.1038/304129a0

Roudenok, V., Gutjar, L., Antipova, V., and Rogov, Y. (2001). Expression of vasoactive intestinal polypeptide and calcitonin gene-related peptide in human stellate ganglia after acute myocardial infarction. *Ann. Anat.* 183, 341–344. doi: 10.1016/S0940-9602(01)80176-X

Routledge, S. J., Simms, J., Clark, A., Yeung, H. Y., Wigglesworth, M. J., Dickerson, I. M., et al. (2020). Receptor component protein, an endogenous allosteric modulator of family B G protein coupled receptors. *Biochim. Biophys. Acta Biomembr.* 1862:183174. doi: 10.1016/j.bbamem.2019.183174

Rovero, P., Giuliani, S., and Maggi, C. A. (1992). CGRP antagonist activity of short C-terminal fragments of human alpha CGRP, CGRP(23-37) and CGRP(19-37). *Peptides* 13, 1025–1027. doi: 10.1016/0196-9781(92)90067-d

Russell, F. A., King, R., Smillie, S. J., Kodji, X., and Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* 94, 1099–1142.

Sabharwal, R., Zhang, Z., Lu, Y., Abboud, F. M., Russo, A. F., and Chapleau, M. W. (2010). Receptor activity-modifying protein 1 increases baroreflex sensitivity and attenuates angiotensin-induced hypertension. *Hypertension* 55, 627–635. doi: 10.1161/HYPERTENSIONAHA.109.148171

Salio, C., Averill, S., Priestley, J. V., and Merighi, A. (2007). Costorage of BDNF and neuropeptides within individual dense-core vesicles in central and peripheral neurons. *Dev. Neurobiol.* 67, 326–338. doi: 10.1002/dneu.20358

Serafin, D. S., Harris, N. R., Nielsen, N. R., Mackie, D. I., and Caron, K. M. (2020). Dawn of a new RAMPage. *Trends Pharmacol. Sci.* 41, 249–265. doi: 10.1016/j.tips.2020.01.009

Sexton, P. M., Mckenzie, J. S., Mason, R. T., Moseley, J. M., Martin, T. J., and Mendelsohn, F. A. (1986). Localization of binding sites for calcitonin gene-related peptide in rat brain by in vitro autoradiography. *Neuroscience* 19, 1235–1245. doi: 10.1016/0306-4522(86)90137-5

Shekhar, Y. C., Anand, I. S., Sarma, R., Ferrari, R., Wahi, P. L., and Poole-Wilson, P. A. (1991). Effects of prolonged infusion of human alpha calcitonin gene-related peptide on hemodynamics, renal blood flow and hormone levels in congestive heart failure. *Am. J. Cardiol.* 67, 732–736. doi: 10.1016/0002-9149(91)90531-o

Shi, L., Lehto, S. G., Zhu, D. X., Sun, H., Zhang, J., Smith, B. P., et al. (2016). Pharmacologic characterization of AMG 334, a potent and selective human monoclonal antibody against the calcitonin gene-related peptide receptor. *J. Pharmacol. Exp. Ther.* 356, 223–231. doi: 10.1124/jpet.115.227793

Silberstein, S. D., Dodick, D. W., Bigal, M. E., Yeung, P. P., Goadsby, P. J., Blankenbiller, T., et al. (2017). Fremanezumab for the preventive treatment of chronic migraine. *N. Engl. J. Med.* 377, 2113–2122.

Smillie, S. J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., et al. (2014). An ongoing role of alpha-calcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. *Hypertension* 63, 1056–1062. doi: 10.1161/HYPERTENSIONAHA.113.02517

Smith, T. R., Janelidze, M., Chakhava, G., Cady, R., Hirman, J., Allan, B., et al. (2020). Eptinezumab for the prevention of episodic migraine: sustained effect through 1 year of treatment in the PROMISE-1 study. *Clin. Ther.* 42, 2254–2265.e3.

Song, S. W., Guo, K. J., Shi, R., Cheng, Y., and Liu, Y. F. (2009). Pretreatment with calcitonin gene-related peptide attenuates hepatic ischemia/reperfusion injury in rats. *Transplant. Proc.* 41, 1493–1498. doi: 10.1016/j.transproceed.2009.03.056

Stauffer, V. L., Dodick, D. W., Zhang, Q., Carter, J. N., Ailani, J., and Conley, R. R. (2018). Evaluation of galcanezumab for the prevention of episodic migraine: the EVOLVE-1 randomized clinical trial. *JAMA Neurol.* 75, 1080–1088. doi: 10.1001/jamaneurol.2018.1212

Steenbergh, P. H., Hoppener, J. W., Zandberg, J., Lips, C. J., and Jansz, H. S. (1985). A second human calcitonin/CGRP gene. *FEBS Lett.* 183, 403–407.

Steenbergh, P. H., Hoppener, J. W., Zandberg, J., Visser, A., Lips, C. J., and Jansz, H. S. (1986). Structure and expression of the human calcitonin/CGRP genes. *FEBS Lett.* 209, 97–103. doi: 10.1016/0014-5793(86)81091-2

Steiner, S., Muff, R., Gujer, R., Fischer, J. A., and Born, W. (2002). The transmembrane domain of receptor-activity-modifying protein 1 is essential for the functional expression of a calcitonin gene-related peptide receptor. *Biochemistry* 41, 11398–11404. doi: 10.1021/bi020279r

Strecker, T., Reeh, P. W., Weyand, M., and Messlinger, K. (2006). Release of calcitonin gene-related peptide from the isolated mouse heart: methodological validation of a new model. *Neuropeptides* 40, 107–113.

Struthers, A. D., Brown, M. J., Macdonald, D. W., Beacham, J. L., Stevenson, J. C., Morris, H. R., et al. (1986). Human calcitonin gene related peptide: a potent endogenous vasodilator in man. *Clin. Sci.* 70, 389–393. doi: 10.1042/cs0700389

Supowit, S. C., Ramana, C. V., Westlund, K. N., and Dipette, D. J. (1993). Calcitonin gene-related peptide gene expression in the spontaneously hypertensive rat. *Hypertension* 21, 1010–1014.

Supowit, S. C., Rao, A., Bowers, M. C., Zhao, H., Fink, G., Steficek, B., et al. (2005). Calcitonin gene-related peptide protects against hypertension-induced heart and kidney damage. *Hypertension* 45, 109–114. doi: 10.1161/01.HYP.0000151130.34874.fa

Supowit, S. C., Zhao, H., and Dipette, D. J. (2001). Nerve growth factor enhances calcitonin gene-related peptide expression in the spontaneously hypertensive rat. *Hypertension* 37, 728–732. doi: 10.1161/01.hyp.37.2.728

Supowit, S. C., Zhao, H., Hallman, D. M., and Dipette, D. J. (1997). Calcitonin gene-related peptide is a depressor of deoxycorticosterone-salt hypertension in the rat. *Hypertension* 29, 945–950. doi: 10.1161/01.hyp.29.4.945

Supowit, S. C., Zhao, H., Hallman, D. M., and Dipette, D. J. (1998). Calcitonin gene-related peptide is a depressor in subtotal nephrectomy hypertension. *Hypertension* 31, 391–396. doi: 10.1161/01.hyp.31.1.391

Supowit, S. C., Zhao, H., Katki, K. A., Gupta, P., and Dipette, D. J. (2011). Bradykinin and prostaglandin E(1) regulate calcitonin gene-related peptide expression in cultured rat sensory neurons. *Regul. Pept.* 167, 105–111.

Supowit, S. C., Zhao, H., Wang, D. H., and Dipette, D. J. (1995b). Regulation of neuronal calcitonin gene-related peptide expression. Role of increased blood pressure. *Hypertension* 26, 1177–1180. doi: 10.1161/01.hyp.26.6.1177

Supowit, S. C., Gururaj, A., Ramana, C. V., Westlund, K. N., and Dipette, D. J. (1995a). Enhanced neuronal expression of calcitonin gene-related peptide in mineralocorticoid-salt hypertension. *Hypertension* 25, 1333–1338. doi: 10.1161/01.hyp.25.6.1333

Tepper, S., Ashina, M., Reuter, U., Brandes, J. L., Dolezil, D., Silberstein, S., et al. (2017). Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol.* 16, 425–434. doi: 10.1016/S1474-4422(17)30083-2

Tjen, A. L. S., Ekman, R., Lippton, H., Cary, J., and Keith, I. (1992). CGRP and somatostatin modulate chronic hypoxic pulmonary hypertension. *Am. J. Physiol.* 263, H681–H690. doi: 10.1152/ajpheart.1992.263.3.H681

Tomoda, Y., Kikumoto, K., Isumi, Y., Katafuchi, T., Tanaka, A., Kangawa, K., et al. (2001). Cardiac fibroblasts are major production and target cells of adrenomedullin in the heart in vitro. *Cardiovasc. Res.* 49, 721–730. doi: 10.1016/s0008-6363(00)00291-1

Tortorella, C., Macchi, C., Forneris, M., and Nussdorfer, G. G. (2001). Calcitonin gene-related peptide (CGRP), acting via CGRP type 1 receptors, inhibits potassium-stimulated aldosterone secretion and enhances basal catecholamine secretion from rat adrenal gland. *Int. J. Mol. Med.* 8, 261–264. doi: 10.3892/ijmm.8.3.261

Tsujikawa, K., Yayama, K., Hayashi, T., Matsushita, H., Yamaguchi, T., Shigeno, T., et al. (2007). Hypertension and dysregulated proinflammatory cytokine

production in receptor activity-modifying protein 1-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16702–16707. doi: 10.1073/pnas.0705974104

Udawela, M., Christopoulos, G., Morfis, M., Christopoulos, A., Ye, S., Tilakaratne, N., et al. (2006). A critical role for the short intracellular C terminus in receptor activity-modifying protein function. *Mol. Pharmacol.* 70, 1750–1760.

Uddman, R., Edvinsson, L., Ekblad, E., Hakanson, R., and Sundler, F. (1986). Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. *Regul. Pept.* 15, 1–23. doi: 10.1016/0167-0115(86)90071-6

Urits, I., Jones, M. R., Gress, K., Charipova, K., Fiocchi, J., Kaye, A. D., et al. (2019). CGRP antagonists for the treatment of chronic migraines: a comprehensive review. *Curr. Pain Headache Rep.* 23:29. doi: 10.1007/s11916-019-0768-y

van Rossum, D., Hanisch, U. K., and Quirion, R. (1997). Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci. Biobehav. Rev.* 21, 649–678. doi: 10.1016/s0149-7634(96)00023-1

Walker, C. S., Conner, A. C., Poyner, D. R., and Hay, D. L. (2010). Regulation of signal transduction by calcitonin gene-related peptide receptors. *Trends Pharmacol. Sci.* 31, 476–483. doi: 10.1016/j.tips.2010.06.006

Walker, C. S., Eftekhari, S., Bower, R. L., Wilderman, A., Insel, P. A., Edvinsson, L., et al. (2015). A second trigeminal CGRP receptor: function and expression of the AMY1 receptor. *Ann. Clin. Transl. Neurol.* 2, 595–608. doi: 10.1002/acn3.197

Walker, C. S., Raddant, A. C., Woolley, M. J., Russo, A. F., and Hay, D. L. (2018). CGRP receptor antagonist activity of olcegepant depends on the signalling pathway measured. *Cephalalgia* 38, 437–451. doi: 10.1177/0333102417691762

Wang, H., Xing, L., Li, W., Hou, L., Guo, J., and Wang, X. (2002). Production and secretion of calcitonin gene-related peptide from human lymphocytes. *J. Neuroimmunol.* 130, 155–162. doi: 10.1016/s0165-5728(02)00221-7

Wang, X. L., Cui, L. W., Liu, Z., Gao, Y. M., Wang, S., Li, H., et al. (2019). Effects of TRPA1 activation and inhibition on TRPA1 and CGRP expression in dorsal root ganglion neurons. *Neural Regen. Res.* 14, 140–148. doi: 10.4103/1673-5374.243719

Warfvinge, K., and Edvinsson, L. (2019). Distribution of CGRP and CGRP receptor components in the rat brain. *Cephalalgia* 39, 342–353.

Watkins, H. A., Rathbone, D. L., Barwell, J., Hay, D. L., and Poyner, D. R. (2013). Structure-activity relationships for alpha-calcitonin gene-related peptide. *Br. J. Pharmacol.* 170, 1308–1322. doi: 10.1111/bph.12072

Wattiez, A. S., Sowers, L. P., and Russo, A. F. (2020). Calcitonin gene-related peptide (CGRP): role in migraine pathophysiology and therapeutic targeting. *Expert Opin. Ther. Targets* 24, 91–100. doi: 10.1080/14728222.2020.1724285

Wimalawansa, S. J., and MacIntyre, I. (1988). Calcitonin gene-related peptide and its specific binding sites in the cardiovascular system of rat. *Int. J. Cardiol.* 20, 29–37. doi: 10.1016/0167-5273(88)90313-0

Wolfrum, S., Nienstedt, J., Heidbreder, M., Schneider, K., Dominiak, P., and Dendorfer, A. (2005). Calcitonin gene related peptide mediates cardioprotection by remote preconditioning. *Regul. Pept.* 127, 217–224. doi: 10.1016/j.regpep.2004.12.008

Wu, D. M., van Zwieten, P. A., and Doods, H. N. (2001). Effects of calcitonin gene-related peptide and BIBN4096BS on myocardial ischemia in anesthetized rats. *Acta Pharmacol. Sin.* 22, 588–594.

Zhang, Y., Wei, W., Lv, P., Wang, L., and Ma, G. (2011). Preparation and evaluation of alginate-chitosan microspheres for oral delivery of insulin. *Eur. J. Pharm. Biopharm.* 77, 11–19. doi: 10.1016/j.ejpb.2010.09.016

Zhao, Q., Liu, Z., Wang, Z., Yang, C., Liu, J., and Lu, J. (2007). Effect of prepro-calcitonin gene-related peptide-expressing endothelial progenitor cells on pulmonary hypertension. *Ann. Thorac. Surg.* 84, 544–552. doi: 10.1016/j.athoracsur.2007.03.067

Zheng, L. R., Han, J., Yao, L., Sun, Y. L., Jiang, D. M., Hu, S. J., et al. (2012). Up-regulation of calcitonin gene-related peptide protects streptozotocin-induced diabetic hearts from ischemia/reperfusion injury. *Int. J. Cardiol.* 156, 192–198. doi: 10.1016/j.ijcard.2011.04.009

Zhong, B., Rubinstein, J., Ma, S., and Wang, D. H. (2018). Genetic ablation of TRPV1 exacerbates pressure overload-induced cardiac hypertrophy. *Biomed. Pharmacother.* 99, 261–270. doi: 10.1016/j.biopha.2018.01.065

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The Vascular-Dependent and -Independent Actions of Calcitonin Gene-Related Peptide in Cardiovascular Disease

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The treatment of hypertension and heart failure remains a major challenge to healthcare providers. Despite therapeutic advances, heart failure affects more than 26 million people worldwide and is increasing in prevalence due to an ageing population. Similarly, despite an improvement in blood pressure management, largely due to pharmacological interventions, hypertension remains a silent killer. This is in part due to its ability to contribute to heart failure. Development of novel therapies will likely be at the forefront of future cardiovascular studies to address these unmet needs. Calcitonin gene-related peptide (CGRP) is a 37 amino acid potent vasodilator with positive-ionotropic and -chronotropic effects. It has been reported to have beneficial effects in hypertensive and heart failure patients. Interestingly, changes in plasma CGRP concentration in patients after myocardial infarction, heart failure, and in some forms of hypertension, also support a role for CGRP on hemodynamic functions. Rodent studies have played an important role thus far in delineating mechanisms involved in CGRP-induced cardioprotection. However, due to the short plasma half-life of CGRP, these well documented beneficial effects have often proven to be acute and transient. Recent development of longer lasting CGRP agonists may therefore offer a practical solution to investigating CGRP further in cardiovascular disease *in vivo*. Furthermore, pre-clinical murine studies have hinted at the prospect of cardioprotective mechanisms of CGRP which is independent of its hypotensive effect. Here, we discuss past and present evidence of vascular-dependent and -independent processes by which CGRP could protect the vasculature and myocardium against cardiovascular dysfunction.

Keywords: CGRP, cardiovascular, heart, mouse, nitric oxide, hypertension, heart failure

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INTRODUCTION

The discovery of Calcitonin gene-related peptide (CGRP) mRNA in the rat hypothalamus by Amara et al. (1982) sparked a series of studies exploring the effect of CGRP in the central and peripheral systems, where it is widely distributed. The authors discovered that human CALCA gene, which codes for the thyroid gland hormone calcitonin, can also produce CGRP *via* alternative splicing in neural tissues. CGRP is a member of the calcitonin family of peptides, that include adrenomedullin,

adrenomedullin 2/intermedin and amylin (Russell et al., 2014). Soon after, it was found that CGRP has two structurally similar isoforms – the α and β, which are encoded by two distinct genes (Amara et al., 1985). Both isoforms are primarily located in sensory C- and Aδ-fibers (Gibson et al., 1984), and it is generally accepted that despite 94% sequence identity between the two isoforms, β-CGRP synthesis and expression is concentrated around the enteric nervous system, immune cells and pituitary gland (Steenbergh et al., 1985; Brain and Grant, 2004) whereas α-CGRP is primarily involved in the central and peripheral nervous systems, and consequently, is the more extensively studied isoform in cardiovascular studies. This review aims to discuss past and present literature on CGRP in hypertension and heart failure to stimulate thought on the future of CGRP and cardiovascular research, with a particular focus on α-CGRP.

The current consensus is that, upon neuronal depolarization, α- or β-CGRP is released from sensory neurons *via* calcium-dependent exocytosis to bind to its receptor situated on the plasma membrane of several cell types including, but not limited to, smooth muscle cells (Argunhan et al., 2021), endothelial cells (Gray and Marshall, 1992a) and cardiomyocytes (Clark et al., 2021). The receptor complex is composed of a seven domain G-protein coupled receptor (GPCR) known as calcitonin receptor-like receptor (CRLR) and a single transmembrane protein recognized as receptor activity modifying protein-1 (RAMP1). RAMP1 is required for trafficking of the receptor to the cell surface to form a heterodimer with CRLR and mediate high-affinity binding to CGRP. Upon activation of the CRLR/RAMP1 receptor, G-protein induced signaling cascade is initiated, with $G\alpha_s$-induced cyclic adenosine monophosphate (cAMP) being the major secondary messenger involved (McLatchie et al., 1998; Pioszak and Hay, 2020). The CGRP family of receptors also comprise two other RAMP proteins; RAMP2 and RAMP3. There is evidence that CGRP may be able to signal *via* receptors including these components. The receptors involved comprise the calcitonin receptor (CTR) interacting with RAMP1, commonly called the amylin receptor and CRLR interacting with RAMP2, the adrenomedullin receptor (Hay et al., 2018). The selectivity of CGRP for receptors other than CRLR/RAMP1 is under investigation.

Due to the CRLR/RAMP1 receptor complex being expressed in the plasma membrane of smooth muscle cells and endothelial cells, CGRP-induced vasodilation can manifest *via* two distinct but related mechanisms. The CGRP ligand can bind directly to the receptor complex in vascular smooth muscle cells to induce PKA-mediated smooth muscle relaxation, or it can interact with its receptor complex in endothelial cells to induce endothelial-dependent relaxation *via* nitric oxide signaling (Crossman et al., 1990; Gray and Marshall, 1992b). In vascular smooth muscle cells, CGRP-induced PKA phosphorylation leads to: reduced intracellular Ca^{2+} concentration; reduced binding affinity of myosin light chain kinase (MLCK) to Ca^{2+}-calmodulin complex; and activation of ion channels such as ATP-sensitive potassium channels (K_{ATP}). All of these contribute to smooth muscle cell relaxation, and thus vasodilation (Brain and Grant, 2004; **Figure 1A**). In endothelial cells, PKA phosphorylation of eNOS results in NO generation, which is known to diffuse into neighboring vascular smooth muscles and mediate smooth muscle cell relaxation *via* guanyl cyclase and protein kinase G (PKG) signaling induction pathways (Russell et al., 2014; **Figure 1A**). Additionally, there is some evidence for PKC-mediated responses post-CGRP receptor activation (Walker et al., 2010) but the cAMP response is the most established and understood signaling pathway to date. However, it is important to acknowledge the body of *in vitro* evidence suggesting that the CGRP receptor can couple with other G proteins (Weston et al., 2016). It is also noteworthy that, in addition to its canonical receptor, CGRP has been shown to interact with other receptors from the CGRP family of peptides, albeit with lower affinity (Hay et al., 2018). Recently, the structure and dynamics of the canonical CGRP receptor has been investigated using single-particle cryo-EM (Liang et al., 2018; Josephs et al., 2021), and agonist bias studies have revealed physiological consequences for the CRLR-RAMP1 complex in different cell types (Clark et al., 2021). Thus, this is an area of much interest that is continually evolving, with RAMP proteins being the subject of investigation.

CALCITONIN GENE-RELATED PEPTIDE AND VASCULAR TONE – SMALL BUT MIGHTY

Fisher et al. (1983) were the first to demonstrate the hypotensive effects of systemically administered CGRP in rats, whereas Brain et al. (1985, 1986) showed that intradermal injection of CGRP at femtomole doses induces arteriole dilation, thus increasing blood flow locally in animal and human skin. These studies and others indicated that CGRP is a potent vasodilator, leading researchers to further explore its beneficial potential within the cardiovascular system. As a microvascular vasodilator, the potency of CGRP is approximately 10-fold higher than prostaglandins, up to 100 times greater than other well-established vasodilators such as acetylcholine, thus making CGRP the most potent peripheral vasodilator discovered to date (Russell et al., 2014).

Gennari and Fischer (1985) were first to determine the cardiovascular actions of CGRP on hemodynamic parameters in healthy humans. In addition to CGRP's intrinsic ability to cause vasodilation and consequently hypotension, the authors revealed that CGRP possesses positive chronotropic and inotropic activities. CGRP was reported to increase the force of contractility by stimulating the sympathetic nervous system, which was further supported by Gennari et al. (1990) when patients with congestive heart failure (CHF) demonstrated improved cardiac contractility after receiving β-CGRP infusion for 24 h (12.5 ug/h). *Ex vivo* experiments reinforce these findings, although the mechanism behind the positive inotropic effect is still to be fully elucidated. Franco-Cereceda et al. (1987) were the first to report the effect of CGRP treatment in isolated human auricles, and interestingly, the authors found both α- and β- isoforms of CGRP to be equally potent in affecting positive inotropy.

Similarly, Struthers et al. (1986) infused hCGRP into healthy patients (545 pmol/min), which, as expected, caused a significant

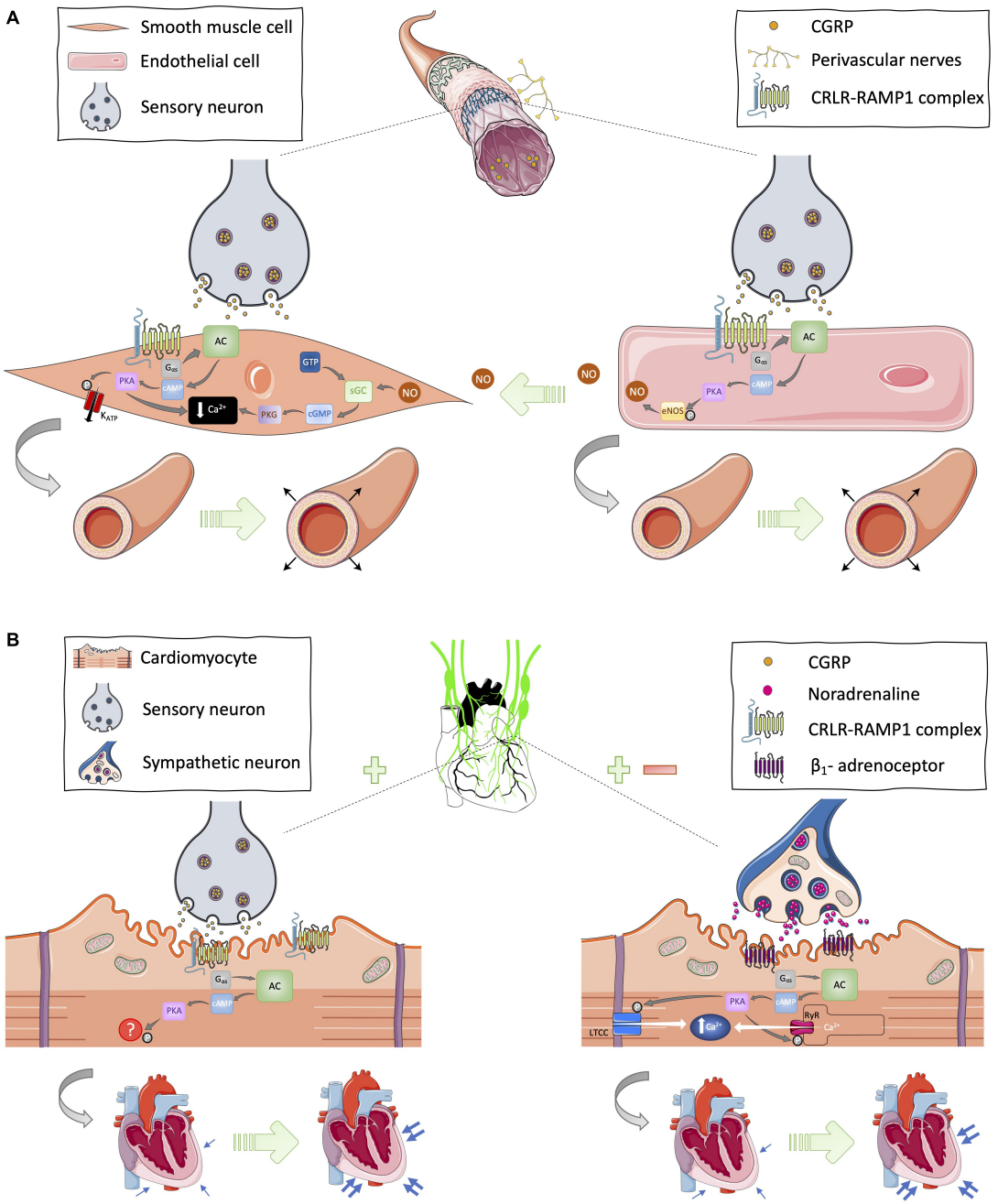

FIGURE 1 | Proposed cardioprotective mechanisms of CGRP in the **(A)** vasculature and **(B)** myocardium. **(A)** In the vasculature, CGRP circulating in the blood or released from sensory neurons can bind on to its canonical receptor, the CRLR-RAMP1 complex, expressed in the plasma membrane of vascular smooth muscle and endothelial cells to initiate $G\alpha_s$-protein signal transduction and subsequently cause relaxation of smooth muscle cells *via* nitric oxide (NO)-dependent and -independent mechanisms thus leading to vasodilation. **(B)** In cardiac tissues, there is evidence for CGRP-stimulated modulation of sympathetic outflow and expression of the CRLR-RAMP1 complex on cardiomyocytes. Hence, CGRP has the potential to induce $G\alpha_s$-protein signaling from sensory and sympathetic nerves leading to increased cardiac contractility, thus positive inotropy and chronotropy. AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; GTP, guanosine triphosphate; K_{ATP}, ATP-sensitive potassium channels; LTCC, L-type calcium channel; NO, nitric oxide; P, phosphate; PKA, protein kinase A; RyR, ryanodine receptor; sGC, soluble guanylate cyclase. [Images were obtained from smart.servier.com under a Creative Commons Attribution 3.0 Unported License].

decrease in diastolic pressure accompanied with an increase in heart rate. The authors suggested a potential role for CGRP in modulating vascular tone in humans, and this was supported by findings from Girgis et al. (1985) demonstrating that CGRP concentration is fivefold higher than calcitonin. Given that it is widely expressed in the human body, these results raised

the possibility that CGRP is a critical physiological regulator of vascular tone and hemodynamics.

Collectively, these studies laid the foundation for elucidating the biological actions of CGRP, which recently reached fruition in the field of migraine research where CGRP blockers are now used therapeutically (Edvinsson et al., 2018; Kee et al., 2018). Generally, the use of migraine blockers have not been associated with significant cardiovascular effects, indicating that CGRP does not play a major role in cardiovascular regulation in individuals with normal blood pressure (Bigal et al., 2015). Separately, there has been increasing research into elucidating the role of CGRP and of exogenous CGRP, in the cardiovascular system. However, the potential biological importance and underlying mechanisms, particularly with respect to heart failure, will be discussed in later sections.

CALCITONIN GENE-RELATED PEPTIDE IN HYPERTENSION – VASCULAR-DEPENDENT EFFECTS

Shekhar et al. (1991) was one of the first to demonstrate the cardiovascular effects of prolonged CGRP infusion (8 ng/kg/min for 8 h) in CHF patients. In agreement with others, they reported significant decreases in cardiac and arterial pressures, as well as in pulmonary and systemic vascular resistance, and increases in cardiac output and stroke volume. Accordingly, the reported hemodynamic changes were absent 30 min after discontinuation of CGRP infusion, consistent with the short half-life of CGRP (approximately 7–30 min) (Kraenzlin et al., 1985).

Concomitantly, preclinical data from rodents have facilitated human studies. Research in the 1990s focusing on the role of CGRP in blood pressure control was dominated by preclinical hypertension studies carried out in rats that were spontaneously hypertensive (SHR), treated with hypertensive agents, or hypertensive due to surgical intervention. These studies and others reported *in vivo* evidence that CGRP can protect against hypertension and the vasoconstrictor effects of hypertensive agents including angiotensin-II (AngII) (Itabashi et al., 1988; Fujioka et al., 1991), noradrenaline (Fujioka et al., 1991), deoxycorticosterone-salt (Supowit et al., 1997; Supowit et al., 2005), and the nitric oxide synthase (NOS) inhibitor L-NAME (Gardiner et al., 1991; Gangula et al., 1997), making progress toward understanding the mechanism of action of CGRP (Kumar et al., 2019a). Kawasaki et al. (1990) found that reduced CGRP-containing nerves in SHRs contribute to the development and maintenance of hypertension, further supporting a protective role for CGRP in hypertension. Furthermore, *ex vivo* experiments, particularly in isolated arterial and mesenteric resistance vessels, have complemented the *in vivo* data to-date (Nelson et al., 1990; Kawasaki et al., 1998; Kawasaki et al., 1999).

It has been difficult to study α-CGRP *in vivo* due to its peptide nature, therefore the use of genetically engineered mice has been fitting. Genetic deletion of CGRP has been reported to cause elevated baseline blood pressure in some (Gangula et al., 2000; Oh-hashi et al., 2001; Li et al., 2004; Mai et al., 2014) but not all studies (Smillie et al., 2014; Argunhan et al., 2021). This is likely due to differences in methodology, such as differences in the precise genetic deletion site when generating knockout (KO) mouse lines, particular strains preserving unique hemodynamic phenotypes, and the utilization of various different blood pressure measurement techniques. Most importantly, most preclinical hypertension studies conclude that genetic deletion of CGRP is detrimental, if not in a naïve state, then evidently in a stressed or hypertensive setting. This indicates that endogenous CGRP may only be functionally active in cardiovascular dysfunction. CGRP blockers (antibodies and now CGRP receptor antagonists) have been studied in humans with migraine over several years now (Al-Hassany and Van Den Brink, 2020). The majority of findings from recent clinical trials report little or no change in blood pressure from migraine patients who received anti-CGRP therapy. Additionally, very few cardiovascular side-effects have been observed in migraine patients taking CGRP blockers (Tepper, 2019). Thus, it is not yet known whether CGRP has a functionally important cardiovascular role in humans, apart from the neurogenic vasodilator response observed typically in patients with migraine (Tepper, 2019). However, due to CGRP being widely expressed and the increasing evidence for a cardioprotective role, there are concerns regarding long-term blockade of CGRP in migraine patients who may also suffer from cardiovascular complications (Rubio-Beltran and Maassen van den Brink, 2019). The observation of cardiovascular adverse effects with CGRP blockers in migraine has not been commonly observed. However, a recent study conducted a retrospective analysis of cases reporting a CGRP receptor antagonist (erenumab) associated with elevated blood pressure (BP) (Saely et al., 2021). The authors identified 61 cases of elevated BP between May 2018 and April 2020, of which the median systolic BP increase was 39 mm Hg. Interestingly, 44% of reported cases required anti-hypertensive medication and the elevated BP occurred most frequently within a week of commencing erenumab treatment. Most importantly, the prescribing information for erenumab/Aimovig now includes hypertension. This further supports the need to continue monitoring cardiovascular parameters in those receiving anti-CGRP therapy for migraine, in addition to continuing research within the CGRP and cardiovascular field (Saely et al., 2021).

Much like CGRP KO mice, RAMP1 KO mice develop high blood pressure (Tsujikawa et al., 2007) and knock-in or overexpression of human RAMP1 in all (Sabharwal et al., 2010) or solely neural tissues (Sabharwal et al., 2019) potentiates CGRP-dependent blood pressure reduction in AngII-induced hypertension. Additionally, in the periphery, in tissues such as skin, it is clear that CGRP is a potent vasodilator and is well placed to play a regulatory role, for example in the recovery of blood flow in the cold-induced vascular response (Aubdool et al., 2014), a response that diminishes as aging occurs (Thapa et al., 2021).

Our research group has reported that, despite a lack of vascular tone modulation at baseline, α-CGRP-specific KO mice display elevated blood pressure after AngII or L-NAME treatment (Smillie et al., 2014; Argunhan et al., 2021). α-CGRP KO mice present with hypertrophic vascular remodeling in their aortic tissues and increased mRNA levels of inflammatory and oxidative

stress markers after 14 or 28 days of AngII treatment (Smillie et al., 2014). These results indicate a protective role for CGRP in AngII-induced pathophysiology, where the benefits are not limited to one system, but instead multiple processes in order to attenuate the pathophysiological changes induced by AngII.

Intriguingly, Smillie et al. (2014) also showed that AngII-treated α-CGRP KO mice display a significant reduction in protein and mRNA expression of endothelial nitric oxide synthase (eNOS). These findings probed us to investigate whether α-CGRP can protect against hypertension independently of eNOS *in vivo*. The decreased production of NO in pathological states disrupts the endothelial equilibrium thus leading to endothelial dysfunction. CGRP receptors are expressed in the plasma membrane of both endothelial and vascular smooth muscle cells, hence have the capacity to induce vasodilation *via* NO-dependent and -independent pathways. However, there is very limited evidence of this *in vivo*. We demonstrated that α-CGRP KO mice develop exacerbated hypertension and present with dysfunctional blood flow recovery in mesenteric vessels *in vivo* after chronic L-NAME administration (Argunhan et al., 2021) indicating that α-CGRP can induce vasodilation and hence attenuate hypertension independently of NOS. These findings suggest that α-CGRP may be able to offer protection to compensate for pathophysiological processes such as endothelial dysfunction, which contributes to hypertension and cardiovascular disease. We also found that 2 weeks of α-CGRP infusion (165 ug/kg/day) *via* osmotic minipumps was able to reverse L-NAME-induced hypertension, left ventricular heart weight gain, and associated increases in mRNA expression of hypertrophic markers in α-CGRP KO mice, providing evidence for the antihypertrophic effects of CGRP. Conversely, this means that the nitric oxide vasodilator pathway theoretically could compensate, in terms of vasodilation, when CGRP is inhibited.

Antihypertrophic effects of CGRP have also been investigated recently by Skaria et al. (2019). The authors examined whether endogenous, physical activity-induced α-CGRP has blood pressure-independent cardioprotective effects in mice which had 1 kidney 1 clip (1k1c) surgery and hence developed chronic hypertension. The authors claimed that exercise has cardioprotective effects in chronic hypertension, which is mediated at least partially through endogenous α-CGRP signaling. They demonstrated αCGRP concentration in plasma is significantly elevated after 7 min of running in hypertensive mice and showed that chronic exogenous CGRP infusion *via* osmotic minipumps can alleviate hypertension-induced hypertrophy and cardiac dysfunction by suppressing pathological cardiac growth and interstitial fibrosis. Importantly, CGRP was infused at a sub-pressor dose (4 nM/h), suggesting that CGRP administration can help to preserve cardiac function in chronic hypertension independent of its blood pressure lowering effect.

LONG-LASTING AGONIST OF CALCITONIN GENE-RELATED PEPTIDE

While the protective effects of CGRP discussed thus far are detailed and mostly consistent between research groups, they do not constitute a complete record of documented studies demonstrating a cardioprotective role of CGRP. It is apparent that the beneficial effects of CGRP have been limited due to its short peptide half-life. Our group was fortunate to investigate the therapeutic potential of a longer lasting CGRP agonist in AngII-induced hypertension and mice that underwent abdominal aortic constriction surgery, which eventually caused heart failure *via* increased pressure-overload on the heart (Aubdool et al., 2017). The acylated α-CGRP analog had been characterized by Nilsson et al. (2016) and has a half-life of >7 h in rodents. Our group demonstrated that daily administration of the CGRP analog [50 nmol/kg/day, *subcutaneous* injection *(s.c.)]* for 2 weeks in AngII-treated mice led to significant attenuation of AngII-induced hypertension and protected against vascular, renal and cardiac dysfunction. α-CGRP analog-treated mice presented with attenuated hypertrophic and fibrotic markers as well as reduced inflammation and oxidative stress. Furthermore, the α-CGRP analog was effective in preserving ejection fraction, a measure of cardiac function, and protecting against fibrosis and apoptosis in cardiac tissues of mice that had undergone abdominal aortic constriction surgery and consequently developed heart failure. Moreover, α-CGRP-treated mice presented with better vascularization in their hearts and expressed reduced mRNA and protein expression of biomarkers for hypertrophy, apoptosis, oxidative stress and inflammation. These findings agree with other *in vivo* heart failure studies (Li et al., 2013; Kumar et al., 2019b; Kumar et al., 2020). *In vitro* experiments have demonstrated similar findings in different cell types. CGRP administration has been shown to: stimulate proliferation of endothelial cells (Haegerstrand et al., 1990), supporting CGRP's proangiogenic effects *in vivo*; reduce vascular smooth muscle cell proliferation (Li et al., 1997) and thus vascular hypertrophy; and show antioxidant and antiapoptotic effects in dorsal root ganglion (DRG) neurons (Liu et al., 2019).

However, data from the Aubdool et al. (2017) study suggests these protective effects of CGRP can be long lasting using a novel α-CGRP agonist with an extended half-life. The CGRP agonist can delay the onset and development of hypertension through cardioprotective mechanisms in addition to ameliorating pressure overload-induced heart failure. Interestingly, mice with heart failure that had received the α-CGRP analog or its vehicle presented with comparable blood pressures, indicating that the cardioprotective mechanisms involved are likely to be blood pressure-independent. Furthermore, the same analog has recently been administered to rats that have undergone permanent occlusion of their left coronary artery to investigate coronary perfusion in myocardial infarction. Three injections of the CGRP analog, SAX, at 20 min, 24 and 48 h after coronary ligation, was sufficient to improve myocardial perfusion recovery in rats, indicative of myocardium protection after ischemic damage (Bentsen et al., 2021). An earlier study by Kallner et al. (1998) reported CGRP-mediated improvement in post-ischemic coronary flow early after MI, but whether treatment with the long lasting agonist will increase cardioprotective effects in the long term is still to be investigated.

CALCITONIN GENE-RELATED PEPTIDE IN THE MYOCARDIUM – VASCULAR-INDEPENDENT EFFECTS

The heart is densely innervated by nerve fibers comprised of: the sympathetic trunk, which starts from the base of the skull; parasympathetic nerves, including the right and left vagus nerves; cervical cardiac nerves, which run parallel to the vagus nerves; and the cardiac plexus at the base of the heart. α-CGRP producing sensory nerves have been reported within the perivascular layer of coronary arteries, in the myocardium of ventricles, and within the cardiac conduction system (Gulbenkian et al., 1993). In addition to this, CGRP immunoreactivity has been associated with; myocytes from atria, coronary vessels, local parasympathetic ganglia and with epi-and endocardia (Franco-Cereceda et al., 1987). Therefore, it is likely that CGRP signaling manifests within cardiac cells, in addition to cardiac vessels, and that the sensory, parasympathetic and sympathetic nerves are involved in facilitating CGRP-induced signaling within, and surrounding, the myocardium.

Several *in vivo* studies of hypertension and heart failure have reported an upregulation of RAMP1 and/or CGRP expression in pathological conditions (Supowit et al., 1995; Li and Wang, 2005; Aubdool et al., 2017; Argunhan et al., 2021). In addition to this, Franco-Cereceda and Liska (2000) have previously reported the presence of a subpopulation of capsaicin-sensitive cardiac C-fiber afferents that store CGRP, substance P and neurokinin A. The C-fibers are likely to express transient receptor potential vanilloid 1 (TRPV1) channel, which upon stimulation by capsaicin lead to CGRP release (Franco-Cereceda, 1991). This finding of local efferent release of CGRP in the heart is consistent with the presence of capsaicin-sensitive receptors on the epicardial surface of rat hearts (Zahner et al., 2003). More recently, Moreira et al. (2020) reported elevated CGRP levels in human atrial tissue lysates and atrial cardiomyocytes obtained from patients with atrial fibrillation, in agreement with Franco-Cereceda et al. (1987) who found between threefold and fourfold higher levels of CGRP-like immunoreactivity in atria compared to ventricles. These studies describe and support a structural basis for CGRP signaling within cardiac tissues.

Furthermore, coinciding with its interactions in vascular smooth muscle and endothelial cells, CGRP has similarly been demonstrated to bind to its canonical receptor complex and activate $G\alpha_s$-signaling in cardiomyocytes (Huang et al., 1999; Sueur et al., 2005; Schavinski et al., 2021). It is well established that an increase in cAMP concentration followed by PKA activation leads to phosphorylation of key Ca^{2+}-handling proteins including phospholamban, ryanodine receptor, voltage-gated L-type Ca^{2+} channels, troponin I, and myosin binding protein C; all which play an essential role in cardiac excitability and contraction (Zaccolo, 2009). Earlier studies have reported that activation of the CRLR/RAMP1 complex leads to stimulation of a contractile response in adult rat ventricular cardiomyocytes (Bell and McDermott, 1994; Bell et al., 1995). However, unlike the vascular-dependent effects of CGRP, the precise mechanism of action of CGRP in cardiomyocytes remains to be fully elucidated.

Interestingly, a recent study investigating GPCR agonist bias in CGRP and CGRP-like family peptides demonstrated that, in human ventricular cardiomyocytes, CGRP is more potent than adrenomedullin and adrenomedullin 2/intermedin in generating cAMP (Clark et al., 2021). Acute cAMP elevation is known to compensate for impaired cardiac function by modulating the positive-inotropic, -chronotropic and -lusitropic responses in the heart. Thus, in addition to its vascular dependent actions, it is tempting to speculate that CGRP-induced positive-inotropy and -chronotropy observed in earlier human studies could be a consequence of vascular-independent actions *via* direct interaction with cardiomyocytes. Chronic cAMP activation, however, has been associated with adverse cardiac remodeling (Triposkiadis et al., 2009). Importantly, cAMP is a pleiotropic secondary messenger and thus able to produce several biological outcomes in response to different stimuli. Moreover, locally accumulated cAMP has been shown to ameliorate cardiac hypertrophy *via* cAMP-degrading enzyme phosphodiesterase-2 (PDE2; Zoccarato et al., 2015). The regulation and function of local cAMP-PKA signaling remains to be fully understood, and compartmentalized cAMP-PKA has been suggested to play a key role in cardiac physiology and pathophysiology (Surdo et al., 2017).

Coupling of the CRLR-RAMP1 complex to $G\alpha_s$ can lead to other responses such as phosphorylation of ERK1/2 or cell proliferation; a response which has been suggested to be cell type dependent (Clark et al., 2021). Interestingly, the CGRP family of receptors, including CRLR/RAMP1, can couple to $G\alpha_i$ and $G\alpha_q$ subunits too, although there is little evidence of this in cardiomyocytes (Nishikimi et al., 1998; Walker et al., 2010). These findings further support the need to investigate CGRP-signaling in cardiomyocytes and fibroblasts *in vitro*, which may help to clarify the mechanism of action behind the cardioprotective effects of CGRP reported in whole body physiology studies (Li et al., 2013; Aubdool et al., 2017; Kumar et al., 2019b; Skaria et al., 2019; Kumar et al., 2020; Argunhan et al., 2021), especially in studies that have shown blood pressure independent effects of CGRP (Aubdool et al., 2017; Skaria et al., 2019).

In addition to its direct effect on cardiomyocytes, CGRP has also been shown to modulate sympathetic nervous activity (**Figure 1B**). Activation of β-adrenoceptors *via* increased sympathetic nervous activity leads to $G\alpha_s$-signaling-induced cardiac contractility. Earlier studies suggest that CGRP-induced positive inotropic effects may be, at least partially, due to increased sympathetic activity (Fisher et al., 1983; Gennari and Fischer, 1985; Struthers et al., 1986; Katori et al., 2005). On the other hand, Kawasaki (2002) reported that exogenous CGRP treatment can impair noradrenergic-induced constriction in rat mesenteric vessels. Thus, it is thought the activation or increased sympathetic activity reported in some studies is part of a compensatory reflex system to combat CGRP-induced hypotension. Considering that exogenous administration of CGRP primarily lowers blood pressure, its ability to stimulate the sympathetic nervous system is likely to be minimal and secondary to its primary action of inducing vasodilation. Evidence suggests that stimulation of the sympathetic nervous system may be one of the mechanisms CGRP is able to stimulate in situations where inotropic support is necessary (**Figure 1B**).

It is unclear whether the aforementioned inotropic effects of CGRP are solely a consequence CGRP directly binding to its receptor complex found on myocardial cells (Bell and McDermott, 1994), modulation of the sympathetic nervous system, or an as yet unknown mechanism. A combination of these pathways is likely, with the choice dependent on the pathophysiological setting. It is important to acknowledge that selective and non-selective β-blockers, which target β-adrenoreceptors, constitute well-established therapeutics for cardiovascular diseases such as hypertension, coronary artery disease and severe tachycardia. They decrease contractility, thus reducing cardiac output and oxygen demand (Chatterjee et al., 2013; McDonagh et al., 2021), and are therefore part of the evidence-based long-term management of heart failure with reduced ejection fraction (HFrEF). β-blockers are, however, concurrently used with ACE inhibitors, and in many cases, an aldosterone inhibitor or diuretic, to off-set other symptoms of heart failure. Despite being generally well tolerated and recommended as pharmacological therapeutics for heart failure patients who require long-term management of their medical symptoms, there remains an unmet therapeutic need for better management of HF pathophysiology.

Contrary to the above, increasing inotropy can also be of benefit in heart failure patients (Page et al., 2016). However, this is specifically for those who require inotropic- or short-term hemodynamic support due to suffering with decompensated HFrEF, presenting with low cardiac output and hypotension or evidence of end-organ hypoperfusion (McDonagh et al., 2021). Sympathetic cardiac stimulants such as dopamine and dobutamine are therefore still recommended for use in such cases (Bistola et al., 2019) and vasodilator therapeutics are also first-line agents for acute HF with elevated blood pressure (McDonagh et al., 2021). Human studies suggest that CGRP administration is protective in patients who require short-term inotropic support, whilst long-term pre-clinical studies indicate CGRP can improve cardiovascular function parameters in pressure-overload-induced heart failure.

However, whether a sub-pressor dose of CGRP can protect against the development and progression of hypertension and heart failure needs to be investigated further. If CGRP can indeed protect against cardiovascular disease without lowering blood pressure, this answers one query but raises further questions regarding its mechanism of action. Vasodilators are known to primarily reduce total peripheral resistance *via* vasodilation in blood vessels, thus lowering blood pressure. This in turn acutely enhances sympathetic stimulation due to the baroreceptor reflex, hence increasing heart rate and cardiac contractility (positive-chronotropy and -inotropy) in the short-term. The dilation of venous and arterial vessels also leads to a reduction in venous return to the heart (pre-load), which reduces congestion and after-load, therefore increasing stroke volume, cardiac output and subsequent relief of symptoms. It is for these reasons that vasodilators such as nitrates or nitroprusside are recommended for management of acute heart failure (AHF) in patients with elevated blood pressure (McDonagh et al., 2021). If a dilator such as CGRP does not affect blood pressure but is able to improve cardiac function, this adds complexity to our understanding of how vasodilators can modulate cardiac function. The recent data from Aubdool et al. (2017) and Skaria et al. (2019) suggests that CGRP can regulate cardiac function independently of blood pressure, thus *via* vascular-dependent and -independent pathways.

Collectively, these studies propose vascular-independent mechanisms for CGRP in cardiac tissues and may be the primary mechanism by which CGRP elicits protection in the absence of vascular tone changes. There is evidence supporting that CGRP can act locally on cardiomyocytes to elicit some of its cardioprotective actions, and future studies should aim to clarify the precise mechanism(s) involved in cardiac pathophysiology.

DISCUSSION

A wide spectrum of *in vitro, ex vivo, in vivo* and human studies have highlighted the therapeutic potential of CGRP in various pathophysiological conditions within the cardiovascular system. Genetic tools such as transgenic mouse lines combined with pharmacological agents including CGRP peptide administration systems and delivery of selective receptor antagonists, means that researchers are well-equipped to investigate the effects of reduced, enhanced and lack of CGRP signaling in whole body pathophysiology studies. More recently, anti-CGRP therapies for treatment of migraine have proven to be successful with generally minimal adverse effects reported. Additional follow-up clinical trials will be welcomed by all to clarify whether long-term CGRP blockade leads to hypertension-related side effects. Meanwhile, long lasting agonists have emerged as a promising avenue for CGRP-therapy in cardiovascular disease, which will facilitate research into the intrinsic proliferative and angiogenic characteristics of CGRP, in addition to its anti-inflammatory and anti-apoptotic effects reported *in vivo*. Future studies should aim to investigate the blood pressure-independent cardioprotective mechanisms of CGRP treatment in PO-induced heart failure, and whether treatment with the long-lasting agonist could improve outcome after ischemic heart failure. Collectively, these findings further demonstrate the importance of continuing CGRP research to fully elucidate the physiological influence of CGRP in the cardiovascular system, as well as in migraine pathophysiology.

AUTHOR CONTRIBUTIONS

FA wrote the first draft. Both FA and SDB finalised the manuscript. Both authors contributed to the article and approved the submitted version.

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REFERENCES

Al-Hassany, L., and Van Den Brink, A. M. (2020). Targeting CGRP in migraine: a matter of choice and dose. *Lancet Neurol.* 19, 712–713. doi: 10.1016/S1474-4422(20)30282-9

Amara, S. G., Arriza, J. L., Leff, S. E., Swanson, L. W., Evans, R. M., and Rosenfeld, M. G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science* 229, 1094–1097. doi: 10.1126/science.2994212

Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 298, 240–244. doi: 10.1038/298240a0

Argunhan, F., Thapa, D., Aubdool, A. A., Carlini, E., Arkless, K., Hendrikse, E. R., et al. (2021). Calcitonin gene-related peptide protects against cardiovascular dysfunction independently of nitric oxide In Vivo. *Hypertension* 77, 1178–1190. doi: 10.1161/HYPERTENSIONAHA.120.14851

Aubdool, A. A., Graepel, R., Kodji, X., Alawi, K. M., Bodkin, J. V., Srivastava, S., et al. (2014). TRPA1 is essential for the vascular response to environmental cold exposure. *Nat. Commun.* 5:5732. doi: 10.1038/ncomms6732

Aubdool, A. A., Thakore, P., Argunhan, F., Smillie, S. J., Schnelle, M., Srivastava, S., et al. (2017). A novel alpha-calcitonin gene-related peptide analogue protects against end-organ damage in experimental hypertension, cardiac hypertrophy, and heart failure. *Circulation* 136, 367–383. doi: 10.1161/circulationaha.117.028388

Bell, D., and McDermott, B. J. (1994). Calcitonin gene-related peptide stimulates a positive contractile response in rat ventricular cardiomyocytes. *J. Cardiovasc. Pharmacol.* 23, 1011–1021. doi: 10.1097/00005344-199406000-00021

Bell, D., Schluter, K. D., Zhou, X. J., Mcdermott, B. J., and Piper, H. M. (1995). Hypertrophic effects of calcitonin gene-related peptide (Cgrp) and amylin on adult mammalian ventricular cardiomyocytes. *J. Mol. Cell Cardiol.* 27, 2433–2443. doi: 10.1006/jmcc.1995.0231

Bentsen, S., Sams, A., Hasbak, P., Edvinsson, L., Kjaer, A., and Ripa, R. S. (2021). Myocardial perfusion recovery induced by an alpha-calcitonin gene-related peptide analogue. *J. Nucl. Cardiol.* (in press). doi: 10.1007/s12350-021-02678-8

Bigal, M. E., Walter, S., and Rapoport, A. M. (2015). Therapeutic antibodies against CGRP or its receptor. *Br. J. Clin. Pharmacol.* 79, 886–895.

Bistola, V., Arfaras-Melainis, A., Polyzogopoulou, E., Ikonomidis, I., and Parissis, J. (2019). Inotropes in acute heart failure: from guidelines to practical use: therapeutic options and clinical practice. *Card. Fail. Rev.* 5, 133–139. doi: 10.15420/cfr.2019.11.2

Brain, S. D., and Grant, A. D. (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol. Rev.* 84, 903–934. doi: 10.1152/physrev.00037.2003

Brain, S. D., Tippins, J. R., Morris, H. R., Macintyre, I., and Williams, T. J. (1986). Potent vasodilator activity of calcitonin gene-related peptide in human skin. *J. Invest. Dermatol.* 87, 533–536. doi: 10.1111/1523-1747.ep12455620

Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R., and Macintyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313, 54–56.

Chatterjee, S., Biondi-Zoccai, G., Abbate, A., D'ascenzo, F., Castagno, D., Van Tassell, B., et al. (2013). Benefits of beta blockers in patients with heart failure and reduced ejection fraction: network meta-analysis. *BMJ* 346:f55. doi: 10.1136/bmj.f55

Clark, A. J., Mullooly, N., Safitri, D., Harris, M., De Vries, T., Maassenvandenbrink, A., et al. (2021). CGRP, adrenomedullin and adrenomedullin 2 display endogenous Gpcr agonist bias in primary human cardiovascular cells. *Commun. Biol.* 4:776. doi: 10.1038/s42003-021-02293-w

Crossman, D. C., Dashwood, M. R., Brain, S. D., Mcewan, J., and Pearson, J. D. (1990). Action of calcitonin gene-related peptide upon bovine vascular endothelial and smooth muscle cells grown in isolation and co-culture. *Br. J. Pharmacol.* 99, 71–76. doi: 10.1111/j.1476-5381.1990.tb14656.x

Edvinsson, L., Haanes, K. A., Warfvinge, K., and Krause, D. N. (2018). CGRP as the target of new migraine therapies - successful translation from bench to clinic. *Nat. Rev. Neurol.* 14, 338–350. doi: 10.1038/s41582-018-0003-1

Fisher, L. A., Kikkawa, D. O., Rivier, J. E., Amara, S. G., Evans, R. M., Rosenfeld, M. G., et al. (1983). Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. *Nature* 305, 534–536. doi: 10.1038/305534a0

Franco-Cereceda, A. (1991). Calcitonin gene-related peptide and human epicardial coronary arteries: presence, release and vasodilator effects. *Br. J. Pharmacol.* 102, 506–510. doi: 10.1111/j.1476-5381.1991.tb12201.x

Franco-Cereceda, A., and Liska, J. (2000). Potential of calcitonin gene-related peptide in coronary heart disease. *Pharmacology* 60, 1–8. doi: 10.1159/000028339

Franco-Cereceda, A., Henke, H., Lundberg, J. M., Petermann, J. B., Hokfelt, T., and Fischer, J. A. (1987). Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: distribution and release by capsaicin. *Peptides* 8, 399–410. doi: 10.1016/0196-9781(87)90117-3

Fujioka, S., Sasakawa, O., Kishimoto, H., Tsumura, K., and Morii, H. (1991). The antihypertensive effect of calcitonin gene-related peptide in rats with norepinephrine- and angiotensin II-induced hypertension. *J. Hypertens.* 9, 175–179. doi: 10.1097/00004872-199102000-00013

Gangula, P. R., Supowit, S. C., Wimalawansa, S. J., Zhao, H., Hallman, D. M., Dipette, D. J., et al. (1997). Calcitonin gene-related peptide is a depressor in NG-nitro-L-arginine methyl ester-induced hypertension during pregnancy. *Hypertension* 29, 248–253. doi: 10.1161/01.hyp.29.1.248

Gangula, P. R., Zhao, H., Supowit, S. C., Wimalawansa, S. J., Dipette, D. J., Westlund, K. N., et al. (2000). Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. *Hypertension* 35, 470–475. doi: 10.1161/01.hyp.35.1.470

Gardiner, S. M., Compton, A. M., Kemp, P. A., Bennett, T., Foulkes, R., and Hughes, B. (1991). Haemodynamic effects of human alpha-calcitonin gene-related peptide following administration of endothelin-1 or NG-nitro-L-arginine methyl ester in conscious rats. *Br. J. Pharmacol.* 103, 1256–1262. doi: 10.1111/j.1476-5381.1991.tb12333.x

Gennari, C., and Fischer, J. A. (1985). Cardiovascular action of calcitonin gene-related peptide in humans. *Calcif. Tissue Int.* 37, 581–584. doi: 10.1007/BF02554909

Gennari, C., Nami, R., Agnusdei, D., and Fischer, J. A. (1990). Improved cardiac performance with human calcitonin gene related peptide in patients with congestive heart failure. *Cardiovasc. Res.* 24, 239–241. doi: 10.1093/cvr/24.3.239

Gibson, S. J., Polak, J. M., Bloom, S. R., Sabate, I. M., Mulderry, P. M., Ghatei, M. A., et al. (1984). Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J. Neurosci.* 4, 3101–3111. doi: 10.1523/JNEUROSCI.04-12-03101.1984

Girgis, S. I., Macdonald, D. W., Stevenson, J. C., Bevis, P. J., Lynch, C., Wimalawansa, S. J., et al. (1985). Calcitonin gene-related peptide: potent vasodilator and major product of calcitonin gene. *Lancet* 2, 14–16. doi: 10.1016/s0140-6736(85)90059-5

Gray, D. W., and Marshall, I. (1992a). Human alpha-calcitonin gene-related peptide stimulates adenylate cyclase and guanylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide. *Br. J. Pharmacol.* 107, 691–696. doi: 10.1111/j.1476-5381.1992.tb14508.x

Gray, D. W., and Marshall, I. (1992b). Nitric oxide synthesis inhibitors attenuate calcitonin gene-related peptide endothelium-dependent vasorelaxation in rat aorta. *Eur. J. Pharmacol.* 212, 37–42. doi: 10.1016/0014-2999(92)90069-g

Gulbenkian, S., Saetrum Opgaard, O., Ekman, R., Costa Andrade, N., Wharton, J., Polak, J. M., et al. (1993). Peptidergic innervation of human epicardial coronary arteries. *Circ. Res.* 73, 579–588. doi: 10.1161/01.res.73.3.579

Haegerstrand, A., Dalsgaard, C. J., Jonzon, B., Larsson, O., and Nilsson, J. (1990). Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3299–3303. doi: 10.1073/pnas.87.9.3299

Hay, D. L., Garelja, M. L., Poyner, D. R., and Walker, C. S. (2018). Update on the pharmacology of calcitonin/Cgrp family of peptides: iuphar review 25. *Br. J. Pharmacol.* 175, 3–17. doi: 10.1111/bph.14075

Huang, M. H., Knight, P. R. III, and Izzo, J. L. Jr. (1999). Ca2+-induced Ca2+ release involved in positive inotropic effect mediated by Cgrp in ventricular myocytes. *Am. J. Physiol.* 276, R259–R264. doi: 10.1152/ajpregu.1999.276.1.R259

Itabashi, A., Kashiwabara, H., Shibuya, M., Tanaka, K., Masaoka, H., Katayama, S., et al. (1988). The interaction of calcitonin gene-related peptide with angiotensin Ii on blood pressure and renin release. *J. Hypertens. Suppl.* 6, S418–S420. doi: 10.1097/00004872-198812040-00131

Josephs, T. M., Belousoff, M. J., Liang, Y. L., Piper, S. J., Cao, J., Garama, D. J., et al. (2021). Structure and dynamics of the Cgrp receptor in apo and peptide-bound forms. *Science* 372:eabf7258. doi: 10.1126/science.abf7258

Kallner, G., Gonon, A., and Franco-Cereceda, A. (1998). Calcitonin gene-related peptide in myocardial ischaemia and reperfusion in the pig. *Cardiovasc. Res.* 38, 493–499. doi: 10.1016/s0008-6363(98)00016-9

Katori, T., Hoover, D. B., Ardell, J. L., Helm, R. H., Belardi, D. F., Tocchetti, C. G., et al. (2005). Calcitonin gene-related peptide in vivo positive inotropy is attributable to regional sympatho-stimulation and is blunted in congestive heart failure. *Circ. Res.* 96, 234–243. doi: 10.1161/01.RES.0000152969.42117.ca

Kawasaki H. (2002). Regulation of vascular function by perivascular calcitonin gene-related peptide-containing nerves. *Jpn. J. Pharmacol.* 88, 39–43. doi: 10.1254/JP.88.39

Kawasaki, H., Okazaki, M., Nakatsuma, A., Mimaki, Y., Araki, H., and Gomita, Y. (1999). Long-term treatment with angiotensin converting enzyme inhibitor restores reduced calcitonin gene-related peptide-containing vasodilator nerve function in mesenteric artery of spontaneously hypertensive rats. *Jpn. J. Pharmacol.* 79, 221–229. doi: 10.1254/jjp.79.221

Kawasaki, H., Saito, A., and Takasaki, K. (1990). Age-related decrease of calcitonin gene-related peptide-containing vasodilator innervation in the mesenteric resistance vessel of the spontaneously hypertensive rat. *Circ. Res.* 67, 733–743. doi: 10.1161/01.res.67.3.733

Kawasaki, H., Takenaga, M., Araki, H., Futagami, K., and Gomita, Y. (1998). Angiotensin inhibits neurotransmission of calcitonin gene-related peptide-containing vasodilator nerves in mesenteric artery of spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.* 284, 508–515.

Kee, Z., Kodji, X., and Brain, S. D. (2018). The role of calcitonin gene related peptide (CGRP) in neurogenic vasodilation and its cardioprotective effects. *Front. Physiol.* 9:1249. doi: 10.3389/fphys.2018.01249

Kraenzlin, M. E., Ch'ng, J. L., Mulderry, P. K., Ghatei, M. A., and Bloom, S. R. (1985). Infusion of a novel peptide, calcitonin gene-related peptide (CGRP) in man. Pharmacokinetics and effects on gastric acid secretion and on gastrointestinal hormones. *Regul. Pept.* 10, 189–197. doi: 10.1016/0167-0115(85)90013-8

Kumar, A., Belhaj, M., Dipette, D. J., and Potts, J. D. (2020). A novel alginate-based delivery system for the prevention and treatment of pressure-overload induced heart failure. *Front. Pharmacol.* 11:602952. doi: 10.3389/fphar.2020.602952

Kumar, A., Potts, J. D., and Dipette, D. J. (2019a). Protective role of alpha-calcitonin gene-related peptide in cardiovascular diseases. *Front. Physiol.* 10:821. doi: 10.3389/fphys.2019.00821

Kumar, A., Supowit, S., Potts, J. D., and Dipette, D. J. (2019b). Alpha-calcitonin gene-related peptide prevents pressure-overload induced heart failure: role of apoptosis and oxidative stress. *Physiol. Rep.* 7:e14269. doi: 10.14814/phy2.14269

Li, J., and Wang, D. H. (2005). Development of angiotensin II-induced hypertension: role of CGRP and its receptor. *J. Hypertens.* 23, 113–118. doi: 10.1097/00004872-200501000-00020

Li, J., Levick, S. P., Dipette, D. J., Janicki, J. S., and Supowit, S. C. (2013). Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure. *Regul. Pept.* 185, 20–28. doi: 10.1016/j.regpep.2013.06.008

Li, J., Zhao, H., Supowit, S. C., Dipette, D. J., and Wang, D. H. (2004). Activation of the renin-angiotensin system in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. *J. Hypertens.* 22, 1345–1349. doi: 10.1097/01.hjh.0000125409.50839.f1

Li, Y., Fiscus, R. R., Wu, J., Yang, L., and Wang, X. (1997). The antiproliferative effects of calcitonin gene-related peptide in different passages of cultured vascular smooth muscle cells. *Neuropeptides* 31, 503–509. doi: 10.1016/s0143-4179(97)90046-9

Liang, Y. L., Khoshouei, M., Deganutti, G., Glukhova, A., Koole, C., Peat, T. S., et al. (2018). Cryo-EM structure of the active, Gs-protein complexed, human Cgrp receptor. *Nature* 561, 492–497. doi: 10.1038/s41586-018-0535-y

Liu, Y., Zhang, S., Xue, J., Wei, Z., Ao, P., Shen, B., et al. (2019). Cgrp reduces apoptosis of DRG cells induced by high-glucose oxidative stress injury through PI3K/AKT induction of heme oxygenase-1 and Nrf-2 expression. *Oxid. Med. Cell Longev.* 2019:2053149. doi: 10.1155/2019/2053149

Mai, T. H., Wu, J., Diedrich, A., Garland, E. M., and Robertson, D. (2014). Calcitonin gene-related peptide (CGRP) in autonomic cardiovascular regulation and vascular structure. *J. Am. Soc. Hypertens.* 8, 286–296. doi: 10.1016/j.jash.2014.03.001

McDonagh, T. A., Metra, M., Adamo, M., Gardner, R. S., Baumbach, A., Bohm, M., et al. (2021). 2021 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure. *Eur. Heart J.* 42, 3599–3726.

McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., et al. (1998). RAMPS regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393, 333–339. doi: 10.1038/30666

Moreira, L. M., Takawale, A., Hulsurkar, M., Menassa, D. A., Antanaviciute, A., Lahiri, S. K., et al. (2020). Paracrine signalling by cardiac calcitonin controls atrial fibrogenesis and arrhythmia. *Nature* 587, 460–465. doi: 10.1038/s41586-020-2890-8

Nelson, M. T., Huang, Y., Brayden, J. E., Hescheler, J., and Standen, N. B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K+ channels. *Nature* 344, 770–773. doi: 10.1038/344770a0

Nilsson, C., Hansen, T. K., Rosenquist, C., Hartmann, B., Kodra, J. T., Lau, J. F., et al. (2016). Long acting analogue of the calcitonin gene-related peptide induces positive metabolic effects and secretion of the glucagon-like peptide-1. *Eur. J. Pharmacol.* 773, 24–31. doi: 10.1016/j.ejphar.2016.01.003

Nishikimi, T., Horio, T., Yoshihara, F., Nagaya, N., Matsuo, H., and Kangawa, K. (1998). Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes. *Eur. J. Pharmacol.* 353, 337–344. doi: 10.1016/s0014-2999(98)00400-2

Oh-hashi, Y., Shindo, T., Kurihara, Y., Imai, T., Wang, Y., Morita, H., et al. (2001). Elevated sympathetic nervous activity in mice deficient in alphacgrp. *Circ. Res.* 89, 983–990. doi: 10.1161/hh2301.100812

Page, R. L. II, O'bryant, C. L., Cheng, D., Dow, T. J., Ky, B., Stein, C. M., et al. (2016). Drugs that may cause or exacerbate heart failure: a scientific statement from the american heart association. *Circulation* 134, e32–e69.

Pioszak, A. A., and Hay, D. L. (2020). RAMPs as allosteric modulators of the calcitonin and calcitonin-like class B G protein-coupled receptors. *Adv. Pharmacol.* 88, 115–141. doi: 10.1016/bs.apha.2020.01.001

Rubio-Beltran, E., and Maassen van den Brink, A. (2019). Understanding CGRP and cardiovascular risk. *Handb. Exp. Pharmacol.* 255, 131–140. doi: 10.1007/164_2019_204

Russell, F. A., King, R., Smillie, S. J., Kodji, X., and Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* 94, 1099–1142. doi: 10.1152/physrev.00034.2013

Sabharwal, R., Mason, B. N., Kuburas, A., Abboud, F. M., Russo, A. F., and Chapleau, M. W. (2019). Increased receptor activity-modifying protein 1 in the nervous system is sufficient to protect against autonomic dysregulation and hypertension. *J. Cereb. Blood Flow Metab.* 39, 690–703. doi: 10.1177/0271678X17751352

Sabharwal, R., Zhang, Z., Lu, Y., Abboud, F. M., Russo, A. F., and Chapleau, M. W. (2010). Receptor activity-modifying protein 1 increases baroreflex sensitivity and attenuates Angiotensin-induced hypertension. *Hypertension* 55, 627–635. doi: 10.1161/HYPERTENSIONAHA.109.148171

Saely, S., Croteau, D., Jawidzik, L., Brinker, A., and Kortepeter, C. (2021). Hypertension: A new safety risk for patients treated with erenumab. *Headache* 61, 202–208. doi: 10.1111/head.14051

Schavinski, A. Z., Machado, J., Morgan, H. J. N., Lautherbach, N., Paula-Gomes, S., Kettelhut, I. C., et al. (2021). Calcitonin gene-related peptide exerts inhibitory effects on autophagy in the heart of mice. *Peptides* 146:170677. doi: 10.1016/j.peptides.2021.170677

Shekhar, Y. C., Anand, I. S., Sarma, R., Ferrari, R., Wahi, P. L., and Poole-Wilson, P. A. (1991). Effects of prolonged infusion of human alpha calcitonin gene-related peptide on hemodynamics, renal blood flow and hormone levels in congestive heart failure. *Am. J. Cardiol.* 67, 732–736. doi: 10.1016/0002-9149(91)90531-o

Skaria, T., Mitchell, K. J., Vogel, O., Walchli, T., Gassmann, M., and Vogel, J. (2019). Blood pressure normalization-independent cardioprotective effects of endogenous, physical activity-induced alphaCGRP (alpha calcitonin gene-related peptide) in chronically hypertensive mice. *Circ. Res.* 125, 1124–1140. doi: 10.1161/CIRCRESAHA.119.315429

Smillie, S. J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., et al. (2014). An ongoing role of alpha-calcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. *Hypertension* 63, 1056–1062. doi: 10.1161/HYPERTENSIONAHA.113.02517

Steenbergh, P. H., Hoppener, J. W., Zandberg, J., Lips, C. J., and Jansz, H. S. (1985). A second human calcitonin/CGRP gene. *FEBS Lett.* 183, 403–407.

Struthers, A. D., Brown, M. J., Macdonald, D. W., Beacham, J. L., Stevenson, J. C., Morris, H. R., et al. (1986). Human calcitonin gene related peptide: a potent endogenous vasodilator in man. *Clin. Sci. (Lond)* 70, 389–393. doi: 10.1042/cs0700389

Sueur, S., Pesant, M., Rochette, L., and Connat, J. L. (2005). Antiapoptotic effect of calcitonin gene-related peptide on oxidative stress-induced injury in H9c2 cardiomyocytes via the Ramp1/Crlr complex. *J. Mol. Cell Cardiol.* 39, 955–963. doi: 10.1016/j.yjmcc.2005.09.008

Supowit, S. C., Rao, A., Bowers, M. C., Zhao, H., Fink, G., Steficek, B., et al. (2005). Calcitonin gene-related peptide protects against hypertension-induced heart and kidney damage. *Hypertension* 45, 109–114. doi: 10.1161/01.HYP.0000151130.34874.fa

Supowit, S. C., Zhao, H., Hallman, D. M., and Dipette, D. J. (1997). Calcitonin gene-related peptide is a depressor of deoxycorticosterone-salt hypertension in the rat. *Hypertension* 29, 945–950. doi: 10.1161/01.hyp.29.4.945

Supowit, S. C., Zhao, H., Wang, D. H., and Dipette, D. J. (1995). Regulation of neuronal calcitonin gene-related peptide expression. Role of increased blood pressure. *Hypertension* 26, 1177–1180. doi: 10.1161/01.hyp.26.6.1177

Surdo, N. C., Berrera, M., Koschinski, A., Brescia, M., Machado, M. R., Carr, C., et al. (2017). FRET biosensor uncovers camp nano-domains at beta-adrenergic targets that dictate precise tuning of cardiac contractility. *Nat. Commun.* 8:15031. doi: 10.1038/ncomms15031

Tepper, S. J. (2019). Cgrp and headache: a brief review. *Neurol. Sci.* 40, 99–105. doi: 10.1007/s10072-019-03769-8

Thapa, D., Valente, J. S., Barrett, B., Smith, M. J., Argunhan, F., Lee, S. Y., et al. (2021). Dysfunctional Trpm8 signalling in the vascular response to environmental cold in ageing. *eLife* 10:e70153. doi: 10.7554/eLife.70153

Triposkiadis, F., Karayannis, G., Giamouzis, G., Skoularigis, J., Louridas, G., and Butler, J. (2009). The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. *J. Am. Coll. Cardiol.* 54, 1747–1762. doi: 10.1016/j.jacc.2009.05.015

Tsujikawa, K., Yayama, K., Hayashi, T., Matsushita, H., Yamaguchi, T., Shigeno, T., et al. (2007). Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16702–16707. doi: 10.1073/pnas.0705974104

Walker, C. S., Conner, A. C., Poyner, D. R., and Hay, D. L. (2010). Regulation of signal transduction by calcitonin gene-related peptide receptors. *Trends Pharmacol. Sci.* 31, 476–483. doi: 10.1016/j.tips.2010.06.006

Weston, C., Winfield, I., Harris, M., Hodgson, R., Shah, A., Dowell, S. J., et al. (2016). Receptor activity-modifying protein-directed G protein signaling specificity for the calcitonin gene-related peptide family of receptors. *J. Biol. Chem.* 291, 21925–21944.

Zaccolo, M. (2009). cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *Br. J. Pharmacol.* 158, 50–60. doi: 10.1111/j.1476-5381.2009.00185.x

Zahner, M. R., Li, D. P., Chen, S. R., and Pan, H. L. (2003). Cardiac vanilloid receptor 1-expressing afferent nerves and their role in the cardiogenic sympathetic reflex in rats. *J. Physiol.* 551, 515–523. doi: 10.1113/jphysiol.2003.048207

Zoccarato, A., Surdo, N. C., Aronsen, J. M., Fields, L. A., Mancuso, L., Dodoni, G., et al. (2015). Cardiac hypertrophy is inhibited by a local pool of cAMP regulated by phosphodiesterase 2. *Circ. Res.* 117, 707–719. doi: 10.1161/CIRCRESAHA.114.305892

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Lipidated Calcitonin Gene-Related Peptide (CGRP) Peptide Antagonists Retain CGRP Receptor Activity and Attenuate CGRP Action *In Vivo*

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Signaling through calcitonin gene-related peptide (CGRP) receptors is associated with pain, migraine, and energy expenditure. Small molecule and monoclonal antibody CGRP receptor antagonists that block endogenous CGRP action are in clinical use as anti-migraine therapies. By comparison, the potential utility of peptide antagonists has received less attention due to suboptimal pharmacokinetic properties. Lipidation is an established strategy to increase peptide half-life *in vivo*. This study aimed to explore the feasibility of developing lipidated CGRP peptide antagonists that retain receptor antagonist activity *in vitro* and attenuate endogenous CGRP action *in vivo*. CGRP peptide analogues based on the archetypal CGRP receptor antagonist, $CGRP_{8-37}$, were palmitoylated at the N-terminus, position 24, and near the C-terminus at position 35. The antagonist activities of the lipidated peptide analogues were tested *in vitro* using transfected Cos-7 cells expressing either the human or mouse CGRP receptor, amylin subtype 1 (AMY_1) receptor, adrenomedullin (AM) receptors, or calcitonin receptor. Antagonist activities were also evaluated in SK-N-MC cells that endogenously express the human CGRP receptor. Lipidated peptides were then tested for their ability to antagonize endogenous CGRP action *in vivo* using a capsaicin-induced dermal vasodilation (CIDV) model in C57/BL6J mice. All lipidated peptides except for the C-terminally modified analogue retained potent antagonist activity compared to $CGRP_{8-37}$ towards the CGRP receptor. The lipidated peptides also retained, and sometimes gained, antagonist activities at AMY_1, AM_1 and AM_2 receptors. Several lipidated peptides produced robust inhibition of CIDV in mice. This study demonstrates that selected lipidated peptide antagonists based on $\alpha CGRP_{8-37}$ retain potent antagonist activity at the CGRP receptor and are capable of inhibition of endogenous CGRP action *in vivo*. These findings suggest that lipidation can be applied to peptide antagonists, such as $\alpha CGRP_{8-37}$ and are a potential strategy for antagonizing CGRP action.

Keywords: CGRP, lipidation, AMY_1, peptide, migraine, vasodilation, GPCR

INTRODUCTION

Calcitonin gene related peptide (CGRP) is a 37 amino acid neuropeptide peptide belonging to the calcitonin family of peptides comprising adrenomedullin (AM) 1 and 2, amylin and calcitonin (Hay et al., 2018). CGRP exists as αCGRP and βCGRP isoforms, differing by three amino acids in humans and two amino acids in rodents. Both peptides are expressed in the central and peripheral nervous systems, with βCGRP having a particular prominence in the enteric nervous system (Mulderry et al., 1988; Sternini, 1992).

The receptors that mediate the actions of the calcitonin peptide family are heterodimeric and comprise either the calcitonin receptor-like receptor (CLR) or calcitonin receptor (CTR) in complex with one of the three receptor activity-modifying proteins (RAMPs). The CGRP receptor (CLR: RAMP1) is considered the canonical receptor, and signals primarily through the adenylyl cyclase pathway (Bailey and Hay, 2006). Other combinations produce additional receptors such as AM_1 (CLR/RAMP2), AM_2 (CLR/RAMP3), AMY_1 (CTR/RAMP1), AMY_2 (CTR/RAMP2) and AMY_3 (CTR/RAMP3) receptors (Hay et al., 2018). CGRP also binds and activates the AMY_1 receptor with equal potency to the amylin peptide. By comparison, CGRP is significantly less potent at the AM_1 and AM_2 receptors and has very weak activity at the CTR (Hay et al., 2018; Garelja et al., 2020).

Of the two CGRP isoforms, attention has mostly focused historically on αCGRP and its role in diverse physiological processes such as vasodilation (Greenberg et al., 1987; Gray and Marshall, 1992), inflammation (Brain and Williams, 1985; Basbaum et al., 2009), cardiovascular conditioning (Liu et al., 2011; Mishima et al., 2011; Smillie et al., 2014), energy homeostasis (Walker et al., 2010; Bartelt et al., 2017; Liu et al., 2017) and sensory nerve functions (Walker et al., 2015; Eftekhari et al., 2016). αCGRP is a potent vasodilator, producing skin reddening *in vivo* evoked by intradermal administration of αCGRP (Brain and Williams, 1985). This vasodilatory effect is mediated through cAMP-dependent pathway signaling (Brain and Grant, 2004). Of particular clinical significance is CGRP's etiological role in migraine (Edvinsson, 2018). Systemic administration of αCGRP can provoke migraine-like attacks in migraineurs (Lassen et al., 2002; Hansen et al., 2010; Asghar et al., 2011; Guo et al., 2016).

A range of CGRP antagonist therapeutics comprising monoclonal antibodies, and small molecules are now approved clinically for the treatment of migraine. Currently, there are four approved monoclonal antibodies that block CGRP activity as preventative treatments for migraine. The first approved human monoclonal antibody, erenumab (AMG-334), targets the canonical CGRP receptor (Shi et al., 2016) with clinical efficacy (Sun et al., 2016; Goadsby et al., 2017). This was soon followed by approval of fremanezumab (LBR-101/TEV-48125) targeting CGRP itself (Bigal et al., 2015; Dodick et al., 2018). Two other monoclonal antibodies targeting the CGRP peptide, galcanezumab (LY2951742) and eptinezumab (ALD403) are also approved for preventative treatment of migraine. These antibody therapies are now complemented with the small molecule CGRP receptor antagonists, rimegepant (Croop et al., 2019), ubrogepant (Ailani et al., 2020) and atogepant (Schwedt et al., 2021) as approved acute treatments.

In addition to the development of antibodies and small molecules as CGRP antagonists, there may be opportunities to develop a new class of therapeutics with peptide-based antagonism. CGRP is modified post-translationally with a C-terminal amide and a cysteine-bridge between position 2 and 7 to confer a cyclic N-terminus. Truncation of the first seven amino acid residues of αCGRP yields $αCGRP_{8-37}$, the archetypal competitive peptide antagonist to the CGRP receptor (Chiba et al., 1989). Shorter peptide fragments have also been reported with $αCGRP_{27-37}$ being the shortest that retains antagonist activity at the CGRP receptor (Yan et al., 2011).

Peptide therapeutics that have similar properties to endogenous peptides are a particularly attractive drug class due to their safety profile (Muttenthaler et al., 2021). Nevertheless, there are intrinsic translational difficulties with CGRP peptide antagonists due to the short plasma half-life of CGRP and metabolic instability of $αCGRP_{8-37}$ (Kraenzlin et al., 1985; Miranda et al., 2008; Struthers et al., 1986; Srinivasan et al., 2022). In attempts to overcome these deficiencies, analogues based on $αCGRP_{8-37}$ and $αCGRP_{27-37}$ have been developed ranging from N-terminal modification (Taylor et al., 2006) through to systematic amino acid substitutions, utilization of unnatural amino acids, peptide cyclization (Srinivasan et al., 2022), chimeric CGRP species and PEGylation (Struthers et al., 1986; Miranda et al., 2008). However, despite some reported improvements in pharmacokinetic profile compared to $αCGRP_{8-37}$ (Miranda et al., 2013; Srinivasan et al., 2022), no CGRP peptide-based antagonist therapeutics have progressed to human clinical trials.

Peptide lipidation offers another attractive strategy of extending peptide half-life and has been used successfully in therapeutic development (Davies et al., 2015). In the present study we investigated whether it is possible to develop lipidated analogues based on $CGRP_{8-37}$ that retain antagonist activities at the CGRP receptor *in vitro* and also attenuate CGRP action *in vivo*. We report the characterization of several cysteine-substituted $CGRP_{8-37}$ analogues modified at various positions with a palmitoyl fatty acid sidechain. Our findings show that it is possible to lipidate $αCGRP_{8-37}$ and retain antagonist activity at CGRP and AMY_1 receptors but also increase potency in some cases. We also demonstrate successful antagonism of CGRP action *in vivo* by lipidated $αCGRP_{8-37}$ analogues using a dermal vasodilatory model, suggesting lipidation of peptide antagonists could be a potential strategy to antagonize CGRP action.

MATERIALS AND METHODS

Commercial Peptides and Antagonists

The following peptides were purchased commercially: human (h) and mouse (m) αCGRP, hAM and hAM_{22-52} (American Peptide, Sunnyvale, CA, United States, or Bachem, Bubendorf, Switzerland); calcitonin and salmon (s) $calcitonin_{8-32}$ (sCT_{8-32})

(American Peptide); $\alpha CGRP_{8-37}$ (American Peptide). Commercial $\alpha CGRP_{8-37}$ was used as a control to validate in-house synthesized $\alpha CGRP_{8-37}$. Olcegepant was purchased from AbaChemScene (NJ, United States).

In House-Peptide Synthesis

$h\alpha CGRP_{8-37}$ together with cysteine-substituted analogues were synthesized with an amidated C-terminus using Fmoc solid-phase peptide synthesis (SPPS). Lipidation of peptides was synthesized by Solid-Phase Cysteine Lipidation of Peptides or Amino acids (SP-CLipPA) (Williams et al., 2018) or through the building block method (Lu et al., 2020). $h\beta CGRP_{8-37}$, $h\alpha CGRP_{8-37}$ R11C-palmitate (R11C-palmitate), and $h\beta CGRP_{8-37}$ V8C-palmitate (βV8C-palmitate) synthesis information can also be found in the **Supplementary Data**. All peptides were purified by RP-HPLC to ≥90% purity before lyophilization. For *in vitro* studies, non-lipidated $hCGRP_{8-37}$ analogues and small molecule compounds were reconstituted as stock solutions in water or dimethyl sulfoxide (DMSO). Lipidated $hCGRP_{8-37}$ analogues were reconstituted as stock solutions in 100% DMSO.

Cell Culture and Transfection

Cos-7 cells and SK-N-MC cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 7.5% heat-inactivated fetal bovine serum. Cells were grown in a humidified incubator at 37°C and 5% CO_2 and seeded into 96-well plates at a density of 20,000 cells/well. The following day, Cos-7 cells were transiently transfected using Polyethylenimine (PEI) using a 1:1 ratio of receptor:RAMP DNA. HA-tagged hCLR (Hay et al., 2003) or HA-tagged hCTR ($CT_{(a)}$ splice variant, Udawela et al., 2006) in combination with either myc-tagged hRAMP1 (Qi et al., 2008), FLAG-tagged hRAMP2 (Qi et al., 2013), or untagged hRAMP3 (Hay et al., 2003) were transfected to express the desired calcitonin-family receptor. Untagged $mCT_{(a)}$, mCLR, mRAMP1, mRAMP2 and mRAMP3 were purchased from Origene and transfected in an identical method to the human receptors into Cos-7 cells (Garelja et al., 2021).

cAMP Assay Measurement

Transfected Cos-7 cells were incubated with agonist in the presence or absence of antagonist at 37°C for 15 min. cAMP production was terminated by aspirating all the media in the wells, followed by the addition of 50 µl of ice-cold ethanol. Cell lysates were then prepared for cAMP measurements using LANCE cAMP assay kit (Perkin Elmer, Waltham, MA, United States), as previously described (Woolley et al., 2017).

In one modification of the experimental design, cAMP content was investigated under conditions where the antagonist was added but then removed prior to agonist stimulation by αCGRP. Here, media in the 96-well plate was replaced with 50 µl serum-free DMEM containing 1 mM IBMX and 0.1% w/v bovine serum albumin (BSA) for 30 min at 37°C. After this period, 25 µl of the selected antagonist or media was added and pre-incubated with the transfected Cos-7 cells for 15 min at 37°C. The pre-incubated mixture was then removed by aspirating media from the selected wells, which were then washed once with 50 µl of phosphate-buffered saline and replaced with 75 µl of new serum-free DMEM containing 1 mM IBMX and 0.1% w/v BSA. Finally, 25 µl of the αCGRP agonist was added to each well, to a maximum volume of 100 µl and incubated for a further 15 min at 37°C. The agonist profiles were then compared to the condition-matched antagonist profiles.

Animal Welfare and Ethical Statement

All studies involving animals were approved by the University of Auckland Animal Ethics Committee and conducted in accordance with the New Zealand animal welfare act (1999). Prior to the experiments, mice were housed in environmentally enriched cages under climatically controlled conditions and kept in a 12-h day/night cycle. Mice had ad libitum access to standard chow (Teklad TB 2018; Harlan, Madison, WI, United States) and water.

Capsaicin-Induced Dermal Vasodilation - Laser Doppler Imaging Overview

Experimental design is outlined in **Figure 1**. Male and female C57BL/6J mice were recruited at 10–12 weeks of age at 20–30 g bodyweight. Animals were randomly allocated to each treatment group. Antagonists were diluted from respective stock solutions into 37°C pre-warmed sterile 0.9% saline containing 0.1% BSA and DMSO at a final amount of 3.2%. The vehicle was 0.9% saline supplemented with 0.1% BSA and 3.2% DMSO. Antagonist or vehicle was administered subcutaneously at a volume of 10 ml/kg. The anesthetic was: ketamine at 10 mg/ml and xylazine at 1 mg/ml, dissolved in sterile 0.9% saline. This was administered *via* the intraperitoneal route at 10 ml/kg and the anesthetized mouse placed onto a heating pad to maintain constant body temperature. The head was positioned for the dorsal region of both ears to be aligned to the LDI2-HIR Laser Doppler Imager (Moor Instruments) above it. The imager scanned 40 cm from the ear at a scan speed of 4 ms/pixel, with a scan area of approximately 11.0 cm × 4.1 cm and 256 × 45 pixels resolution. This provided a scan rate of approximately 1 scan/min for both ears. Both ears were simultaneously scanned for 3 min to generate the baseline blood flow prior to capsaicin-challenge (**Figure 1**). Guided by earlier research (Grant et al., 2002), capsaicin (Sigma-Aldrich, St. Louis, MO, United States) dissolved in absolute ethanol was applied topically to the ear (60 µg/ear; 10 µl on both dorsal and ventral side). On the contralateral ear, ethanol was applied as a negative control. Both ears were then immediately scanned with the Laser Doppler imager for a continuous 15-min period to capture changes in blood flow (**Figure 1**). The 15-min duration measurement period was chosen based on pilot trials and the literature (Grant et al., 2002), which indicated that a maximal and sustained increase in blood flow was achieved by this timepoint.

Capsaicin-Induced Dermal Vasodilation – Antagonist Screening and Dose-Ranging Study

Mice were administered subcutaneously with vehicle or antagonist (**Figure 1A**). Ten minutes later mice were

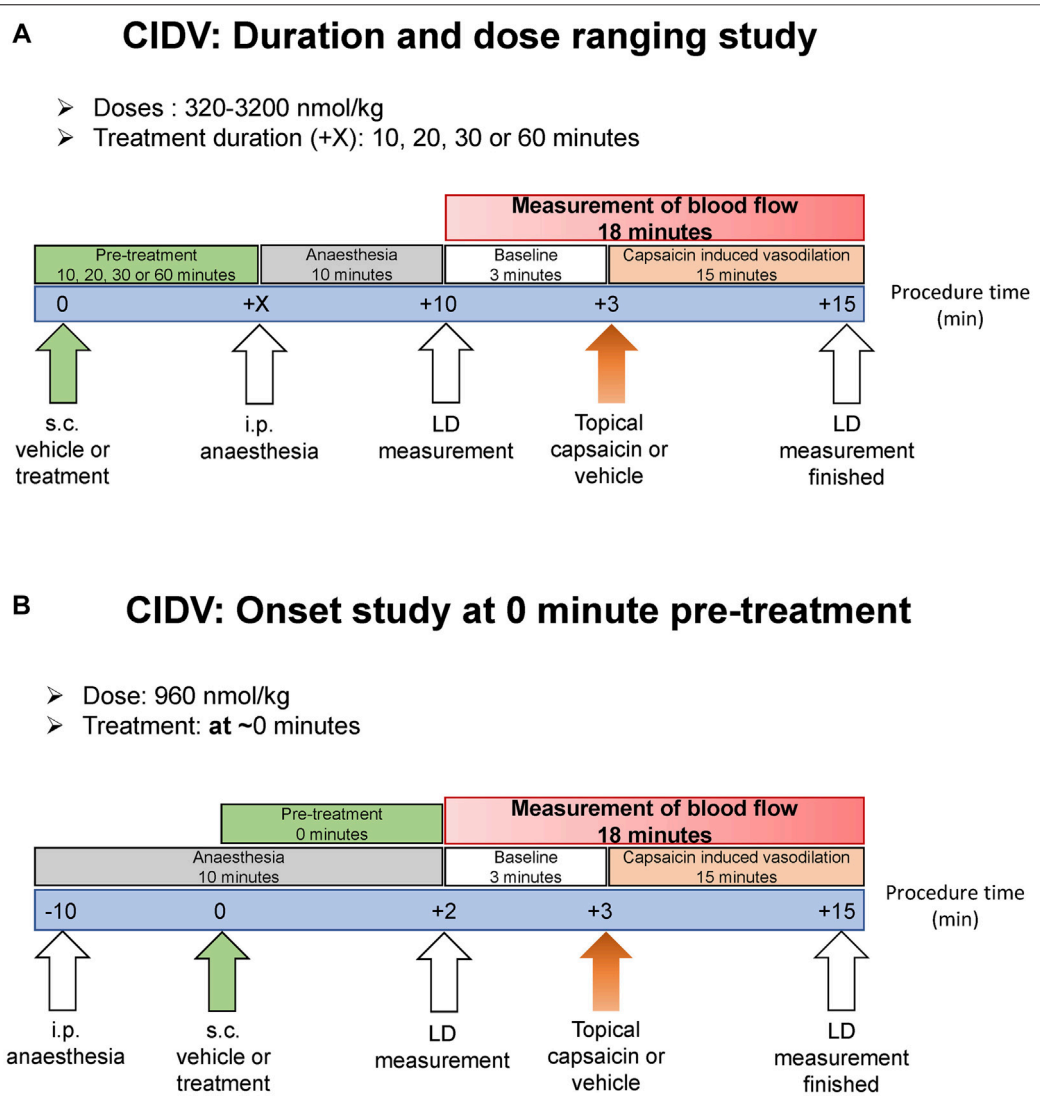

FIGURE 1 | Schematic showing the laser doppler experimental protocol to measure capsaicin-induced dermal vasodilation (CIDV) and antagonist effect of peptide and small molecule antagonists. **(A)** Antagonist screening, dose ranging and dose-duration studies. **(B)** Onset study at 0 min.

anesthetized and placed on a heating pad. Ten minutes after anesthesia induction, measurement of dermal blood-flow commenced. Either a single dose of antagonist as specified in the figures was used or a dose-ranging study was conducted. For the dose-ranging study, either hαCGRP$_{8-37}$ or hαCGRP$_{8-37}$ V8C-palmitate (V8C-palmitate) at a dose of 320, 960 or 3,200 nmol/kg was used. Doses were selected based on existing literature and our own pilot trials to characterize optimal (and maximal/minimal) effective dose within the bounds of solubility limits (Saxen et al., 1994; Grant et al., 2002; Gohin et al., 2015; Aubdool et al., 2016).

Capsaicin-Induced Dermal Vasodilation - Dose-Duration Study

Mice were administered subcutaneously with vehicle or either hαCGRP$_{8-37}$ (960 nmol/kg) or V8C-palmitate (960 nmol/kg) at 10, 20, 30 or 60 min prior to anesthesia and baseline read (**Figure 1A**). Ten minutes after anesthesia induction, measurement of dermal blood-flow began. To study the time of onset of antagonist activity, a shorter period between treatment administration and capsaicin challenge was utilized (T_0) (**Figure 1B**). Here, mice were anesthetized and placed on the heating pad prior to peptide administration. Immediately after peptide administration, measurement of dermal blood-flow began.

Data Analysis

Data were analyzed using GraphPad Prism versions 7–9 (GraphPad Software Inc., San Diego, CA, Unite States). Concentration-response cAMP data were fitted *via* non-linear regression using a four-parameter logistic equation. An extra sum-of-squares F-test was conducted to determine whether the

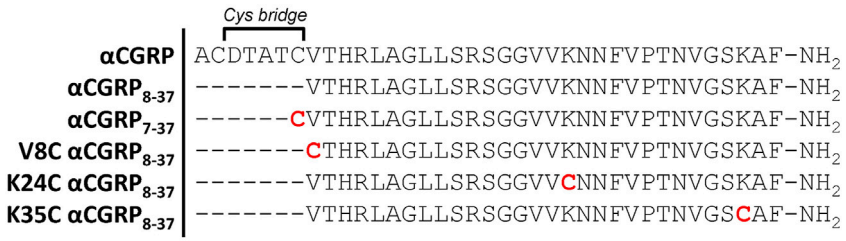

FIGURE 2 | Amino acid sequences of human $\alpha CGRP_{8-37}$ and truncated cysteine-substituted peptide analogues. Red cysteine residues were subsequently modified by palmitoylation to yield the corresponding lipidated $\alpha CGRP_{8-37}$ analogues. $\alpha CGRP_{7-37}$ retains the native cysteine amino acid at position 7.

Hill slope was equal to one. Where it was not significantly different to one, the data were fitted using a three-parameter logistic equation instead. An extra sum-of-squares F-test was also conducted to determine if two curves fitted onto two distinct datasets were significantly different from a single curve fit applied to both datasets to confirm if curve shifts are legitimate. The maximal (E_{max}) and minimal (E_{min}) responses were not constrained between independent experiments to obtain the pEC_{50} value.

For single concentrations of antagonist or global Schild analyses, the data were fitted to a concentration-response curve *via* non-linear regression using the Gaddum/Schild EC_{50} shift equation (Arunlakshana and Schild, 1958). The Hill slope was constrained to one following agonist analyses. The Schild slope was also constrained to one. The pA_2 and pK_B values were obtained from the Schild analysis. For washout experiments, the same method of analysis was applied to the antagonists, but with minor revisions. The Schild analysis relied on the matched control agonist curves i.e. those derived from the same experimental conditions as the agonist + antagonist curves. Normalization was also specific to the matched control agonist curves.

Independent experiments were converted into a combined concentration-response graph by normalizing to control agonist E_{max} as 100% and control agonist E_{min} as 0%. Mean pEC_{50}, pK_B and pA_2 values from at least three independent experiments before normalization were also presented as mean ± SEM. pEC_{50}, pK_B and pA_2 values from independent experiments were grouped and compared by unpaired Student's t-test or by one-way ANOVA. Alternatively, Student's t-test comparisons between washout and no washout pA_2 values of a particular antagonist were paired instead of unpaired, as the two conditions were always tested side-by-side.

For laser doppler imaging (LDI) scans, mean flux values of the whole scanned ear region for both ethanol (control) and capsaicin-treated ears at each time point (per minute) were analyzed using the MoorLDI Review 6.1 software. Mean flux values were normalized to the mean flux values averaged from the three continuous baseline scans and a time course is generated. Area under the curve (AUC) of the % flux mean after capsaicin application ($t \geq 0$) was measured for each animal, grouped and compared between different ears or treatment arms. For statistical analysis and comparisons of treatment or sex, AUC values from different mice were grouped and compared using an unpaired Student's t-test or by one-way ANOVA following a Shapiro-Wilk normality test. Time courses and time points were compared using repeated measures two-way ANOVA with Bonferonni's multiple comparisons test.

RESULTS

We first utilized the human $\alpha CGRP_{8-37}$ peptide backbone as a template to develop palmitoylated derivatives (**Figure 2**). CGRP possesses two native cysteine residues at amino acid positions 2 and 7, which are absent in $\alpha CGRP_{8-37}$. The synthesis route first required the synthesis of cysteine-substituted peptides (**Figure 2**) in order to provide a free thiol group for attachment of the palmitoyl moiety (Williams et al., 2018). We selected three positions on the $\alpha CGRP_{8-37}$ peptide backbone, Val-8, Lys-24, and Lys-35, for cysteine substitution sites and subsequent palmitoylation based on prior data that they could support modification without sacrificing binding affinity (Rist et al., 1999; Watkins et al., 2013; Booe et al., 2015; Liang et al., 2018). In addition, we selected the truncated peptide antagonist, $\alpha CGRP_{7-37}$, which retains the native Cys-7 residue for palmitoylation (**Figure 2**).

$\alpha CGRP_{7-37}$ and Cysteine-Substituted $\alpha CGRP_{8-37}$ Analogues Retain Antagonist Activities at Human CGRP and AMY_1 Receptors

$\alpha CGRP_{8-37}$ is the most characterized peptide antagonist at CGRP-responsive receptors (Hay et al., 2018). We therefore first validated $h\alpha CGRP_{8-37}$ as a reference antagonist with our human assay systems (**Supplementary Table S1**). Agonist stimulation by αCGRP in the presence or absence of $\alpha CGRP_{8-37}$ was performed in Cos-7 cells transfected with either CGRP or AMY_1 receptors. As expected, $\alpha CGRP_{8-37}$ caused a rightward shift in the respective concentration-response curves with no discernible effects on maximal responses, allowing for pA_2 values to be measured. The pEC_{50} and pA_2 values derived for αCGRP agonism and $h\alpha CGRP_{8-37}$ antagonist activity (**Supplementary Table S1**) are consistent with literature values (Bailey and Hay, 2006; Hay et al., 2018). We then investigated antagonist activities

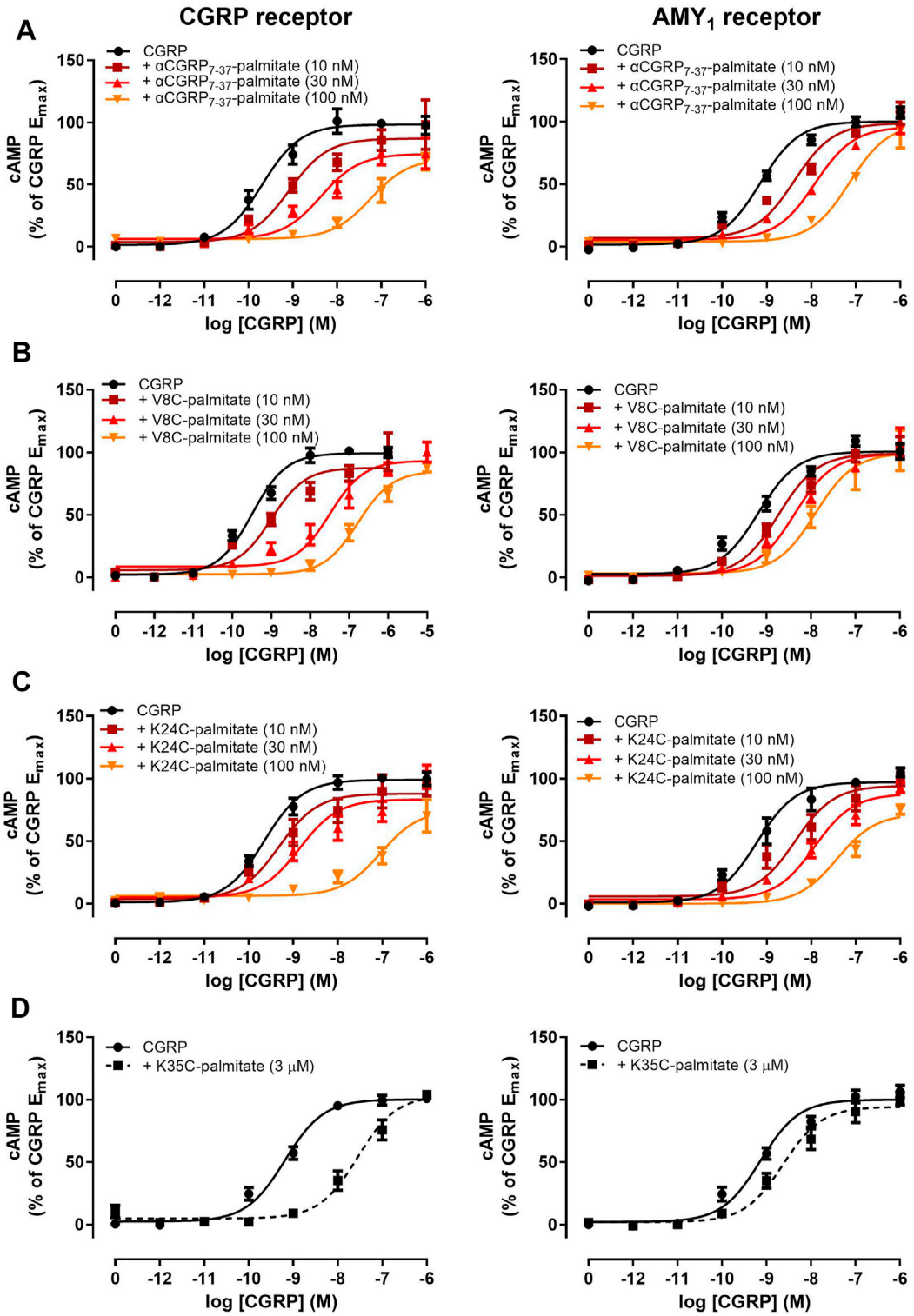

FIGURE 3 | Antagonism of CGRP-stimulated cAMP production by lipidated $\alpha CGRP_{8-37}$ analogues at human CGRP or AMY_1 receptors expressed in Cos-7 cells. Concentration-response curves were generated in the absence or presence of **(A)** $\alpha CGRP_{7-37}$-palmitate, **(B)** V8C-palmitate, **(C)** K24C-palmitate, and **(D)** K35C-palmitate at one or multiple different concentrations. Data points are plotted as a percentage of maximal CGRP-stimulated cAMP production as mean ± SEM of four to five independent experiments.

TABLE 1 | Antagonist activities of lipidated αCGRP$_{8-37}$ analogues at calcitonin-family receptors expressed in Cos-7 cells or SK-N-MC cells with endogenous CGRP receptor expression.

	αCGRP$_{8-37}$	αCGRP$_{7-37}$-palmitate	V8C-palmitate	K24C-palmitate	K35C-palmitate
CGRPr (pK_B)	9.09 ± 0.16 (5)	8.78 ± 0.06 (4)	9.35 ± 0.17 (5)	8.58 ± 0.25 (5)	7.24 ± 0.20 (5)[a]
AMY_1r (pK_B)	7.12 ± 0.13 (5)	8.80 ± 0.18 (5)[a]	8.26 ± 0.21 (5)[a]	8.75 ± 0.10 (5)[a]	6.01 ± 0.12 (5)[a]
AM_1r (pA_2)	7.36 ± 0.11 (9)	8.53 ± 0.17 (5)[a]	8.73 ± 0.17 (5)[a]	9.23 ± 0.16 (5)[a]	6.30 ± 0.14 (5)[a]
AM_2r (pA_2)	7.32 ± 0.20 (9)	8.55 ± 0.16 (5)[a]	8.18 ± 0.13 (5)[b]	9.18 ± 0.18 (5)[a]	6.60 ± 0.21 (5)[b]
CTr (pA_2)	—[c]	7.11 ± 0.10 (5)	6.85 ± 0.13 (5)	6.21 ± 0.19 (5)	5.39 ± 0.30 (3)[d]
SK-N-MC (pA_2)	9.58 ± 0.15 (6)	10.50 ± 0.19 (5)[e]	10.45 ± 0.24 (5)[e]	10.14 ± 0.17 (5)	7.78 ± 0.25 (5)[a]

Values are mean ± SEM of (n) independent experiments. pK_B or pA_2 comparisons to αCGRP$_{8-37}$ were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test.
[a]p < 0.001.
[b]p < 0.05
[c]αCGRP$_{8-37}$ was too weak to generate a pA2 in the pilot experiments and sCT$_{8-32}$ was used as a positive control for the CT receptor.
[d]Denotes that five experiments were conducted but only (n) repeats elicited measurable pA_2 values.
[e]p < 0.01

of each cysteine-substituted analogue together with αCGRP$_{7-37}$ at CGRP and AMY_1 receptors. Each antagonist at a 30 nM concentration evoked a rightward shift in the respective agonist concentration-response curve, again with no significant change in maximal response at either CGRP (**Supplementary Figure S1**) and AMY_1 receptor (**Supplementary Figure S2**). Compared to αCGRP$_{8-37}$, K24C αCGRP$_{8-37}$ and K35C αCGRP$_{8-37}$ showed similar antagonist activities while αCGRP$_{7-37}$ and V8C αCGRP$_{8-37}$ were 4.5-fold and 25-fold less potent, respectively, at the CGRP receptor (**Supplementary Table S1**). At the AMY_1 receptor, only V8C αCGRP$_{8-37}$ displayed reduced antagonist activity compared to αCGRP$_{8-37}$ (**Supplementary Table S1**).

Palmitoylation at the N-Terminus or at Position 24 but not at the C-Terminal Region (Position 35) Retains Antagonist Activities Comparable to αCGRP$_{8-37}$ at CGRP and AMY_1 Receptors

We next proceeded to investigate the antagonist activities of the respective palmitoylated peptides (**Figure 3**). Pilot experiments with a single 30 nM concentration of each lipidated hαCGRP$_{8-37}$ analogue revealed apparent differences in antagonist activities and effects on agonist maximal response (data not shown). Therefore, full Schild analyses were conducted. These experiments confirmed that hαCGRP$_{8-37}$ as the reference antagonist displayed competitive antagonism at both human CGRP and AMY_1 receptors (**Figure 3**). Similarly, all lipidated hαCGRP$_{8-37}$ analogues showed competitive antagonist behavior (**Figure 3**) with derived pK_B values shown in **Table 1**. Attachment of the palmitoyl moiety at the N-terminus (hαCGRP$_{7-37}$-palmitate and hαCGRP$_{8-37}$ V8C-palmitate; V8C-palmitate) or at position 24 (hαCGRP$_{8-37}$ K24C-palmitate; K24C-palmitate) of hαCGRP$_{8-37}$ had little effect on antagonist activity as compared to hαCGRP$_{8-37}$ at the CGRP receptor (**Figures 3A–C** and **Table 1**). At the AMY_1 receptor, these lipidated peptides displayed significantly stronger antagonist activity than hαCGRP$_{8-37}$ (**Figures 3A–C** and **Table 1**). Interestingly, attachment of the palmitoyl moiety near the C-terminus of hαCGRP$_{8-37}$ (hαCGRP$_{8-37}$ K35C-palmitate; K35C-palmitate) substantially decreased antagonist activity as compared to hαCGRP$_{8-37}$ at CGRP (~70-fold reduction) and AMY_1 receptors (~12-fold reduction) (**Figure 3D** and **Table 1**).

Lipidated αCGRP$_{8-37}$ Analogues Display Enhanced Antagonist Activities at the Human CGRP Receptor Endogenously Expressed in SK-N-MC Cells

We measured antagonist activities in human SK-N-MC cells which endogenously express the CGRP receptor (Choksi et al., 2002). The αCGRP agonist pEC_{50} in SK-N-MC cells was 9.26 ± 0.05 (n = 13). The derived pA_2 value for hαCGRP$_{8-37}$ antagonist activity in SK-N-MC cells was similar to the pK_B value calculated in Cos-7 cells expressing the CGRP receptor (**Table 1**). Similar to the findings with transient CGRP receptor expression, all lipidated hαCGRP$_{8-37}$ analogues displayed measurable competitive antagonist activity at a single concentration with no observable decrease in maximal response (**Figure 4**). Interestingly, compared to hαCGRP$_{8-37}$, the derived pA_2 values for αCGRP$_{7-37}$-palmitate and V8C-palmitate were significantly higher than hαCGRP$_{8-37}$ demonstrating increased antagonist activity (**Table 1**). Consistent with data from transient CGRP receptor expression, the derived pA_2 for K35C-palmitate was significantly lower than hαCGRP$_{8-37}$ (**Table 1**).

Lipidated αCGRP$_{8-37}$ Analogues but not αCGRP$_{8-37}$ Exhibit Behavior Consistent With Delayed Dissociation at CGRP and AMY_1 Receptors

As addition of a lipid moiety could affect membrane or receptor residence time (Ray et al., 2017; Fletcher et al., 2021) we investigated whether pre-incubation and wash-out of either hαCGRP$_{8-37}$ or lipidated peptides with either CGRP and AMY_1 receptors transfected in Cos-7 cells affected the observed pharmacology. For these experiments, the peptide antagonist was pre-incubated with cells for 15 min prior to agonist stimulation by αCGRP.

Interestingly, we identified altered pharmacological behaviors unique to αCGRP$_{7-37}$-palmitate, V8C-palmitate, and K24C-

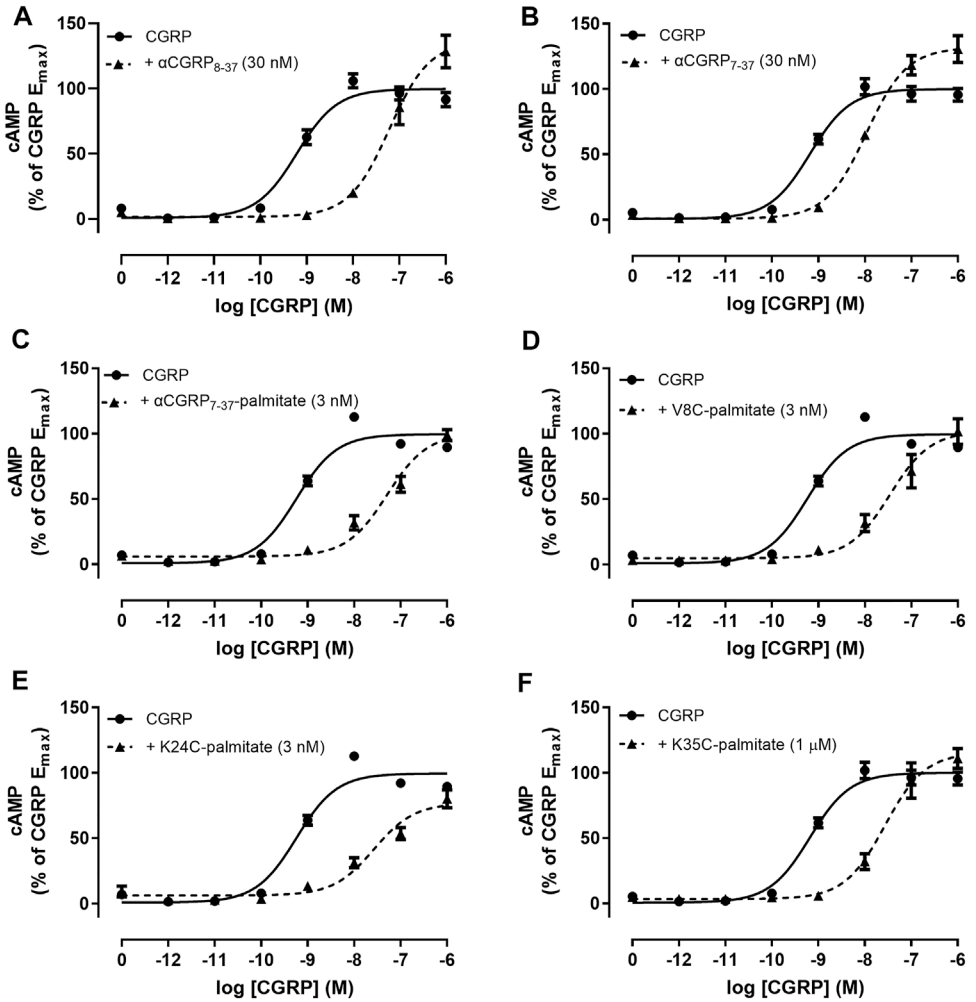

FIGURE 4 | Antagonism by $\alpha CGRP_{8-37}$, $\alpha CGRP_{7-37}$, and lipidated analogues in SK-N-MC cells. CGRP concentration-response curves were generated in the absence or presence of **(A)** $\alpha CGRP_{8-37}$, **(B)** $\alpha CGRP_{7-37}$, **(C)** $\alpha CGRP_{7-37}$-palmitate, **(D)** V8C-palmitate, **(E)** K24C-palmitate, or **(F)** K35C-palmitate. Data points are plotted as a percentage of maximal CGRP-stimulated cAMP production with mean ± SEM of five to six independent experiments.

palmitate but not for K35C-palmitate or $h\alpha CGRP_{8-37}$ (**Figure 5**). Consistent with previous experiments, $h\alpha CGRP_{8-37}$ displayed similar antagonist activity under these experimental conditions (**Figure 5A**, solid red versus black lines; **Table 2**). However, the addition of a 15 min pre-incubation step for either $\alpha CGRP_{7-37}$-palmitate (**Figure 5B**), V8C-palmitate (**Figure 5C**), or K24C-palmitate (**Figure 5D**) resulted in increased antagonist activity at CGRP and AMY_1 receptors compared to previously measured pK_B values (**Table 2** versus **Table 1**).

Most notably, with the exception of K35C-palmitate, significant antagonist activity remained when a washout step was incorporated prior to agonist stimulation (**Figures 5A–E**, dashed red versus black lines). For comparison, the corresponding control agonist in each case also incorporated a wash out step comprising agonist stimulation performed in the absence of antagonist (dashed black line). Despite discernible and persistent antagonism, there was a reduction in pA_2 values for V8C-palmitate and K24C-palmitate at both CGRP and AMY_1 receptors. $\alpha CGRP_{7-37}$-palmitate only had reduction at the CGRP receptor (**Table 2**). By comparison, no retention of antagonist activity occurred for $h\alpha CGRP_{8-37}$ when a washout step was included at CGRP and AMY_1 receptors (**Figure 4A**). These findings suggest that the palmitoyl moiety may delay peptide dissociation from the receptor or membrane.

Lipidated $\alpha CGRP_{8-37}$ Analogues Display Antagonist Activities at AM_1, AM_2, and CT Receptors

Given the pharmacological overlap between the calcitonin family receptors (Hay et al., 2018), we also investigated antagonism by lipidated $\alpha CGRP_{8-37}$ analogues at AM_1, AM_2, and CT receptors. Receptor identities were confirmed pharmacologically with control antagonists (**Table 1** and **Supplementary Figure S3**).

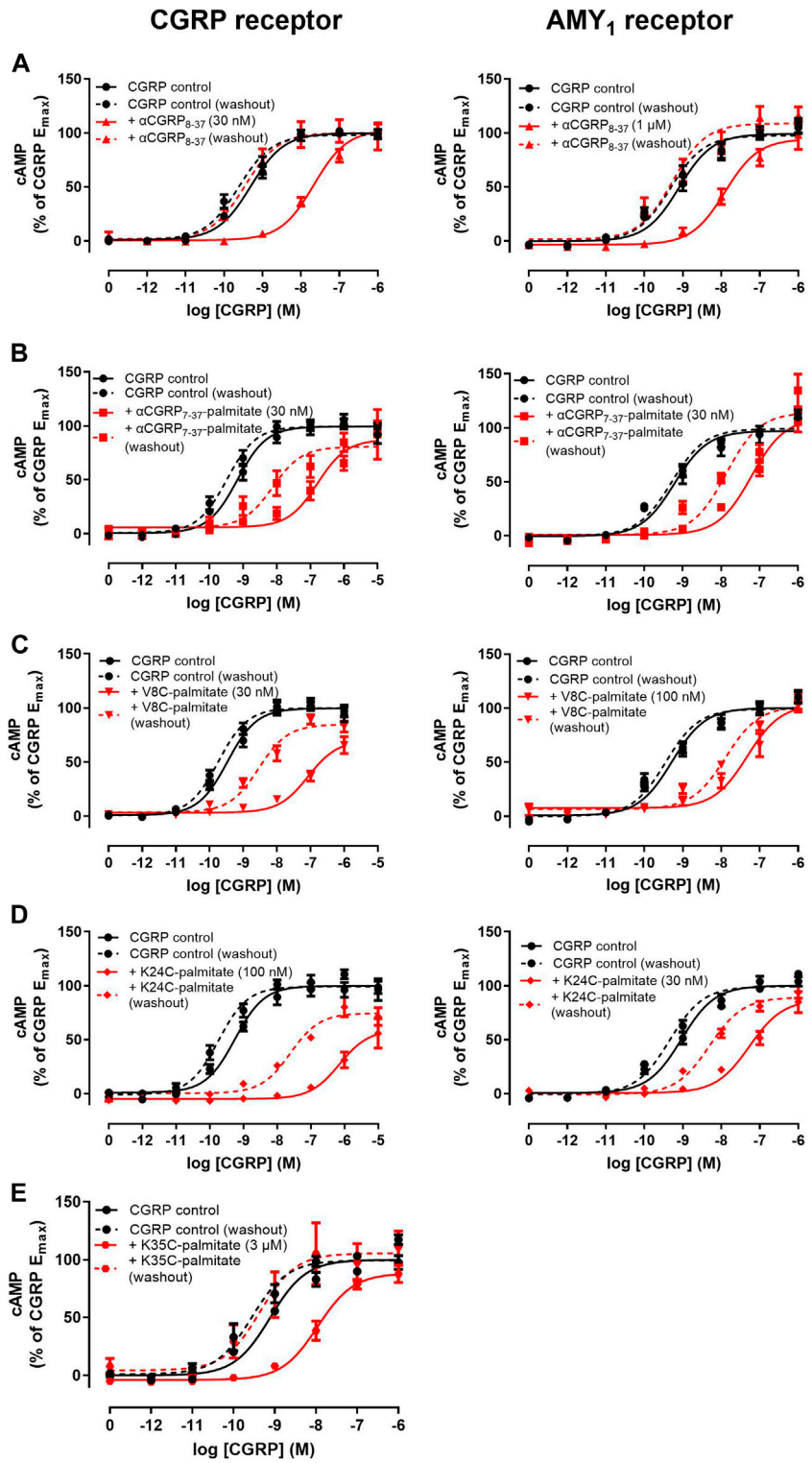

FIGURE 5 | Effect of antagonist with pre-incubation and a washout step on agonist concentration-response curves at human CGRP and AMY_1 receptors expressed in Cos-7 cells. **(A)** $\alpha CGRP_{8-37}$, **(B)** $\alpha CGRP_{7-37}$-palmitate, **(C)** V8C-palmitate, **(D)** K24C-palmitate, **(E)** K35C-palmitate. Solid and dashed lines indicate agonist concentration-response curves performed in the absence or presence of a washout step prior to agonist stimulation. Data points are plotted as a percentage of maximal CGRP-stimulated cAMP production and show mean ± SEM of five independent experiments.

TABLE 2 | Effect of antagonist with pre-incubation and a washout step on pA_2 values for lipidated $\alpha CGRP_{8-37}$ analogues at hCGRP and $hAMY_1$ receptors expressed in Cos-7 cells.

		$\alpha CGRP_{8-37}$	$\alpha CGRP_{7-37}$-palmitate	V8C-palmitate	K24C-palmitate	K35C-palmitate
CGRPr	No washout	9.21 ± 0.21	9.86 ± 0.14	9.85 ± 0.08	10.28 ± 0.12	6.74 ± 0.16
	Washout	<7.5	9.34 ± 0.28[a]	8.67 ± 0.20[b]	9.13 ± 0.14[c]	<5.5[d]
AMY_1r	No washout	7.23 ± 0.10	9.39 ± 0.22	8.98 ± 0.25	9.43 ± 0.20	—
	Washout	<6	8.69 ± 0.27	8.54 ± 0.31[a]	8.45 ± 0.23[a]	—

Values are mean ± SEM of five independent experiments. Comparisons between no washout and washout conditions were analyzed by paired Student's t-test.
[a]$p < 0.05$.
[b]$p < 0.001$.
[c]$p < 0.01$.
[d]*Denotes that only two out of five experiments elicited measurable pA_2 values, averaging 5.60 ± 0.04.*

TABLE 3 | pA_2 values for $\alpha CGRP_{8-37}$ and lipidated $\alpha CGRP_{8-37}$ analogues at mCGRP and AMY_1 receptors expressed in Cos-7 cells.

	$\alpha CGRP_{8-37}$	$\alpha CGRP_{7-37}$-palmitate	V8C-palmitate	K24C-palmitate	K35C-palmitate
mCGRPr	9.00 ± 0.40 (5)	9.89 ± 0.35 (5)	9.70 ± 0.40 (4)[a]	9.03 ± 1.00 (3)[a]	7.56 ± 0.18 (4)[a]
$mAMY_1r$	7.61 ± 0.93 (3)[a]	7.72 ± 0.28 (4)[a]	7.61 ± 0.20 (5)	<6 (3)[b]	6.78 ± 0.14 (3)[a]

Comparisons to $\alpha CGRP_{8-37}$ were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Values are mean ± SEM (n).
[a]*Denotes that five experiments were conducted but only (n) repeats elicited measurable pA_2 values.*
[b]*Two repeats elicited pA_2 values of 7.92 and 5.93.*

In all cases, antagonist activity was observed at a single antagonist concentration (**Table 1** and **Supplementary Figure S4**). At the AM_1 receptor, all lipidated $h\alpha CGRP_{8-37}$ analogues with the exception of K35C-palmitate displayed higher antagonist activities compared to $\alpha CGRP_{8-37}$ (**Table 1**). Likewise, $\alpha CGRP_{7-37}$-palmitate and K24C-palmitate displayed increased antagonist activity at the AM_2 receptor (**Table 1**). Consistent with previous findings, derived pA_2 values for K35C-palmitate were significantly lower than $\alpha CGRP_{8-37}$ at both AM_1 and AM_2 receptors (**Table 1**). At the CTR, the lipidated $h\alpha CGRP_{8-37}$ analogues displayed measurable but otherwise weak antagonist activity (**Table 1**).

Lipidated $\alpha CGRP_{8-37}$ Analogues Display Comparable Antagonist Activities Between Human and Mouse CGRP and AMY_1 Receptors

Antagonist activities of lipidated $h\alpha CGRP_{8-37}$ analogues were also tested at mCGRP and AMY_1 receptors as a bridge to *in vivo* studies in mice. The control agonist in this case, mαCGRP, displayed a potency of 9.59 ± 0.22 (n = 5) at the mCGRP receptor, and 7.82 ± 0.11 (n = 5) at the $mAMY_1$ receptor, which was slightly lower than the mαCGRP pEC_{50} in Garelja et al. (2021). The comparatively lower potency of mαCGRP at the $mAMY_1$ receptor versus the CGRP receptor is different to hαCGRP which is equipotent at both human receptors. However, there is some variation in the potency of mαCGRP at this receptor (Bohn et al., 2017). All lipidated $\alpha CGRP_{8-37}$ analogues displayed antagonist activity at the mCGRP receptor (**Table 3** and **Supplementary Figure S5**). At the $mAMY_1$ receptor, it was difficult to derive measurable pA_2 values for the lipidated $h\alpha CGRP_{8-37}$ analogues due to the relatively low potency of the mCGRP peptide, however antagonism was observed for most of the peptides (**Table 3** and **Supplementary Figure S5**).

Effects of Antagonists on the Capsaicin-Induced Dermal Vasodilatory Response

Establishing the Capsaicin-Induced Dermal Vasodilatory Model

Male and female mice were recruited to measure blood flow as an effect of topical capsaicin. Vehicle was injected subcutaneously followed by induction of anesthesia after 10 min. Capsaicin (in ethanol) evoked a robust increase in dermal blood flow over baseline by approximately five-fold over the 15 min measurement window (**Figures 6A,B**). By comparison, ethanol alone applied to the contralateral ear at the same time yielded no discernible vasodilatory response (**Figures 6A,B**). The rate of increase in vasodilatory responses between male and female mice diverged over the first ~8 min (**Figure 6A**). However, both groups reached the same maximum response, and the overall absolute vasodilatory response was not significantly different as measured by AUC (**Figure 6C**). It is noted that female mice generated greater variability in vasodilatory response.

Lipidated $h\alpha CGRP_{8-37}$ Analogues Antagonize CGRP Action in vivo

Using these conditions we then undertook an investigation of the effect of antagonists on capsaicin-induced dermal vasodilatory (CIDV). Peptide antagonists were tested as well as the small molecule CGRP antagonist, BIBN4096BS (olcegepant) as an additional control. These exploratory studies were also conducted in male and female mice to explore if sex bias is present with respect to their effects on CIDV. Antagonist or vehicle was injected subcutaneously followed by induction of anesthesia after 10 min. For these experiments we used

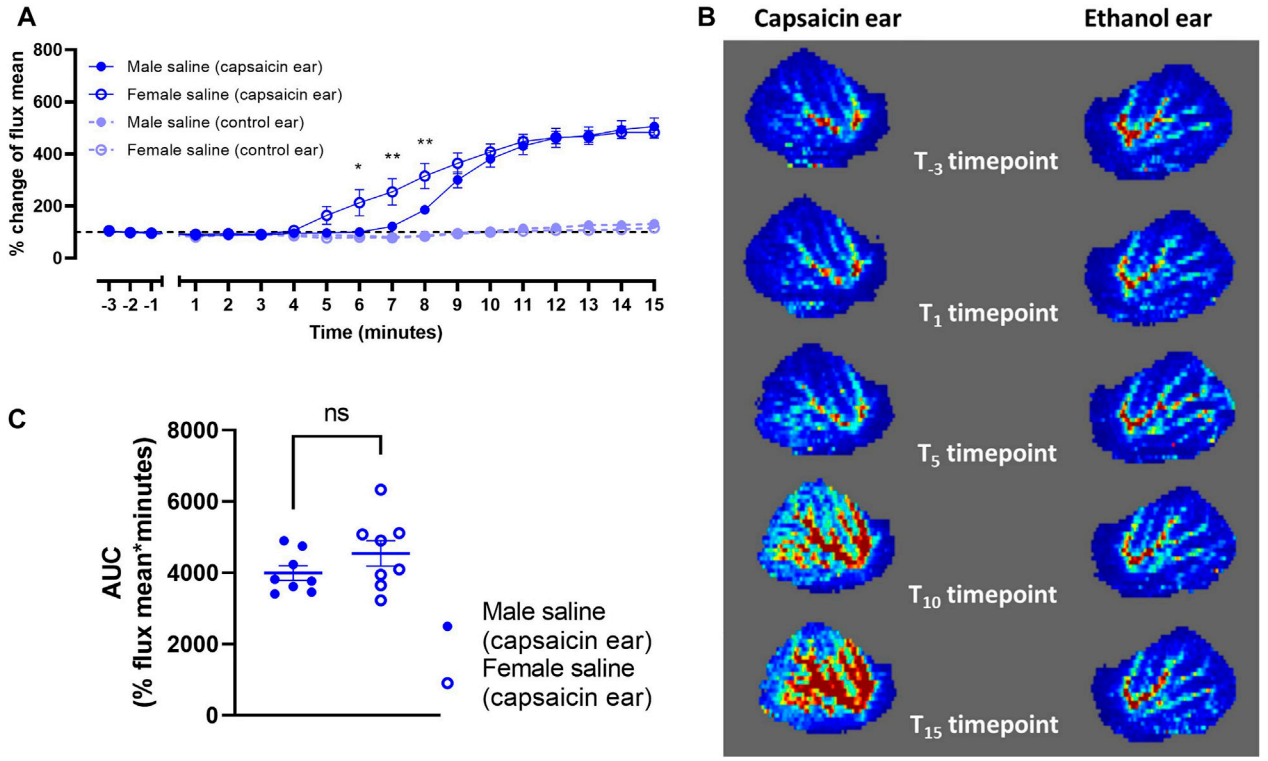

FIGURE 6 | Capsaicin-induced dermal vasodilation in mouse ears of saline-treated male ($n = 8$) and female mice ($n = 8$). **(A)** Vasodilatory responses of ears following application of either capsaicin or ethanol (control). **(B)** Representative laser doppler flux images captured over the 15 min time course. **(C)** Corresponding area-under-curve (AUC) mean values over the 15 min time course for capsaicin-applied ears of male and female mice. Time points are marked where data points differ significantly between sex. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$.

haCGRP$_{8-37}$ (**Figure 7A**), olcegepant (**Figure 7B**), V8C-palmitate (**Figure 7C**), and K24C-palmitate (**Figure 7D**). The K35C-palmitate peptide was not used due to its decreased receptor antagonism. The maximum dose that we could use for K24C-palmitate was 320 nmol/kg based on its limited solubility at high concentrations. All four antagonists attenuated the CIDV response (representative LDI scans shown in **Supplementary Figures S6**), but the effect was more pronounced in male versus female mice as shown by the relative time courses and mean AUC values between saline and antagonist-treated groups (**Figure 7**). Additionally, examination of the raw data indicated that there was no effect on basal blood flow by administration of saline or any of the antagonists.

In parallel, we also developed two further lipidated antagonists. These were R11C-palmitate and βV8C-palmitate. They were tested *in vitro* (**Supplementary Figure S7**) and then in the CIDV model, together with βCGRP$_{8-37}$. These, and subsequent CIDV experiments, were only conducted in male mice due to the apparently greater variance in vasodilatory response in female mice and because our intent was to investigate target engagement which could be achieved from the males. αCGRP$_{7-37}$-palmitate (**Supplementary Figure S8A**), R11C-palmitate (**Supplementary Figure S8B**), βCGRP$_{8-37}$ (**Supplementary Figure S8C**), and βV8C-palmitate (**Supplementary Figure S8D**) were administered at a lower dose (mass-matched dosage) to prior experiments. Compared to V8C-palmitate and K24C-palmitate (**Figure 7**), these peptides displayed weaker antagonist activities versus the matched vehicle group.

Dose-ranging experiments were next conducted with V8C-palmitate, at 320, 960, and 3,200 nmol/kg (**Figure 8**). For comparison, haCGRP$_{8-37}$, was the reference antagonist at molar-matched dosages (**Figure 8**). V8C-palmitate did not have a significant antagonist effect on CIDV at the lowest administered does of 320 nmol/kg (**Figures 8A,D**). However, at 960 nmol/kg, V8C-palmitate significantly reduced CIDV, comparable to haCGRP$_{8-37}$ (**Figures 8B,E**). At the highest dose of 3,200 nmol/kg, V8C-palmitate again had a significant antagonist effect, comparable to that evoked by haCGRP$_{8-37}$ (**Figures 8C,F**). All three doses for both peptides are compared in **Figures 8G,H**.

To determine whether there were any temporal differences between the behavior of these antagonists, haCGRP$_{8-37}$ and V8C-palmitate were each administered at a dose of 960 nmol/kg, at T0, T-10, T-20, T-30, and T-60 timepoints prior to anesthesia and capsaicin application (**Figure 9**). haCGRP$_{8-37}$ attenuated the

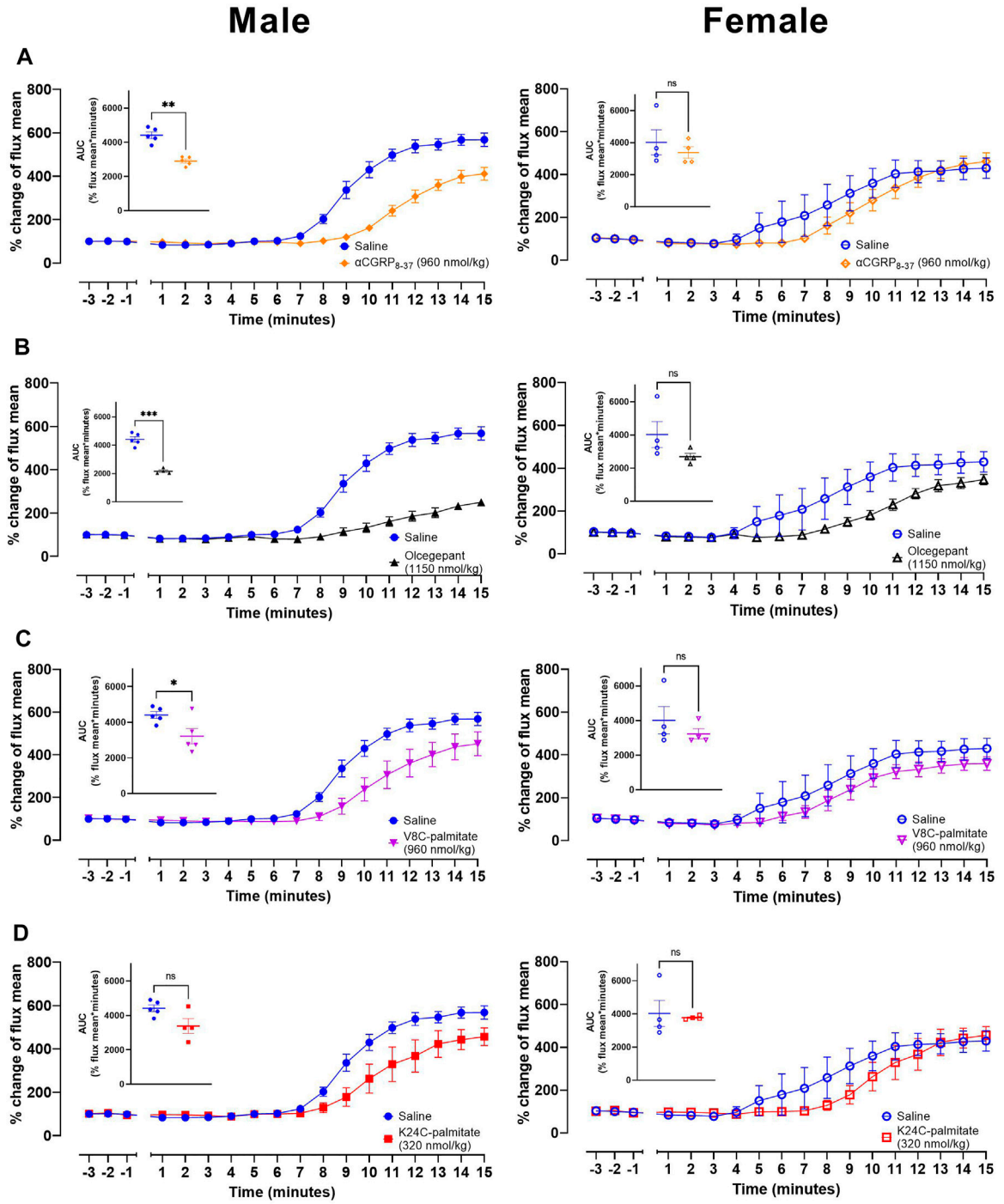

FIGURE 7 | CGRP receptor antagonists attenuate CGRP action *in vivo*. Effects of **(A)** αCGRP$_{8-37}$, **(B)** olcegepant, **(C)** V8C-palmitate and **(D)** K24C-palmitate on CIDV in male (left) and female (right) mice. Inset graphs show corresponding AUC mean values over the 15 min measurement timeframe following capsaicin application to the ear. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Each treatment group comprises four to five mice with corresponding saline controls.

CIDV response immediately at the T0 time point (**Figures 9A,F**) after which antagonist activity waned completely by the T-60 time point (**Figures 9E,J**). By comparison, V8C-palmitate displayed a delayed effect on the CIDV response with attenuation evident only at T-10 (**Figures 9B,G**) before again disappearing by the T-60 timepoint (**Figures 9E,J**).

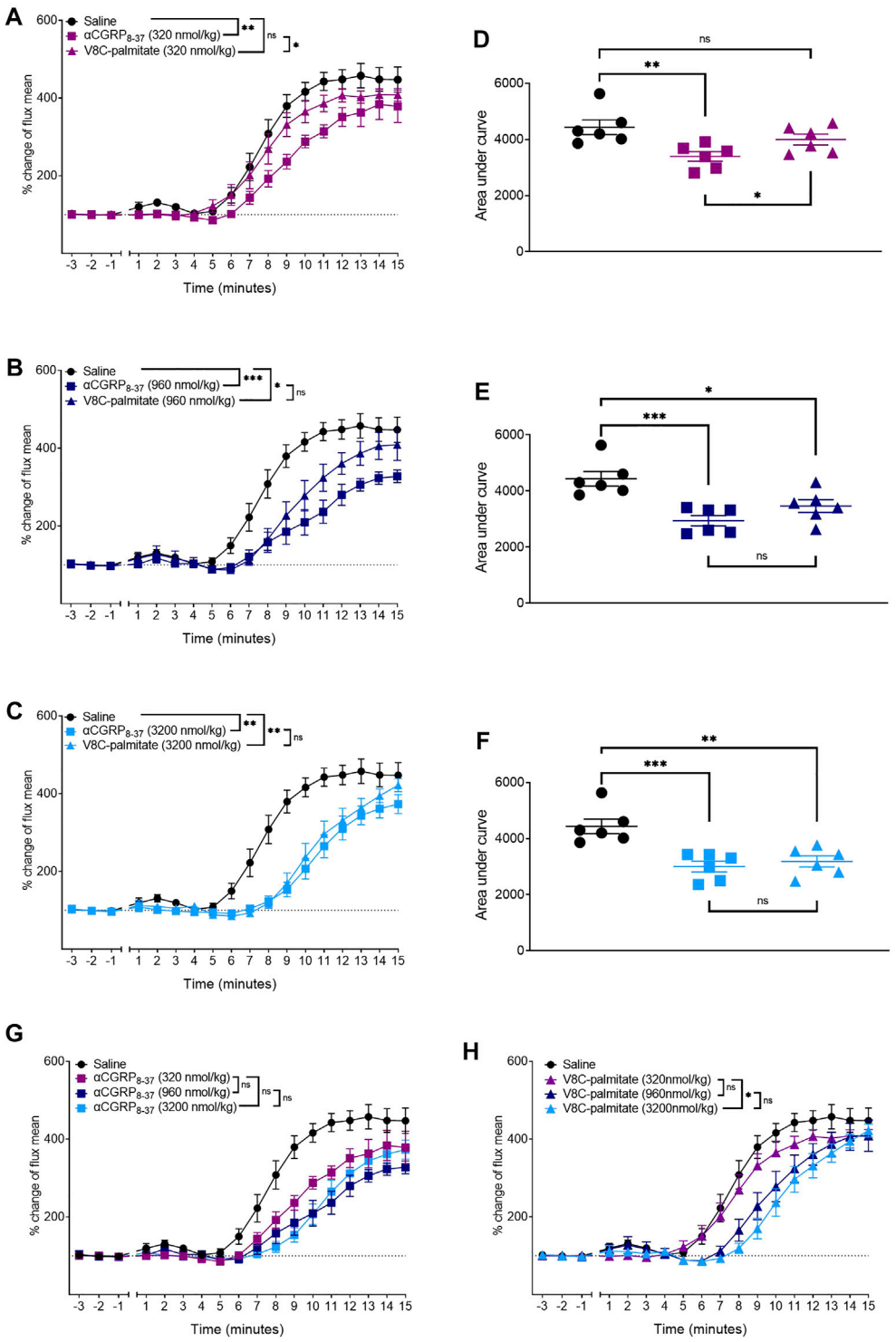

FIGURE 8 | Dose-dependent effects of $\alpha CGRP_{8-37}$ and V8C-palmitate on CIDV in male mice. Peptides were administered at doses of 320 nmol/kg **(A,D)**, 960 nmol/kg, **(B,E)** or 3,200 nmol/kg **(C,F)**. Combined data for $\alpha CGRP_{8-37}$ and V8C-palmitate are shown in **(G,H)**, respectively. ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each treatment group comprised six mice with corresponding saline controls.

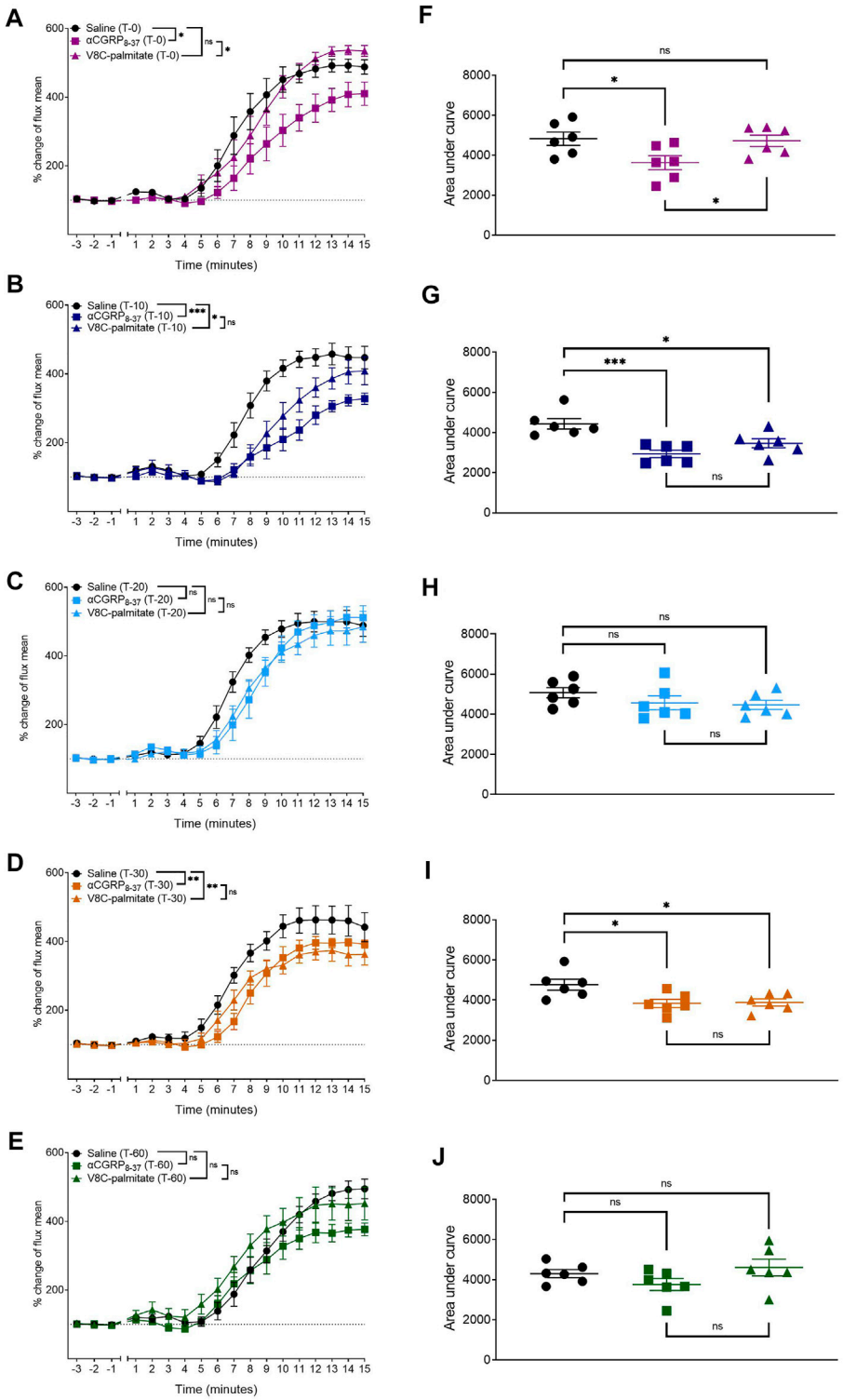

FIGURE 9 | Comparison between $\alpha CGRP_{8-37}$ and V8C-palmitate for antagonism onset of CGRP action *in vivo*. $\alpha CGRP_{8-37}$ (960 nmol/kg) and V8C-palmitate (960 nmol/kg) were administered at 0 min **(A,F)**, 10 min **(B,G)**, 20 min **(C,H)**, 30 min **(D,I)** and 60 min **(E,J)** prior to anesthesia and capsaicin application. ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each treatment group comprised six mice with corresponding saline controls.

Discussion

Lipidated hαCGRP$_{8-37}$ Analogues Retain Antagonist Activity *In Vitro*

Our findings show that it is possible to attach a palmitoyl moiety at selected positions of the CGRP$_{8-37}$ peptide backbone and still preserve antagonist activity. We initially generated hαCGRP$_{8-37}$ cysteine-substituted analogues to assess the effect of cystine-substitution at these residues prior to palmitoylation. The findings suggested that these residues were somewhat amenable to modification with cysteine. However, cysteine-substitution had only limited utility in predicting the effect of cysteine-lipidation (palmitoylation) at the same residues on hαCGRP$_{8-37}$ pharmacology. Except for position 35, the resulting lipidation at cysteine positions 7, 8, and 24, generated analogues that retained antagonist activity at human CGRP and AMY$_1$ receptors and at the human CGRP receptor endogenously expressed in SK-N-MC cells. Our analyses indicated that antagonism of these lipidated hαCGRP$_{8-37}$ analogues at the human CGRP and AMY$_1$ receptor was competitive as the respective E_{max} values were not significantly different. For V8C-palmitate, apparent competitive antagonism was demonstrated directly by increasing the concentration of agonist by an additional log unit to enable full curves to be achieved (**Figure 3B**). Nevertheless, we cannot fully discount the possibility of non-surmountable antagonism by hαCGRP$_{7-37}$-palmitate and K24C-palmitate due to the lipid moiety preventing full dissociation from the receptor. More detailed investigation such as receptor binding studies would be required to resolve the precise mode of antagonism.

We also noted differences in antagonist activity by the lipidated hαCGRP$_{8-37}$ analogues between Cos7 cells expressing the CGRP receptor and SK-N-MC cells expressing endogenous CGRP receptors. We speculate this difference could be due to cellular features specific to each experimental system such as receptor expression and distribution on the cell surface or cell membrane composition affecting lipidated peptide kinetics. Evidence also suggests that antagonist potency can differ depending on the signaling pathway measured (Walker et al., 2018). Thus, if the CGRP receptor couples to a different protein complement of signaling pathways between transfected Cos7 cells and SK-N-MC cells, then measurement of only cAMP could explain the differences in antagonist activity.

The positional effect of palmitoylation can be interpreted by considering the two-domain model of class B G protein-coupled receptor peptide ligand binding and receptor activation, along with receptor structures (Hoare, 2005; Booe et al., 2015; Liang et al., 2018). The peptide C-terminus plays an important role in binding, whereas the N-terminus is crucial for receptor activation. Attachment of a lipid moiety to most positions was able to preserve antagonism, whereas attachment to position 35 in the C-terminus substantially reduced antagonism. Lys-35 faces outwards into unoccupied space when CGRP is bound to the CGRP receptor (Liang et al., 2018) suggesting it is amenable to modification. However, the attached palmitoyl moiety appears to obstruct the peptide from initiating proper contact and binding to the CGRP receptor binding pocket (Booe et al., 2018). The effect of palmitoylation was generally similar between receptors.

Lipidated hαCGRP$_{8-37}$ Peptide Antagonists May Have Altered Receptor Binding Kinetics

Lipidated peptide analogues, except for K35C-palmitate, displayed unique pharmacological characteristics. First, there was increased antagonism when lipidated peptide analogues were pre-incubated with transfected CGRP and AMY$_1$ receptors for a 15-min period prior to stimulation with agonist. This was not observed with hαCGRP$_{8-37}$ which suggests an effect specific to some lipidated peptides. Second, some antagonists retained activity following washing of the cells. These findings suggest that the palmitate moiety may alter the receptor or membrane residence time of the peptide antagonist, which could also potentially explain the observed improved antagonist activities of αCGRP$_{7-37}$-palmitate, V8C-palmitate, and K24C-palmitate, at AM$_1$ and AM$_2$ receptors compared to hαCGRP$_{8-37}$. A similar outcome was observed in the pharmacological characterization of lipidated amylin analogues (Fletcher et al., 2021), where an extended receptor residence time was correlated with their prolonged duration of action. Although receptor residency time and binding kinetics are not the sole contributor to the efficacy of an agonist, they may have a strong influence on antagonist activity. One potential mechanism by which this could occur is through compartmentalization of the lipidated peptide with the cell membrane. Membrane partitioning and membrane trafficking of lipidated proteins have been reported to influence protein activity (Zacharias et al., 2002; Ostrom and Insel, 2004; Ray et al., 2017). Potentially, the palmitoyl moeity facilitates association of the peptide with the cell membrane and increases its local concentration within the vicinity of the target membrane receptors to alter kinetics and receptor residence time, and improve their antagonist activity.

Lipidated αCGRP$_{8-37}$ Peptide Antagonists Attenuate CGRP Action *In Vivo*

We utilized a CIDV animal model to demonstrate *in vivo* target engagement by the lipidated peptide antagonists. The vasodilatory response within the ear following application of capsaicin was robust with minimal response in the ethanol-treated contralateral ear, which was used as an internal control. The magnitude of the CIDV response at the capsaicin dose used (60 μg/ear) is similar to that used in previous studies (Grant et al., 2002; Starr et al., 2008). We validated this experimental system by demonstrating attenuation of the CIDV response by the reference antagonists, hαCGRP$_{8-37}$ and olcegepant, showing successful target engagement with the CGRP receptor *in vivo*.

Female mice appeared to show an earlier vasodilatory onset compared to male mice although the respective total integrated responses as analyzed by AUC were comparable. This observation is consistent with reported differences in CGRP activity and vascular responses between male and female mice (Lee et al., 2003; Peng et al., 2011). Female mice also display a greater endothelium-dependent vasodilatory response to acetylcholine (Zuloaga et al., 2014). It is possible that female mice respond physiologically to capsaicin differently due to differences in CGRP receptor or peptide expression. Alternatively, responses could be influenced by the oestrus cycle phase. There is evidence that hormonal variations as part

of the oestrus cycle modulates TRPV1, which is the major cation channel responsible for the capsaicin-evoked vasodilatory response (Artero-Morales et al., 2018). Thus, hormonal levels could potentially modulate intrinsic vasodilatory responses through TRPV1 expression and activity, and subsequently, affect the release of neurotransmitters or neuropeptides, including CGRP. Additionally, CIDV is an indirect measurement of CGRP activity, since CGRP release is dependent upon TRPV1 activation. It is possible that other substances are involved in the CIDV response, which could influence the effect of CGRP antagonists on CIDV.

The cellular mechanisms of CGRP vasodilatory effect can primarily be divided into endothelium-independent vasodilation or endothelium-dependent vasodilation (Brain and Grant, 2004). This adds a layer of complexity as the literature suggests differences in tissue and species specificity for these two mechanisms (Sohn et al., 2020). It is therefore possible that the temporal and sex differences observed stem from differential CGRP activity. Sex-specific differences in CGRP receptor expression could also impact on CGRP action *in vivo*. The receptor component protein (RCP) expression, which is a component of the CGRP receptor signaling complex (Ji et al., 2019), as well as CLR and RAMP1 expression have been shown to differ between male and female rodents (Stucky et al., 2011). Our study did not account for oestrus cycling or potential differences in CGRP receptor expression, so these remain interesting parameters to explore for future vasodilatory studies involving CGRP.

Peptide lipidation is a useful tool for developing efficacious peptide therapeutics by increasing peptide half-life and decreasing elimination rate. Peptide lipidation as a strategy has been explored previously with CGRP. Here, a modified αCGRP analogue with an albumin binding fatty acid moiety showed protracted pharmacokinetic properties (Nilsson et al., 2016; Sheykhzade et al., 2018) and demonstrated positive utility in alleviating or reversing cardiovascular disease in rodents (Aubdool et al., 2017). Another study reported modification of αCGRP with a fatty acid-dibenzylcyclooctyne (DIBO) moiety at position 24, which improved its plasma stability (Demin, 2018).

By comparison, the current lipidation strategy produced the V8C-palmitate and K24C-palmitate analogues that attenuated CIDV response *in vivo*. We confirmed that the mouse model was an appropriate translational model as the antagonist activities of hαCGRP$_{8-37}$ and the lipidated hαCGRP$_{8-37}$ analogues were comparable across mouse and human CGRP receptors. Dose-ranging experiments showed that a higher dose of V8C-palmitate was required compared to hαCGRP$_{8-37}$ to reach a similar attenuation of the CIDV response. Likewise, time to onset experiments indicated that V8C-palmitate displayed a longer onset of action compared to hαCGRP$_{8-37}$, although attenuation of CIDV was lost after 60 min in both cases. These observations for V8C-palmitate could reflect a depot effect resulting in delayed drug absorption into the blood through the subcutaneous injection route or a slower distribution to the tissue region from plasma compared to hαCGRP$_{8-37}$. This raises the possibility of sustained slow release of the V8C-palmitate analogue into the systemic circulation and/or site of action compared to hαCGRP$_{8-37}$.

Conclusion

hCGRP$_{8-37}$ peptide analogues were palmitoylated at different locations on the peptide sequence. Excluding the C-terminally modified analogue, K35C-palmitate, the lipidated analogues behaved as competitive antagonists at the CGRP and AMY$_1$ receptor *in vitro*. There was evidence that the palmitoyl moiety on the peptide antagonist confers altered residence time compared to hαCGRP$_{8-37}$ as observed by increased antagonist activity with prolonged incubation of lipidated peptide analogues with CGRP and AMY$_1$ receptors prior to agonist stimulation and retention of antagonist activity when a washout step was included prior to agonist stimulation.

For the translational studies in mice, V8C-palmitate and K24C-palmitate significantly attenuated the CIDV response, demonstrating successful target engagement of the CGRP receptor *in vivo*. However, there was a difference in dose-response profile and onset indicating the presence of a depot effect for the lipidated analogue. Overall, these findings show it is possible to generate palmitoylated peptides based on the hαCGRP$_{8-37}$ peptide backbone that retain both antagonist activity at CGRP and AMY$_1$ receptors, and attenuate CGRP action *in vivo*. Although dedicated pharmacokinetic studies are required, these findings suggest that lipidation may offer a route to develop a new class of CGRP peptide antagonists as therapeutics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Auckland Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

AJ, CC, EW, AS, SY, JP, RB, and SC: collected data, performed analyses, critical revision of paper. PH, MB, CW, DH, and KL: analysed data, performed analyses, critical revision of paper. MB, CW, DH, and KL: conceived and designed work. AJ, CC, CW, DH, and KL: wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.832589/full#supplementary-material

REFERENCES

Ailani, J., Lipton, R. B., Hutchinson, S., Knievel, K., Lu, K., Butler, M., et al. (2020). Long-Term Safety Evaluation of Ubrogepant for the Acute Treatment of Migraine: Phase 3, Randomized, 52-Week Extension Trial. *Headache* 60 (1), 141–152. doi:10.1111/head.13682

Artero-Morales, M., González-Rodríguez, S., and Ferrer-Montiel, A. (2018). TRP Channels as Potential Targets for Sex-Related Differences in Migraine Pain. *Front. Mol. Biosci.* 5, 73. doi:10.3389/fmolb.2018.00073

Arunlakshana, O., and Schild, H. O. (1958). Some Quantitative Uses of Drug Antagonists. 1958. *Br. J. Pharmacol.* 120 (4 Suppl. l), 151–161. doi:10.1111/j.1476-5381.1997.tb06793.x

Asghar, M. S., Hansen, A. E., Amin, F. M., van der Geest, R. J., Koning, Pv., Larsson, H. B., et al. (2011). Evidence for a Vascular Factor in Migraine. *Ann. Neurol.* 69 (4), 635–645. doi:10.1002/ana.22292

Aubdool, A. A., Kodji, X., Abdul-Kader, N., Heads, R., Fernandes, E. S., Bevan, S., et al. (2016). TRPA1 Activation Leads to Neurogenic Vasodilatation: Involvement of Reactive Oxygen Nitrogen Species in Addition to CGRP and NO. *Br. J. Pharmacol.* 173 (15), 2419–2433. doi:10.1111/bph.13519

Aubdool, A. A., Thakore, P., Argunhan, F., Smillie, S. J., Schnelle, M., Srivastava, S., et al. (2017). A Novel α-Calcitonin Gene-Related Peptide Analogue Protects against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure. *Circulation* 136 (4), 367–383. doi:10.1161/circulationaha.117.028388

Bailey, R. J., and Hay, D. L. (2006). Pharmacology of the Human CGRP1 Receptor in cos 7 Cells. *Peptides* 27 (6), 1367–1375. doi:10.1016/j.peptides.2005.11.014

Bartelt, A., Jeschke, A., Müller, B., Gaziano, I., Morales, M., Yorgan, T., et al. (2017). Differential Effects of Calca-Derived Peptides in Male Mice with Diet-Induced Obesity. *PloS one* 12 (6), e0180547. doi:10.1371/journal.pone.0180547

Basbaum, A. I., Bautista, D. M., Scherrer, G., and Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell* 139 (2), 267–284. doi:10.1016/j.cell.2009.09.028

Bigal, M. E., Dodick, D. W., Rapoport, A. M., Silberstein, S. D., Ma, Y., Yang, R., et al. (2015). Safety, Tolerability, and Efficacy of TEV-48125 for Preventive Treatment of High-Frequency Episodic Migraine: a Multicentre, Randomised, Double-Blind, Placebo-Controlled, Phase 2b Study. *Lancet Neurol.* 14 (11), 1081–1090. doi:10.1016/s1474-4422(15)00249-5

Bohn, K. J., Li, B., Huang, X., Mason, B. N., Wattiez, A. S., Kuburas, A., et al. (2017). CGRP Receptor Activity in Mice with Global Expression of Human Receptor Activity Modifying Protein 1. *Br. J. Pharmacol.* 174 (12), 1826–1840. doi:10.1111/bph.13783

Booe, J. M., Walker, C. S., Barwell, J., Kuteyi, G., Simms, J., Jamaluddin, M. A., et al. (2015). Structural Basis for Receptor Activity-Modifying Protein-dependent Selective Peptide Recognition by a G Protein-Coupled Receptor. *Mol. Cel* 58 (6), 1040–1052. doi:10.1016/j.molcel.2015.04.018

Booe, J. M., Warner, M. L., Roehrkasse, A. M., Hay, D. L., and Pioszak, A. A. (2018). Probing the Mechanism of Receptor Activity-Modifying Protein Modulation of GPCR Ligand Selectivity through Rational Design of Potent Adrenomedullin and Calcitonin Gene-Related Peptide Antagonists. *Mol. Pharmacol.* 93 (4), 355–367. doi:10.1124/mol.117.110916

Brain, S. D., and Grant, A. D. (2004). Vascular Actions of Calcitonin Gene-Related Peptide and Adrenomedullin. *Physiol. Rev.* 84 (3), 903–934. doi:10.1152/physrev.00037.2003

Brain, S. D., and Williams, T. J. (1985). Inflammatory Oedema Induced by Synergism between Calcitonin Gene-Related Peptide (CGRP) and Mediators of Increased Vascular Permeability. *Br. J. Pharmacol.* 86 (4), 855–860. doi:10.1111/j.1476-5381.1985.tb11107.x

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., et al. (1989). Calcitonin Gene-Related Peptide Receptor Antagonist Human CGRP-(8-37). *Am. J. Physiol.* 256 (2 Pt 1), E331–E335. doi:10.1152/ajpendo.1989.256.2.E331

Choksi, T., Hay, D. L., Legon, S., Poyner, D. R., Hagner, S., Bloom, S. R., et al. (2002). Comparison of the Expression of Calcitonin Receptor-like Receptor (CRLR) and Receptor Activity Modifying Proteins (RAMPs) with CGRP and Adrenomedullin Binding in Cell Lines. *Br. J. Pharmacol.* 136 (5), 784–792. doi:10.1038/sj.bjp.0704761

Croop, R., Goadsby, P. J., Stock, D. A., Conway, C. M., Forshaw, M., Stock, E. G., et al. (2019). Efficacy, Safety, and Tolerability of Rimegepant Orally Disintegrating Tablet for the Acute Treatment of Migraine: a Randomised, Phase 3, Double-Blind, Placebo-Controlled Trial. *Lancet* 394 (10200), 737–745. doi:10.1016/s0140-6736(19)31606-x

Davies, M. J., Bergenstal, R., Bode, B., Kushner, R. F., Lewin, A., Skjøth, T. V., et al. (2015). Efficacy of Liraglutide for Weight Loss Among Patients with Type 2 Diabetes: the SCALE Diabetes Randomized Clinical Trial. *Jama* 314 (7), 687–699. doi:10.1001/jama.2015.9676

Demin, Z. (2018). Modification at Lys24 with Fatty Acid Generates Active Calcitonin Gene-Related Peptide with Much Improved Plasma Stability. *J. Chin. Pharm. Sci.* 27 (9), 589–599. doi:10.5246/jcps.2018.09.060

Dodick, D. W., Silberstein, S. D., Bigal, M. E., Yeung, P. P., Goadsby, P. J., Blankenbiller, T., et al. (2018). Effect of Fremanezumab Compared with Placebo for Prevention of Episodic Migraine: A Randomized Clinical Trial. *Jama* 319 (19), 1999–2008. doi:10.1001/jama.2018.4853

Edvinsson, L. (2018). The CGRP Pathway in Migraine as a Viable Target for Therapies. *Headache* 58 (Suppl. 1), 33–47. doi:10.1111/head.13305

Eftekhari, S., Gaspar, R. C., Roberts, R., Chen, T. B., Zeng, Z., Villarreal, S., et al. (2016). Localization of CGRP Receptor Components and Receptor Binding Sites in Rhesus Monkey Brainstem: A Detailed Study Using *In Situ* Hybridization, Immunofluorescence, and Autoradiography. *J. Comp. Neurol.* 524 (1), 90–118. doi:10.1002/cne.23828

Fletcher, M. M., Keov, P., Truong, T. T., Mennen, G., Hick, C. A., Zhao, P., et al. (2021). AM833 Is a Novel Agonist of Calcitonin Family G Protein-Coupled Receptors: Pharmacological Comparison with Six Selective and Nonselective Agonists. *J. Pharmacol. Exp. Ther.* 377 (3), 417–440. doi:10.1124/jpet.121.000567

Garelja, M. L., Au, M., Brimble, M. A., Gingell, J. J., Hendrikse, E. R., Lovell, A., et al. (2020). Molecular Mechanisms of Class B GPCR Activation: Insights from Adrenomedullin Receptors. *ACS Pharmacol. Transl Sci.* 3 (2), 246–262. doi:10.1021/acsptsci.9b00083

Garelja, M. L., Bower, R. L., Brimble, M. A., Chand, S., Harris, P. W. R., Jamaluddin, M. A., et al. (2021). Pharmacological Characterisation of Mouse Calcitonin and Calcitonin Receptor-like Receptors Reveals Differences Compared with Human Receptors. *Br. J Pharmacol.* 179, 416–434. doi:10.1111/bph.15628

Goadsby, P. J., Reuter, U., Hallström, Y., Broessner, G., Bonner, J. H., Zhang, F., et al. (2017). A Controlled Trial of Erenumab for Episodic Migraine. *N. Engl. J. Med.* 377 (22), 2123–2132. doi:10.1056/NEJMoa1705848

Gohin, S., Decorps, J., Sigaudo-Roussel, D., and Fromy, B. (2015). Sensory Nerves Contribute to Cutaneous Vasodilator Response to Cathodal Stimulation in Healthy Rats. *Microvasc. Res.* 101, 103–110. doi:10.1016/j.mvr.2015.06.010

Grant, A. D., Gerard, N. P., and Brain, S. D. (2002). Evidence of a Role for NK1 and CGRP Receptors in Mediating Neurogenic Vasodilatation in the Mouse Ear. *Br. J. Pharmacol.* 135 (2), 356–362. doi:10.1038/sj.bjp.0704485

Gray, D. W., and Marshall, I. (1992). Nitric Oxide Synthesis Inhibitors Attenuate Calcitonin Gene-Related Peptide Endothelium-dependent Vasorelaxation in Rat Aorta. *Eur. J. Pharmacol.* 212 (1), 37–42. doi:10.1016/0014-2999(92)90069-g

Greenberg, B., Rhoden, K., and Barnes, P. (1987). Calcitonin Gene-Related Peptide (CGRP) Is a Potent Non-endothelium-dependent Inhibitor of Coronary Vasomotor Tone. *Br. J. Pharmacol.* 92 (4), 789–794. doi:10.1111/j.1476-5381.1987.tb11382.x

Guo, S., Vollesen, A. L., Olesen, J., and Ashina, M. (2016). Premonitory and Nonheadache Symptoms Induced by CGRP and PACAP38 in Patients with Migraine. *Pain* 157 (12), 2773–2781. doi:10.1097/j.pain.0000000000000702

Hansen, J. M., Hauge, A. W., Olesen, J., and Ashina, M. (2010). Calcitonin Gene-Related Peptide Triggers Migraine-like Attacks in Patients with Migraine with Aura. *Cephalalgia* 30 (10), 1179–1186. doi:10.1177/0333102410368444

Hay, D. L., Garelja, M. L., Poyner, D. R., and Walker, C. S. (2018). Update on the Pharmacology of Calcitonin/CGRP Family of Peptides: IUPHAR Review 25. *Br. J. Pharmacol.* 175 (1), 3–17. doi:10.1111/bph.14075

Hay, D. L., Howitt, S. G., Conner, A. C., Schindler, M., Smith, D. M., and Poyner, D. R. (2003). CL/RAMP2 and CL/RAMP3 Produce Pharmacologically Distinct Adrenomedullin Receptors: a Comparison of Effects of Adrenomedullin22-52, CGRP8-37 and BIBN4096BS. *Br. J. Pharmacol.* 140 (3), 477–486. doi:10.1038/sj.bjp.0705472

Hoare, S. R. (2005). Mechanisms of Peptide and Nonpeptide Ligand Binding to Class B G-Protein-Coupled Receptors. *Drug Discov. Today* 10 (6), 417–427. doi:10.1016/S1359-6446(05)03370-2

Ji, Y., Rizk, A., Voulalas, P., Aljohani, H., Akerman, S., Dussor, G., et al. (2019). Sex Differences in the Expression of Calcitonin Gene-Related Peptide Receptor Components in the Spinal Trigeminal Nucleus. *Neurobiol. Pain* 6, 100031. doi:10.1016/j.ynpai.2019.100031

Kraenzlin, M. E., Ch'ng, J. L., Mulderry, P. K., Ghatei, M. A., and Bloom, S. R. (1985). Infusion of a Novel Peptide, Calcitonin Gene-Related Peptide (CGRP) in Man. Pharmacokinetics and Effects on Gastric Acid Secretion and on Gastrointestinal Hormones. *Regul. Pept.* 10 (2-3), 189–197. doi:10.1016/0167-0115(85)90013-8

Lassen, L. H., Haderslev, P. A., Jacobsen, V. B., Iversen, H. K., Sperling, B., and Olesen, J. (2002). CGRP May Play a Causative Role in Migraine. *Cephalalgia* 22 (1), 54–61. doi:10.1046/j.1468-2982.2002.00310.x

Lee, W. I., Xu, Y., Fung, S. M., and Fung, H. L. (2003). eNOS-dependent Vascular Interaction between Nitric Oxide and Calcitonin Gene-Related Peptide in Mice: Gender Selectivity and Effects on Blood Aggregation. *Regul. Pept.* 110 (2), 115–122. doi:10.1016/s0167-0115(02)00210-0

Liang, Y. L., Khoshouei, M., Deganutti, G., Glukhova, A., Koole, C., Peat, T. S., et al. (2018). Cryo-EM Structure of the Active, Gs-Protein Complexed, Human CGRP Receptor. *Nature* 561 (7724), 492–497. doi:10.1038/s41586-018-0535-y

Liu, T., Kamiyoshi, A., Sakurai, T., Ichikawa-Shindo, Y., Kawate, H., Yang, L., et al. (2017). Endogenous Calcitonin Gene-Related Peptide Regulates Lipid Metabolism and Energy Homeostasis in Male Mice. *Endocrinology* 158 (5), 1194–1206. doi:10.1210/en.2016-1510

Liu, Z., Liu, Q., Cai, H., Xu, C., Liu, G., and Li, Z. (2011). Calcitonin Gene-Related Peptide Prevents Blood-Brain Barrier Injury and Brain Edema Induced by Focal Cerebral Ischemia Reperfusion. *Regul. Pept.* 171 (1-3), 19–25. doi:10.1016/j.regpep.2011.05.014

Lu, B. L., Loomes, K. M., Hay, D. L., Harris, P. W. R., and Brimble, M. A. (2020). Synthesis of Isotopically Labelled αCGRP8-37 and its Lipidated Analogue. *J. Labelled Comp. Radiopharm.* 63 (7), 325–332. doi:10.1002/jlcr.3838

Miranda, L. P., Holder, J. R., Shi, L., Bennett, B., Aral, J., Gegg, C. V., et al. (2008). Identification of Potent, Selective, and Metabolically Stable Peptide Antagonists to the Calcitonin Gene-Related Peptide (CGRP) Receptor. *J. Med. Chem.* 51 (24), 7889–7897. doi:10.1021/jm8009298

Miranda, L. P., Shi, L., Holder, J. R., Wright, M., Gegg, C. V., Johnson, E., et al. (2013). Peptide Antagonists of the Calcitonin Gene-Related Peptide (CGRP) Receptor with Improved Pharmacokinetics and Pharmacodynamics. *Biopolymers* 100 (4), 422–430. doi:10.1002/bip.22270

Mishima, T., Ito, Y., Hosono, K., Tamura, Y., Uchida, Y., Hirata, M., et al. (2011). Calcitonin Gene-Related Peptide Facilitates Revascularization during Hindlimb Ischemia in Mice. *Am. J. Physiol. Heart Circ. Physiol.* 300 (2), H431–H439. doi:10.1152/ajpheart.00466.2010

Mulderry, P. K., Ghatei, M. A., Spokes, R. A., Jones, P. M., Pierson, A. M., Hamid, Q. A., et al. (1988). Differential Expression of Alpha-CGRP and Beta-CGRP by Primary Sensory Neurons and Enteric Autonomic Neurons of the Rat. *Neuroscience* 25 (1), 195–205. doi:10.1016/0306-4522(88)90018-8

Muttenthaler, M., King, G. F., Adams, D. J., and Alewood, P. F. (2021). Trends in Peptide Drug Discovery. *Nat. Rev. Drug Discov.* 20 (4), 309–325. doi:10.1038/s41573-020-00135-8

Nilsson, C., Hansen, T. K., Rosenquist, C., Hartmann, B., Kodra, J. T., Lau, J. F., et al. (2016). Long Acting Analogue of the Calcitonin Gene-Related Peptide Induces Positive Metabolic Effects and Secretion of the Glucagon-like Peptide-1. *Eur. J. Pharmacol.* 773, 24–31. doi:10.1016/j.ejphar.2016.01.003

Ostrom, R. S., and Insel, P. A. (2004). The Evolving Role of Lipid Rafts and Caveolae in G Protein-Coupled Receptor Signaling: Implications for Molecular Pharmacology. *Br. J. Pharmacol.* 143 (2), 235–245. doi:10.1038/sj.bjp.0705930

Peng, X., Wang, J., Lassance-Soares, R. M., Najafi, A. H., Sood, S., Aghili, N., et al. (2011). Gender Differences Affect Blood Flow Recovery in a Mouse Model of Hindlimb Ischemia. *Am. J. Physiol. Heart Circ. Physiol.* 300 (6), H2027–H2034. doi:10.1152/ajpheart.00004.2011

Qi, T., Christopoulos, G., Bailey, R. J., Christopoulos, A., Sexton, P. M., and Hay, D. L. (2008). Identification of N-Terminal Receptor Activity-Modifying Protein Residues Important for Calcitonin Gene-Related Peptide, Adrenomedullin, and Amylin Receptor Function. *Mol. Pharmacol.* 74 (4), 1059–1071. doi:10.1124/mol.108.047142

Qi, T., Dong, M., Watkins, H. A., Wootten, D., Miller, L. J., and Hay, D. L. (2013). Receptor Activity-Modifying Protein-dependent Impairment of Calcitonin Receptor Splice Variant Δ(1-47)hCT((a)) Function. *Br. J. Pharmacol.* 168 (3), 644–657. doi:10.1111/j.1476-5381.2012.02197.x

Ray, A., Jatana, N., and Thukral, L. (2017). Lipidated Proteins: Spotlight on Protein-Membrane Binding Interfaces. *Prog. Biophys. Mol. Biol.* 128, 74–84. doi:10.1016/j.pbiomolbio.2017.01.002

Rist, B., Lacroix, J. S., Entzeroth, M., Doods, H. N., and Beck-Sickinger, A. G. (1999). CGRP 27-37 Analogues with High Affinity to the CGRP1 Receptor Show Antagonistic Properties in a Rat Blood Flow Assay. *Regul. Pept.* 79 (2-3), 153–158. doi:10.1016/s0167-0115(98)00159-1

Saxen, M. A., Smith, F. L., Dunlow, L. D., Dombrowski, D. S., and Welch, S. P. (1994). The Hypothermic and Antinociceptive Effects of Intrathecal Injection of CGRP (8-37) in Mice. *Life Sci.* 55 (21), 1665–1674. doi:10.1016/0024-3205(94)00334-3

Schwedt, T. J., Lipton, R. B., Ailani, J., Silberstein, S. D., Tassorelli, C., Guo, H., et al. (2021). Time Course of Efficacy of Atogepant for the Preventive Treatment of Migraine: Results from the Randomized, Double-Blind ADVANCE Trial. *Cephalalgia* 42, 3–11. doi:10.1177/03331024211042385

Sheykhzade, M., Abdolalizadeh, B., Koole, C., Pickering, D. S., Dreisig, K., Johansson, S. E., et al. (2018). Vascular and Molecular Pharmacology of the Metabolically Stable CGRP Analogue, SAX. *Eur. J. Pharmacol.* 829, 85–92. doi:10.1016/j.ejphar.2018.04.007

Shi, L., Lehto, S. G., Zhu, D. X., Sun, H., Zhang, J., Smith, B. P., et al. (2016). Pharmacologic Characterization of AMG 334, a Potent and Selective Human Monoclonal Antibody against the Calcitonin Gene-Related Peptide Receptor. *J. Pharmacol. Exp. Ther.* 356 (1), 223–231. doi:10.1124/jpet.115.227793

Smillie, S. J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., et al. (2014). An Ongoing Role of α-calcitonin Gene-Related Peptide as Part of a Protective Network against Hypertension, Vascular Hypertrophy, and Oxidative Stress. *Hypertension* 63 (5), 1056–1062. doi:10.1161/hypertensionaha.113.02517

Sohn, I., Sheykhzade, M., Edvinsson, L., and Sams, A. (2020). The Effects of CGRP in Vascular Tissue - Classical Vasodilation, Shadowed Effects and Systemic Dilemmas. *Eur. J. Pharmacol.* 881, 173205. doi:10.1016/j.ejphar.2020.173205

Srinivasan, K., Kozminski, K., Zhang, Y., Wisniewski, K., Kohout, T., Wisniewska, H., et al. (2022). Pharmacological, Pharmacokinetic, Pharmacodynamic and Physicochemical Characterization of FE 205030: A Potent, Fast Acting, Injectable CGRP Receptor Antagonist for the Treatment of Acute Episodic Migraine. *J. Pharm. Sci.* 111 (1), 247–261. doi:10.1016/j.xphs.2021.06.034

Starr, A., Graepel, R., Keeble, J., Schmidhuber, S., Clark, N., Grant, A., et al. (2008). A Reactive Oxygen Species-Mediated Component in Neurogenic Vasodilatation. *Cardiovasc. Res.* 78 (1), 139–147. doi:10.1093/cvr/cvn012

Sternini, C. (1992). Enteric and Visceral Afferent CGRP Neurons. Targets of Innervation and Differential Expression Patterns. *Ann. N. Y Acad. Sci.* 657, 170–186. doi:10.1111/j.1749-6632.1992.tb22766.x

Struthers, A. D., Brown, M. J., Macdonald, D. W., Beacham, J. L., Stevenson, J. C., Morris, H. R., et al. (1986). Human Calcitonin Gene Related Peptide: a Potent Endogenous Vasodilator in Man. *Clin. Sci. (Lond)* 70 (4), 389–393. doi:10.1042/cs0700389

Stucky, N. L., Gregory, E., Winter, M. K., He, Y. Y., Hamilton, E. S., McCarson, K. E., et al. (2011). Sex Differences in Behavior and Expression of CGRP-Related Genes in a Rodent Model of Chronic Migraine. *Headache* 51 (5), 674–692. doi:10.1111/j.1526-4610.2011.01882.x

Sun, H., Dodick, D. W., Silberstein, S., Goadsby, P. J., Reuter, U., Ashina, M., et al. (2016). Safety and Efficacy of AMG 334 for Prevention of Episodic Migraine: a Randomised, Double-Blind, Placebo-Controlled, Phase 2 Trial. *Lancet Neurol.* 15 (4), 382–390. doi:10.1016/s1474-4422(16)00019-3

Taylor, C. K., Smith, D. D., Hulce, M., and Abel, P. W. (2006). Pharmacological Characterization of Novel Alpha-Calcitonin Gene-Related Peptide (CGRP) Receptor Peptide Antagonists that Are Selective for Human CGRP Receptors. *J. Pharmacol. Exp. Ther.* 319 (2), 749–757. doi:10.1124/jpet.106.108316

Udawela, M., Christopoulos, G., Tilakaratne, N., Christopoulos, A., Albiston, A., and Sexton, P. M. (2006). Distinct Receptor Activity-Modifying Protein Domains Differentially Modulate Interaction with Calcitonin Receptors. *Mol. Pharmacol.* 69 (6), 1984–1989. doi:10.1124/mol.105.021915

Walker, C. S., Eftekhari, S., Bower, R. L., Wilderman, A., Insel, P. A., Edvinsson, L., et al. (2015). A Second Trigeminal CGRP Receptor: Function and Expression of the AMY1 Receptor. *Ann. Clin. Transl Neurol.* 2 (6), 595–608. doi:10.1002/acn3.197

Walker, C. S., Li, X., Whiting, L., Glyn-Jones, S., Zhang, S., Hickey, A. J., et al. (2010). Mice Lacking the Neuropeptide Alpha-Calcitonin Gene-Related Peptide Are Protected against Diet-Induced Obesity. *Endocrinology* 151 (9), 4257–4269. doi:10.1210/en.2010-0284

Walker, C. S., Raddant, A. C., Woolley, M. J., Russo, A. F., and Hay, D. L. (2018). CGRP Receptor Antagonist Activity of Olcegepant Depends on the Signalling Pathway Measured. *Cephalalgia* 38 (3), 437–451. doi:10.1177/0333102417691762

Watkins, H. A., Au, M., Bobby, R., Archbold, J. K., Abdul-Manan, N., Moore, J. M., et al. (2013). Identification of Key Residues Involved in Adrenomedullin Binding to the AM1 Receptor. *Br. J. Pharmacol.* 169 (1), 143–155. doi:10.1111/bph.12118

Williams, E. T., Harris, P. W. R., Jamaluddin, M. A., Loomes, K. M., Hay, D. L., and Brimble, M. A. (2018). Solid-Phase Thiol-Ene Lipidation of Peptides for the Synthesis of a Potent CGRP Receptor Antagonist. *Angew. Chem. Int. Ed. Engl.* 57 (36), 11640–11643. doi:10.1002/anie.201805208

Woolley, M. J., Reynolds, C. A., Simms, J., Walker, C. S., Mobarec, J. C., Garelja, M. L., et al. (2017). Receptor Activity-Modifying Protein Dependent and Independent Activation Mechanisms in the Coupling of Calcitonin Gene-Related Peptide and Adrenomedullin Receptors to Gs. *Biochem. Pharmacol.* 142, 96–110. doi:10.1016/j.bcp.2017.07.005

Yan, L. Z., Johnson, K. W., Rothstein, E., Flora, D., Edwards, P., Li, B., et al. (2011). Discovery of Potent, Cyclic Calcitonin Gene-Related Peptide Receptor Antagonists. *J. Pept. Sci.* 17 (5), 383–386. doi:10.1002/psc.1358

Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002). Partitioning of Lipid-Modified Monomeric GFPs into Membrane Microdomains of Live Cells. *Science* 296 (5569), 913–916. doi:10.1126/science.1068539

Zuloaga, K. L., Davis, C. M., Zhang, W., and Alkayed, N. J. (2014). Role of Aromatase in Sex-specific Cerebrovascular Endothelial Function in Mice. *Am. J. Physiol. Heart Circ. Physiol.* 306 (7), H929–H937. doi:10.1152/ajpheart.00698.2013

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Determining the Effects of Differential Expression of GRKs and β-arrestins on CLR-RAMP Agonist Bias

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Signalling of the calcitonin-like receptor (CLR) is multifaceted, due to its interaction with receptor activity modifying proteins (RAMPs), and three endogenous peptide agonists. Previous studies have focused on the bias of G protein signalling mediated by the receptor and receptor internalisation of the CLR-RAMP complex has been assumed to follow the same pattern as other Class B1 G Protein-Coupled Receptors (GPCRs). Here we sought to measure desensitisation of the three CLR-RAMP complexes in response to the three peptide agonists, through the measurement of β-arrestin recruitment and internalisation. We then delved further into the mechanism of desensitisation through modulation of β-arrestin activity and the expression of GPCR kinases (GRKs), a key component of homologous GPCR desensitisation. First, we have shown that CLR-RAMP1 is capable of potently recruiting β-arrestin1 and 2, subsequently undergoing rapid endocytosis, and that CLR-RAMP2 and -RAMP3 also utilise these pathways, although to a lesser extent. Following this we have shown that agonist-dependent internalisation of CLR is β-arrestin dependent, but not required for full agonism. Overexpression of GRK2-6 was then found to decrease receptor signalling, due to an agonist-independent reduction in surface expression of the CLR-RAMP complex. These results represent the first systematic analysis of the importance of β-arrestins and GRKs in CLR-RAMP signal transduction and pave the way for further investigation regarding other Class B1 GPCRs.

Keywords: GPCRs (G protein-coupled receptors), signalling bias, CLR, β-arrestins, RAMPs, internalisation, GRK (G protein receptor kinase)

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INTRODUCTION

Most G protein-coupled receptors (GPCRs) are capable of trafficking to the plasma membrane and signalling in the absence of accessory proteins. The calcitonin like receptor (CLR), however, requires one of three receptor activity modifying proteins (RAMP1-3) for functional membrane expression. Each combination forms a distinct receptor, with a different signalling profile (Weston et al., 2016). RAMP and CLR expression vary across different tissues, creating a diverse profile of signalling from just one GPCR. These CLR-based receptors can respond to three endogenous agonists: calcitonin gene-related peptide (CGRP), adrenomedullin (AM), and adrenomedullin 2 (AM2) (Clark et al., 2021). CLR in complex with RAMP1 generates the CGRP receptor (CGRPR), as CGRP, an abundant neuropeptide that also plays roles in the cardiovascular system, is the most potent agonist for this

receptor in generating cAMP. CLR-RAMP2 generates the adrenomedullin 1 receptor (AM1R), with AM (a potent vasodilator) being the most potent at this receptor, and CLR-RAMP3 produces the AM receptor 2 (AM2R), where AM and AM2 are equipotent agonists. The cognate receptor for AM2 is unknown but the peptide, analogous to AM and also a vasodilator, is highly expressed in the heart (Clark et al., 2021).

The G protein and downstream signalling bias in response to all three agonists has been well documented for all CLR-RAMP complexes (Weston et al., 2016; Clark et al., 2021). However, the signalling of GPCRs is not limited to membrane G protein signalling. There is significant evidence that GPCRs are able to signal via β-arrestins, originally identified as terminators of G protein signalling (reviewed Gurevich and Gurevich, 2019). Furthermore, GPCRs, including CLR, are able to signal once internalised, from endosomes (Cahill et al., 2017; Yarwood et al., 2017; Nguyen et al., 2019). It is therefore important to consider β-arrestin recruitment and receptor internalisation when investigating the signalling bias of CLR.

Previously, GPCR signalling was thought of as binary, with agonists activating the receptor before being "turned off"; this is now deemed an oversimplification of the process. G protein-coupled receptor kinases (GRKs), which phosphorylate the receptor following prolonged stimulation, enable the recruitment of β-arrestins. This forms the start of the pathway for homologous desensitisation, leading to receptor internalisation via clathrin-coated pits. There are 7 GRKs, with GRK2-6 ubiquitously expressed and GRK1/7 restricted to photoreceptors. The extent to which each promotes β-arrestin recruitment at different receptors varies. Each phosphorylates different serine and threonine residues on the intracellular region of the GPCR (primarily the C terminal tail), leading to the so-called phosphorylation barcode (Nobles et al., 2011). This barcode, together with the recruitment of β-arrestins, can mediate β-arrestin-dependent signalling. However, it is not possible to predict the pattern in which GRKs phosphorylate receptors, as the consensus sequence for these phosphorylation sites is not fully established. Different patterns of phosphorylation are thought to convey different receptor conformational states, correlating with different downstream signalling pathways (Liggett, 2011). Similarly, activation of different G proteins can correlate with receptor phosphorylation by different GRKs; Gαq activity has recently been inversely linked with GRK5/6-mediated phosphorylation of the angiotensin receptor 1 (AT1R) (Kawakami et al., 2022). In addition to their canonical function, GRKs can be activated by GPCRs but subsequently phosphorylate non-GPCR targets, leading to further signalling cascades (Gurevich et al., 2012; reviewed; Gurevich and Gurevich, 2019). Furthermore, some GRKs have been shown to phosphorylate and sequester the β_2-adrenoceptor in an agonist independent manner, primarily GRK4, 5, and 6 (Ménard et al., 1996; Andresen, 2010).

To add further complexity to the mechanism of receptor desensitisation, there are two β-arrestin proteins, each with different signalling profiles. Following recruitment to the GPCR, β-arrestins were classically thought to sterically hinder the G protein, blocking further signalling mediated by this pathway and promote desensitisation through clathrin-mediated internalisation. However, recently it has become apparent that some GPCRs can continue to signal once internalised from the endosome. It is now appreciated that the GPCR-β-arrestin complex can assume two distinct conformations. The first is where the β-arrestin only binds to the phosphorylated tail of the GPCR, so facilitating internalisation without blocking G protein signalling; a so called GPCR-G protein-β-arrestin megaplex (Thomsen et al., 2016; Cahill et al., 2017; Nguyen et al., 2019). In the second conformation, the β-arrestin adopts a closed conformation binding to the intracellular core of the GPCR, blocking G protein access (Nguyen et al., 2019). Beyond their role in blocking G protein signalling and mediating internalisation, β-arrestins are believed to act as scaffold proteins, eliciting further signalling pathways. These signalling pathways are distinct from those which are G protein-mediated, and in some cases therapeutically favourable. Some orthosteric (Wisler et al., 2007) and allosteric (Slosky et al., 2020) ligands for GPCRs exploit this β-arrestin-biased signalling in order to exert their favourable pharmacological profiles.

The role of each β-arrestin and GRK in GPCR signalling is poorly understood. Some characterisation of the different isoforms' roles has been performed, primarily on certain class A GPCRs (Palczewski et al., 1995; Fredericks et al., 1996; Nash et al., 2018; Møller et al., 2020). Recently work has looked at the cannabinoid 2 (CB2) receptor, identifying very little effect when expressing each GRK in turn on the signalling capacity of this receptor (Patel et al., 2021). Previous work has shown roles for GRK2, 3, and 4 in increasing CLR-RAMP2 internalisation when stimulated with AM (Kuwasako et al., 2010) and a role for GRK6 in mediating desensitisation of CLR-RAMP1 (Aiyar et al., 2000), but no investigation of the role of GRKs on desensitisation of CLR-RAMP3 has been conducted. We therefore sought to fully characterise the β-arrestin recruitment and internalisation profile of CLR in complex with each of the RAMPs and identify the role of GRKs in the aforementioned.

Here, we examined signalling bias for cAMP, β-arrestin recruitment, and internalisation for each CLR-RAMP complex. Furthermore, we investigated the role of β-arrestins in agonist dependent internalisation, through chemical inhibition with barbadin, and genetic manipulation of β-arrestin expression. The effects of barbadin were inconclusive, with no effect on receptor internalisation, but a significant decrease in cAMP accumulation. We determined that agonist-stimulated internalisation is dependent on β-arrestins, but cAMP accumulation appeared relatively independent of any internalisation. We then looked at increasing GRK expression, in an attempt to increase β-arrestin recruitment. However, we found that increasing GRK expression (in particular GRK4, 5, and 6) led to an agonist-independent decrease in receptor surface expression, likely due to constitutive phosphorylation and internalisation.

METHODS

Materials

CGRP, AM, and AM2 were purchased from Bachem and dissolved at 1 mM in water with 0.1% BSA *w/v*. Barbadin

(Aobious, Gloucestershire, United Kingdom) was dissolved at 10 mM in DMSO.

Constructs and Sources

CLR containing a direct C-terminal inframe fusion to NanoLuc was generated in pcDNA3.1(−) (pcDNA3.1(−)-CLR-Nluc) using standard molecular cloning techniques by Sabrina Carvalho (University of Cambridge). pcDNA3.1(+)FLAG-RAMPs and pcDNA3.1-HA-CLR have been described previously (Weston et al., 2016; Harris et al., 2021). pcDNA3.1(+)-hGRKs (Patel et al., 2020) were donated by Professor Michelle Glass and Dr David Finlay (University of Otago). β-arrestin1/2-YFP (Mackie et al., 2019) were donated by Professor Kathleen Caron (Chappell Hill, North Carolina). RIT-Venus (Jimenez-Vargas et al., 2018), Rab5a-Venus, Rab7-Venus, and Rab11-Venus (Jimenez-Vargas et al., 2020) were donated by Luke Pattinson (University of Cambridge).

Transfection and Cell Culture

HEK293T, HEK293, and HEK293Δβ-arrestin1/2 (donated by Dr Asoka Inoue, Tokyo University) cells were grown in DMEM/F12 Glutamax (ThermoScientific) supplemented with 1% antibiotic-antimycotic solution (AA) and 10% Foetal Bovine Serum (FBS) *v/v*. Cells were grown at 37°C with 5% CO_2 in a humidified incubator. Cells were transfected using polyethylenimine (PEI, Polyscience Inc.), at a 6:1 ratio of PEI:DNA *v/w*, diluted in 150 mM NaCl.

Measurement of Intracellular cAMP Accumulation

cAMP accumulation was measured in the HEK293 cell lines as previously described (Knight et al., 2016; Weston et al., 2016). Briefly, cells transfected with CLR-Nluc, FLAG-RAMP1/2/3, GRK2/3/4/5/6, and β-arrestin1/2-YFP (or pcDNA3.1(+) substitutes) in a 1:1:4:5 ratio were harvested using Trypsin-EDTA to bring into single cell suspension, before being resuspended in stimulation buffer (SB, phosphate buffered saline containing 0.1% BSA *w/v*). Cells were plated at 500 cells per well of a 384-well optiplate (PerkinElmer) and stimulated with agonist for 30 min cAMP accumulation was detected using the LANCE *ultra* cAMP detection kit on a Mithras LB 940 multimode microplate reader (Berthold Technologies). For experiments looking at changing β-arrestin expression, in HEK293T, HEK293 or HEK293Δβ-arrestin1/2 cells, cells were incubated in stimulation buffer containing 500 μM isobutylmethylxanthine (IBMX). For experiments utilising barbadin, cells were preincubated with 10 μM barbadin, or 1% DMSO *v/v* for 30 min prior to stimulation. Data were normalised to the maximal level of cAMP accumulation from cells stimulated with 100 μM Forskolin (Sigma).

Quantification of β-arrestin Recruitment to CLR-RAMP Complexes

β-arrestin recruitment assays were performed as previously described (Marti-Solano et al., 2020). Briefly, HEK293T cells were transfected with CLR-Nluc, FLAG-RAMP1/2/3, GRK2/3/4/5/6, and β-arrestin1/2-YFP (or pcDNA3.1(+) substitutes) in a 1:1:4:5 ratio. 24 h later cells were seeded onto 0.01% Poly-L-lysine coated white 96-well CulturPlates (Perkin Elmer) at 50,000 cells per well in growth media. After 24 h, media was removed, and cells washed in PBS. Cells were then incubated in the dark in buffer containing PBS, 0.49 mM $MgCl_2.6H_2O$, 0.9 mM $CaCl_2.2H_2O$, 0.1% BSA *w/v* and 5 μM of coelenterazine-*h* (Nanolight Technology) for 10 min, before addition of agonist in the range 1 μM to 10 pM. β-arrestin recruitment was recorded for at least 20 min, at 60 s intervals, and measured via a change in the BRET ratio between the donor (λ = 460 nm) and acceptor (λ = 530 nm), using a Mithras LB 940 multimode plate reader.

Quantification of CLR-RAMP Internalisation and Localisation to Endosomal Compartments

HEK293T, HEK293, or HEK293Δβ-arrestin1/2 cells were transfected with CLR-Nluc, FLAG-RAMP1/2/3, and a Venus tagged membrane GTPase (RIT), or endosomal markers Rab5a, Rab7, or Rab11, at a 1:1:5 ratio. After 24 h, cells were seeded onto 0.01% Poly-L-lysine coated white 96-well CulturPlates in growth media. Following a further 24 h, media was removed, and cells washed with Krebs (125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$) containing 0.1% BSA w/v before being incubated in 0.1% NanoGlo reagent (Promega) v/v for 5 min. Where barbadin was used, cells were incubated in KREBs buffer containing 10 μM inhibitor for 30 min prior to addition of NanoGlo reagent. Agonists were added in the range 1 μM to 0.1 nM and internalisation measured over 60 min at 120 s intervals via a change in the BRET ratio between the donor (λ = 460 nm) and acceptor (λ = 530 nm), using a Mithras LB 940 multimode plate reader.

Reverse Transcriptase PCR to Determine Endogenous Expression of GRKs in HEK293 Cells

RNA was extracted from HEK293 and HEK293T cells using RNeasy mini kit (Qiagen) as per the manufacturer's protocol. Complementary DNA was generated using the QuantiTect reverse transcription kit (Qiagen) following the manufacturer's instructions with minus Reverse Transcriptase negative controls performed simultaneously. PCR amplification was performed as previously described (Bailey et al., 2019; Safitri et al., 2020) using the following gene-specific primers: GADPH, forward (5′-TGC ACCACCAACTGCTTAGC-3′) and reverse (5′-GGCATGGAC TGTGGTCATGAG-3′); GRK1, forward (5′-GGAGTTTGAGAG TGTGTGCTT-3′) and reverse (5′-GCTTCTCTGCCGATTGTA GGA-3′); GRK2, forward (5′-TCCAGCCATACATCGAAG AGA-3′) and reverse (5′-CAAAACCGTGTGAACTTATCGC-3′); GRK3, forward (5′-CCGATGTCAGTTACCTGATGGC-3′) and reverse (5′-GCAGGACGATCCTCTTGCT-3′); GRK4, forward (5′-GGAAAGGCAACCCGTAACAAA-3′) and reverse (5′-AGGCGCAAACCTCTCCAAATC-3′); GRK5

forward (5′-CCAACACGGTCTTGCTGAAAG-3′) and reverse (5′-TCTCTGTCTATGGTCCTTCGG-3′); GRK6, forward (5′-GAGAACATCGTAGCGAACACG-3′) and reverse (5′-CAG GCTGTGATAGTCACGCTC-3′); β-arrestin1, forward (5′-AAAGGGACCCGAGTGTTCAAG-3′) and reverse (5′-CGT CACATAGACTCTCCGCT-3′); β-arrestin2, forward (5′-TCC ATGCTCCGTCACACTG-3′) and reverse (5′-ACAGAAGGC TCGAATCTCAAAG-3′); RAMP1, forward (5′-CTGCCAGGA GGCTAACTACG-3′) and reverse (5′-GACCACGATGAAGGG GTAGA-3′); RAMP2, forward (5′- GGGGGACGGTGAAGA ACTAT-3′) and reverse (5′-GTTGGCAAAGTGGATCTGGT-3′); RAMP3, forward (5′-AACTTCTCCCGTTGCTGCT-3′) and reverse (5′- GACGGGTATAACGATCAGCG-3′); CLR, forward (5′-ACCAGGCCTTAGTAGCCACA-3′) and reverse (5′-ACAAATTGGGCCATGGATAA-3′). Products were resolved on a 2% agarose gel and imaged using a G:Box iChemi gel documentation system. Densitometry was performed using GeneTools (Syngene) and data were normalized to GAPDH expression.

Quantification of Cell Surface Expression

For CLR surface expression, HEK293 or HEK293Δβ-arrestin1/2 cells were transfected with HA-CLR, FLAG-RAMP1/2/3 and GRK2/3/4/5/6 at a 1:1:4 ratio. After 48 h, 300,000 cells were washed twice with FACS buffer (PBS supplemented with 1% BSA and 0.03% sodium azide) before and after incubation with phycoerythrin (PE)-conjugated mouse anti-HA monoclonal antibody (BioLegend, diluted 1:200 in FACS buffer) for 1 h at room temperature in the dark. Samples were analysed using a BD Accuri C6 flow cytometer, Ex. λ 488 nm and Em. λ 585 nm. Data were normalised to the median APC intensity of cells transfected with pcDNA3.1 as 0% and HA-CLR + FLAG-RAMP1+ pcDNA3.1 as 100%. For RAMP cell surface expression in the presence and absence of agonist, HEK293T cells were transfected with either HA-CLR or CLR-Nluc and FLAG-RAMP1/2/3 at a 1:1 ratio. After 48 h, cells were washed and treated with appropriate agonist (CGRP for CLR-RAMP1 and AM for CLR-RAMP2/3) or vehicle for 30 min. Cells were then washed with ice cold PBS, harvested, assayed as described above and kept at 4°C throughout. Data were normalized to the median APC intensity of cells transfected with pcDNA3.1 as 0% and vehicle treated HA-CLR + FLAG-RAMP2 cells as 100%. Percentage internalisation is expressed relative to vehicle treated cells and cells expressing pcDNA3.1 + FLAG-RAMP.

Data Analysis

Pharmacological data was analysed in GraphPad Prism v9.0 (GraphPad Software, San Diego). Data were fitted using the three-parameter logistic equation to obtain concentration-response curves and estimates for values of Emax and pEC_{50}. Emax was constrained to below 100 for cAMP accumulation assays, and to below the highest response observed in β-arrestin and internalisation assays, which corresponds to CGRP at CLR-RAMP1. Statistical differences were analysed using either a one-way ANOVA followed by Dunnett's *post-hoc*, a Kruskal-Wallis One-Way ANOVA test with Dunn's post-test was used or a two-way ANOVA followed by a Tukey's multiple comparisons test (for comparisons amongst more than two groups) as appropriate. Where comparisons are made between two groups a two-tailed Student's t-test was used. cAMP data was normalised to 100 μM forskolin stimulation. The means of individual experiments were combined to generate the concentration-response curves displayed in the figures. Heatmaps were generated using the pEC_{50} values calculated from concentration-response curves of the mean of the data, or using the Emax values from the same data, normalised to the response of the cognate ligand at a given receptor. Where no response was observed in the absence of GRK, the maximal change in pEC_{50} was assumed, and the normalised Emax value used.

RESULTS

Quantifying Agonist-dependent Desensitisation Bias at the Three CLR-RAMP Complexes

G protein-mediated signalling bias has been well documented for CLR when co-expressed with each of the RAMPs. However, we wanted to examine the bias pattern regarding β-arrestin recruitment and internalisation, as both are important factors in functionality of Class B1 GPCRs, and there is yet to be a global study looking at the desensitisation of each CLR-RAMP complex with all three agonists. We have previously demonstrated HEK293T cells endogenously express low levels of CLR and RAMPs (Bailey et al., 2019), which yields a small response to CGRP and AM (and an even lesser response to AM2), at a far lower potency than would be expected of CLR in complex with any of the three RAMPs (**Supplementary Figure S1A**). We then determined the suitability of the C-terminal Nluc-tagged CLR with individual FLAG-tagged RAMPs for measuring cAMP accumulation. FLAG-RAMPs have previously been shown to signal comparably to other N terminally tagged RAMPs when coexpressed with CLR (Harris et al., 2021). When compared to HA-CLR, which has previously been used to characterise G protein signalling of the CLR-RAMP complexes (Weston et al., 2016; Harris et al., 2021), CLR-Nluc displayed a reduced potency (~10-fold compared to HA-CLR), but the same rank order of potency of the three peptides at the different RAMP complexes was observed (Compare **Figure 1A** with **Supplementary Figure S1B**).

Given the suitably similar potency profile observed with CLR-Nluc, we next quantified cAMP accumulation, β-arrestin1/2 recruitment (using arrestins each containing an in-frame fusion with a C-terminal YFP) and agonist-dependent internalisation (assayed using a Venus-YFP tagged plasma membrane GTPase RIT) of CLR-Nluc expressed with each FLAG-RAMP (**Figures 1A–D**; **Supplementary Figure S2**; **Supplementary Table S1**). Consistent with our previous reports for cAMP accumulation, CGRP was the most potent agonist at the CGRPR and AM at the AM1R, although all three peptides were observed to be reasonably equipotent at the AM2R. Significantly, these rank orders of potency also translated to both β-arrestins with CGRP being the most potent at RAMP1 (pEC_{50} of 7.56 ± 0.06 at β-arrestin1 and 7.49 ± 0.06 at β-arrestin2), AM at RAMP2 (pEC_{50} of 7.57 ± 0.17 at β-arrestin1 and 7.48 ± 0.09 at β-arrestin2), and all three agonists being equipotent at β-arrestin2

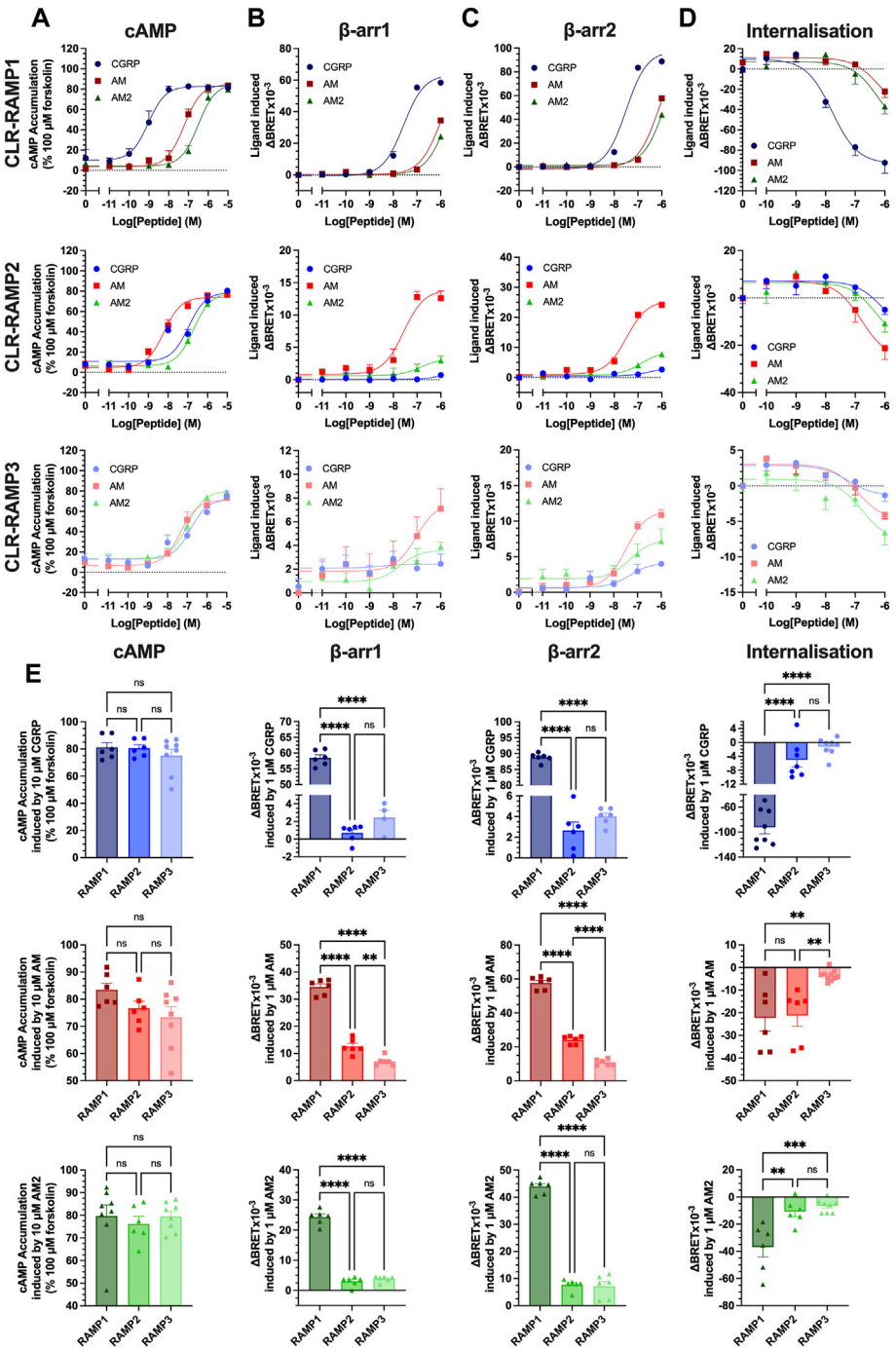

FIGURE 1 | Only cognate agonists of CLR-RAMP complexes recruit β-arrestins and induce internalisation. HEK293T cells expressing CLR-RAMP complexes were assayed for cAMP accumulation **(A)**, β-arrestin1 **(B)**, β-arrestin2 **(C)** recruitment, and internalisation **(D)** at CLR in complex with RAMP1, RAMP2, and RAMP3. **(E)** Responses to 10 μM or 1 μM CGRP, AM, and AM2 at cAMP, β-arrestin1, β-arrestin2, and internalisation. Statistical significance between RAMPs for each peptide was determined, at $p < 0.05$, through One-Way ANOVA with Dunnett's post-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Data are shown as mean with error bars indicating the SEM of *n* repeats where *n* ranges between 3 and 5 duplicates.

recruitment at RAMP3 (pEC_{50} of 7.31 ± 0.45, 7.49 ± 0.14, and 7.31 ± 0.46 for CGRP, AM, and AM2 respectively). Unlike cAMP accumulation assays, not all agonists were able to elicit β-arrestin recruitment at all CLR-RAMP complexes, with no response observed for CGRP at CLR-RAMP2, and no β-arrestin1 recruitment observed for CGRP at CLR-RAMP3.

In contrast to the cAMP accumulation assays where each CGPR-based peptide was able to act as a full agonist irrespective

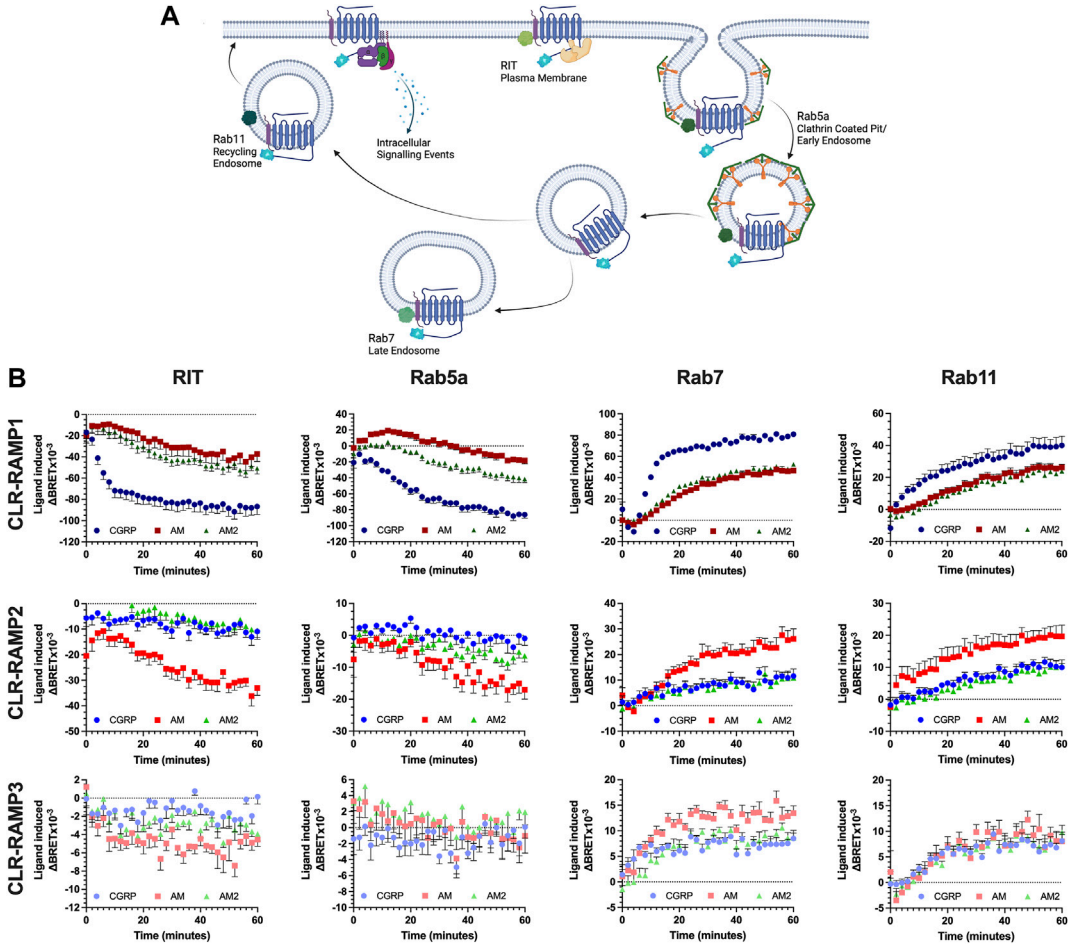

FIGURE 2 | CLR-RAMP1 in the only complex to undergo appreciable internalisation and trafficking, leading towards degradation. Schematic (created with BioRender.com) showing the internalisation and subcellular trafficking of CLR in complex with a RAMP, identifying the different membrane GTPases used **(A)**. Change in colocalisation of CLR over 60 min stimulation with each peptide as determined in HEK293T cells, for RIT, Rab5a, Rab7, and Rab11 when in complex with RAMP1, RAMP2, and RAMP3 **(B)**. Data are shown as mean with error bars indicating the SEM of *n* repeats where *n* = 3 duplicates.

of the CLR-RAMP complex expressed, only the cognate agonists were able to elicit full β-arrestin recruitment within the concentrations tested. Furthermore, comparison of the overall magnitude of agonist-mediated β-arrestin1/2 recruitment at the respective CLR-RAMP complexes highlighted that all three agonists at CLR-RAMP1 induced significantly higher maximal responses compared to the RAMP2 and RAMP3 complexes ($p < 0.0001$) (**Figure 1E**). This trend continued in the agonist-induced CLR internalisation assays, suggesting a direct correlation between β-arrestin recruitment and receptor internalisation.

In order to validate the observed internalisation of the three CLR-RAMP complexes, we measured the internalisation of FLAG-RAMP1-3 when co-expressed with HA-CLR or CLR-Nluc. CLR-RAMP complexes are thought to exist in a 1:1 stoichiometry (Hilairet et al., 2001), and internalise as a complex, as such FLAG-RAMP surface expression can be considered a proxy for CLR internalisation. Although FLAG-RAMP1 displayed a higher cell surface expression when co-expressed with HA-CLR than CLR-Nluc, each complex displayed significant internalisation, which was broadly similar across all three RAMPs (**Supplementary Figure S3**).

Tracking CLR-RAMP Subcellular Trafficking With Endosomal Markers

Previous studies have focused on the internalisation and trafficking of a single CLR-RAMP complex. CLR-RAMP1 has been shown to internalise as a complex in a β-arrestin dependent manner (Hilairet et al., 2001; Gingell et al., 2020). Similarly, in response to AM, CLR-RAMP2 underwent internalisation, in a manner dependent on the C terminal tail of the receptor, implicating GRKs and β-arrestins in its internalisation (Kuwasako et al., 2010). The role of the PDZ-interacting domain, found at the C-terminus of RAMP3, on CLR subcellular trafficking has been studied previously; this region is able to interact with subcellular proteins such as the Na+/H+ exchanger regulatory factor-1 (NHERF-1) (Bomberger et al., 2005b) and N-ethylmaleimide-sensitive factor (NSF)

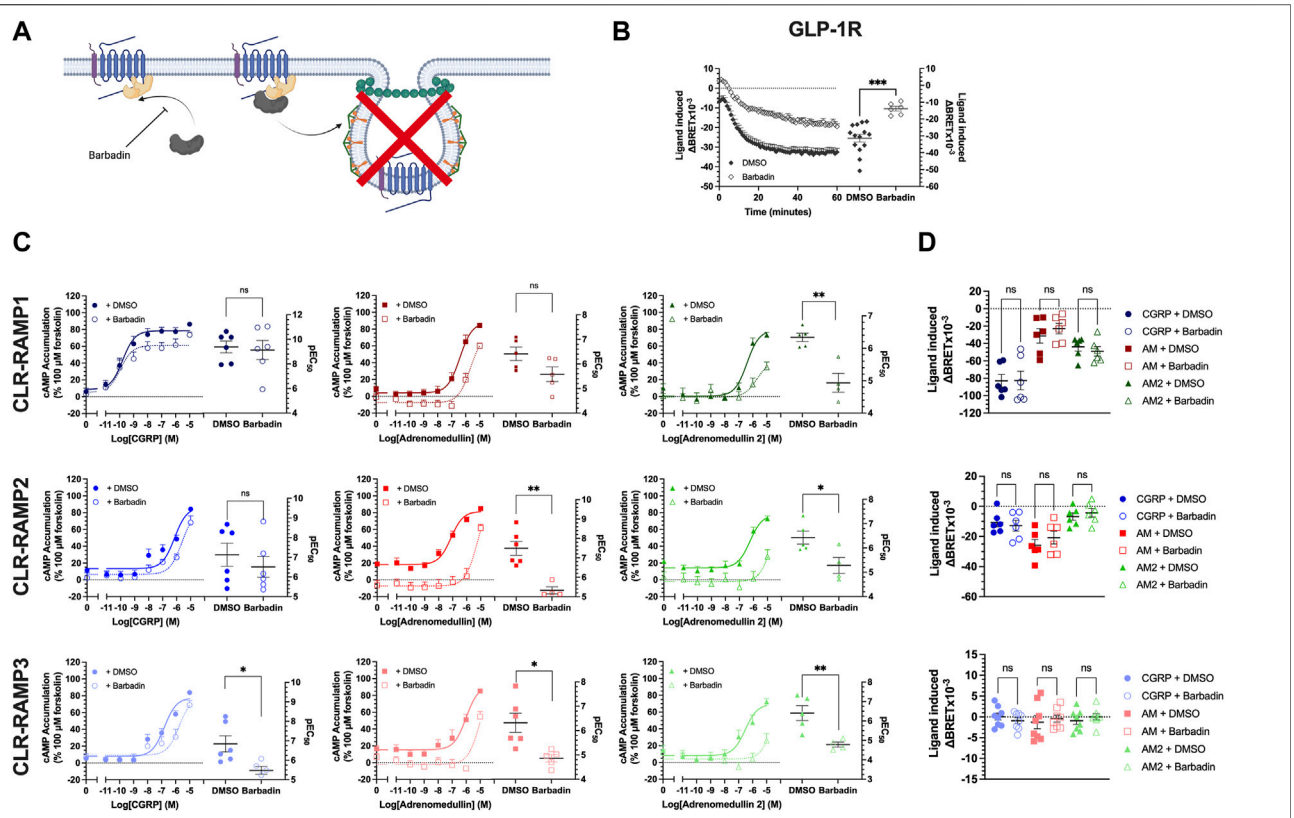

FIGURE 3 | Barbadin significantly impairs cAMP accumulation at CLR-RAMP complexes in an internalisation independent manner. **(A)** Schematic (created with BioRender.com) indicating the role of barbadin, a small molecule inhibitor of the β-arrestin-AP2 complex. **(B)** Effects of barbadin on another Class B1 GPCR, the GLP1R. **(C)** cAMP accumulation mediated by each peptide agonist following a 30 min pretreatment with DMSO (closed symbols, solid line) or 100 μM barbadin (open symbol, dotted line), for RAMP1, RAMP2, and RAMP3. Differences in, and potency values are reported on the adjoining scatter plots. **(D)** Effect of 30 min pretreatment with DMSO (closed symbols) or barbadin (open symbols) on internalisation of CLR-RAMP1, RAMP2, or RAMP3. Statistical significance between vehicle (DMSO) and barbadin treated cells was determined, at $p < 0.05$, using Student's t-test (*, $p < 0.05$; **, $p < 0.01$). Data are shown as mean with error bars indicating the SEM of n repeats where n = ranges between 3 and 6 duplicates.

(Bomberger et al., 2005a) to regulate internalisation and recycling of the CLR-RAMP3 complex respectively. Comparisons looking at the relative internalisation and trafficking of each CLR-RAMP complex in response to all three peptides have yet to be performed. We utilised RIT as a marker for the plasma membrane, the early endosomal marker Rab5a, as well as the late endosomal marker Rab7 and the recycling endosomal marker Rab11 (**Figure 2A**), each tagged with Venus-YFP to measure colocalisation with the C-terminal Nluc-tagged CLR.

Consistent with the signalling data, the cognate ligand for each CLR-RAMP complex displayed the greatest internalisation (as quantified by loss of a BRET signal between CLR-Nluc and Venus YFP-RIT) and subcellular trafficking, as determined through colocalization with each GTPase in turn (**Figure 2B**). This translated across all compartments. Due to the dynamic nature of the passage through the early endosome (rapid entry and departure), only for the cognate agonists at CLR-RAMP1 and -RAMP2 could any substantial change in colocalisation over time be observed. The increase in colocalization with Rab7 suggests CLR is degraded as a means of desensitisation, although colocalization with Rab11 indicates it is capable of recycling back to the membrane. The AM2R (RAMP3-CLR complex) displayed very little change in colocalization with RIT at the plasma membrane or with Rab5a when stimulated with any of the peptides, however some increase in colocalization with Rab7 and Rab11 was observed, indicating a very small level of internalisation does occur.

Use of the Small Molecular Inhibitor Barbadin Reduces cAMP Accumulation Independently of Internalisation

The canonical role of β-arrestins is the desensitisation of G protein signalling; steric hindrance to disrupt G protein-receptor association and acting as scaffolds for proteins which decrease the G protein activity and output e.g. phosphodiesterases (PDEs) (Perry et al., 2002). Additionally, β-arrestins mediate agonist dependent internalisation at many GPCRs, through the recruitment of β2-adaptin (AP2) and subsequently clathrin, to mediate endocytosis. Hence, we attempted to decouple these two mechanisms, using the small molecule inhibitor barbadin. Barbadin is an inhibitor of the β-arrestin-AP2 interacting

domain, therefore inhibiting clathrin-mediated endocytosis without inhibiting β-arrestin recruitment (Beautrait et al., 2017) (**Figure 3A**). In order to validate barbadin, we measured its effects on internalisation of the Glucagon-like peptide-1 receptor (GLP-1R), another Class B1 GPCR which undergoes clathrin-mediated endocytosis following β-arrestin recruitment (Fletcher et al., 2018). A 30 min preincubation with 100 μM barbadin was found to inhibit GLP-1R internalisation (**Figure 3**), therefore showing it is active at this concentration (**Figure 3B**).

Barbadin appeared to reduce cAMP accumulation for all agonists at the three CLR-RAMP complexes, although in some instances (e.g. for CGRP at the CLR-RAMP1 complex) these effects did not reach significance (**Figure 3C**, **Supplementary Table S3**). At the CLR-RAMP1 complex, the biggest differences were observed when AM2 was used as the stimulating agonist, with barbadin inhibiting signalling, and reducing both the potency and maximal response (pEC_{50} from 6.34 ± 0.13 to 4.93 ± 0.29; $p = 0.0021$, Emax from 79.8 ± 4.5 to 47.2 ± 8.9; $p = 0.02$). When looking at CLR-RAMP2, although the response to CGRP was again unaltered, the response to AM was significantly impaired (pEC_{50} reduced from 7.49 ± 0.36 to 5.33 ± 0.18; $p = 0.0017$). Again, the response to AM2 was far smaller when pretreated with barbadin. At CLR-RAMP3, all three agonists showed reduced potencies at stimulating cAMP accumulation when treated with barbadin (**Figure 3C**; **Supplementary Table S3**).

Finally, we then investigated the actions of barbadin treatment of CLR-RAMP complex internalisation (**Figure 3D**, **Supplementary Table S4**). Surprisingly, and in contrast to the actions observed at cAMP accumulation, barbadin treatment did not block agonist-mediated CLR internalisation for any CLR-RAMP complex when used at the concentration that blocked GLP-1R internalisation (**Figure 3B**). This data would suggest that barbadin could not be used to decouple CLR-RAMP internalisation from β-arrestin recruitment.

CLR Requires β-arrestins to Internalise, and This Internalisation Is Important for the Receptor to Achieve Maximal Signalling

Due to the inconclusive nature of the effects of barbadin, we then looked at the signalling in cells genetically modified to express different levels of β-arrestins. We used HEK293 cells and a modified line devoid of β-arrestin1 or 2 (O'Hayre et al., 2017), which displayed a similar expression of CLR and RAMPs as HEK293T cells (**Supplementary Figure S4**). No agonist-induced internalisation was observed in this cell line, leading us to conclude that CLR-RAMP internalisation is β-arrestin-dependent, despite observing no effect of barbadin (**Figure 4A**, **Supplementary Figure S5A**, **Supplementary Table S5**). We repeated the cAMP accumulation experiments in the presence of barbadin in these β-arrestin KO HEK293 cells, and an agonist dependent reduction in cAMP accumulation was again observed (**Supplementary Figure S5B**), suggesting at least part of the effects of barbadin occur independently of the β-arrestin.

Having considered the effects of deleting β-arrestins on CLR-RAMP internalisation, we next considered their impact on agonist-dependent cAMP accumulation. In HEK293 cells with β-arrestin knocked out, there was a significant trend towards increasing the potency when β-arrestins were knocked out ($p < 0.0001$) (**Figure 4B**, **Supplementary Table S6**). This was the greatest for CGRP at CLR-RAMP2 and CLR-RAMP3 (RAMP2, $p = 0.002$, RAMP3, $p = 0.007$). Finally, we determined the effects of overexpression of either β-arrestins on cAMP accumulation from the three CLR-RAMP complexes (**Figure 4C Supplementary Figure S5C**, **Supplementary Table S7**). Overall, there was a significant trend towards β-arrestin overexpression decreasing the potency of cAMP accumulation, indicating a small increase in desensitisation ($p < 0.0001$). Whilst this was not significant in most individual cases, there was a significant decrease in potency observed for AM2 at CLR-RAMP2 when β-arrestin2 was overexpressed ($p = 0.02$). It is likely that these effects are only small as HEK293T cells endogenously express high levels of β-arrestin1/2 (**Supplementary Figure S6A**). Overall, our data is supportive of the notion that RAMP-CLR complexes require β-arrestins to undergo receptor internalisation, and that modulation of β-arrestin expression can influence the potency and magnitude of the signalling response when stimulated with the CGRP-family of peptide agonists.

Overexpression of GRKs Induces Agonist-independent Internalisation of CLR-RAMP Complexes

There is growing evidence to suggest that different GRKs are responsible for mediating different levels of β-arrestin recruitment. Thus, having established that β-arrestin expression is important for CLR-RAMP complex signalling, we sought to determine which GRKs may be responsible for mediating these effects (**Figure 5A**). Analysis of GRK expression, using semi-quantitative rt-PCR, indicated that GRK2/3/4/5/6 were all expressed in all HEK293 cell lines in the study (**Supplementary Figure S6B**). Since both GRK1 and GRK7 expression is restricted to the retina neither was included in our analysis, nor were they included in the functional studies. We then strove to determine the effects of GRK overexpression on cAMP accumulation and β-arrestin recruitment at each CLR-RAMP complex with all three agonists (**Supplementary Figures S7–S9**). Strikingly, and somewhat surprisingly, overexpression of many of the GRKs appeared to significantly attenuate cAMP accumulation and recruitment of β-arrestins to the CLR-RAMP complexes upon agonist stimulation (**Figure 5B**, **Supplementary Table S8–S10**). Of the three CLR-RAMP complexes, CLR-RAMP1 appeared most resistant to GRK-mediated attenuation of signalling. The negative effects of GRK expression were most pronounced for GRK5 and GRK6 at all three CLR-RAMP complexes, with recruitment of β-arrestins to the CLR-RAMP2 or -RAMP3 complexes being abolished. The effects were least prevalent for GRK2 and GRK3, which in some cases enhanced signalling. In general, the cAMP responses were more resistant to GRK overexpression than β-arrestin-recruitment. This is not surprising, as accumulation of cAMP is a result of signal amplification from the agonist-activated GPCR, while β-arrestin

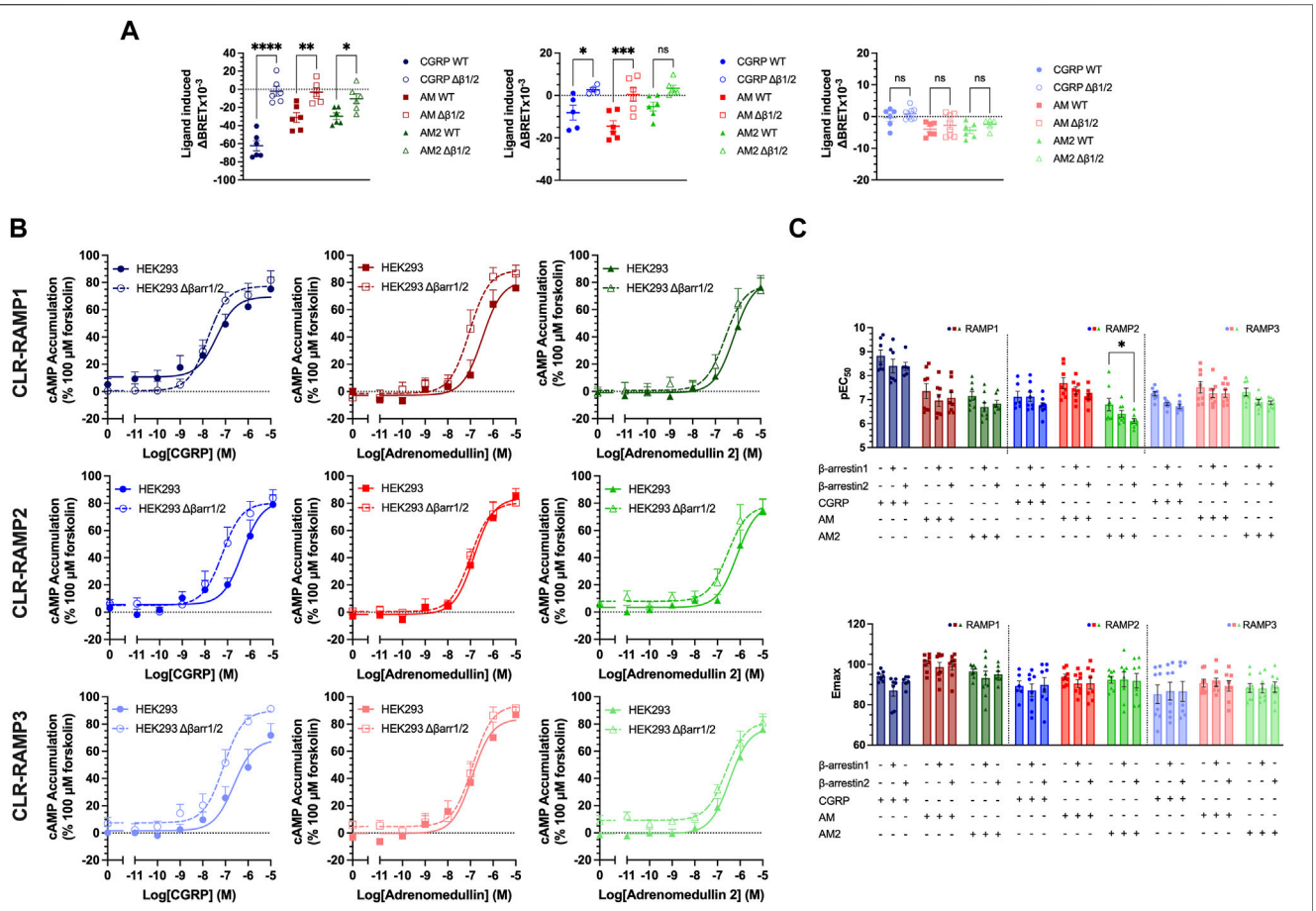

FIGURE 4 | Expression of β-arrestins reduced potency and efficacy of cAMP signalling for all CLR-RAMP complexes. **(A)** Changes in CLR internalisation in complex with RAMP1, RAMP2, or RAMP3 in HEK293 cells expressing (closed symbols) or lacking (open symbols) β-arrestin1/2. Statistical significance between responses in WT and β-arrestin1/2 KO cells was determined, at $p < 0.05$, through Student's t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). **(B)** Effect of knocking out β-arrestin1/2 on cAMP in cells expressing CLR-RAMP1, RAMP2, or RAMP3. **(C)** Bar charts showing the potency and maximal response of the peptides at CLR with each RAMP in turn, in cells overexpressing β-arrestin1 or 2. All data are mean ± SEM of *n* repeats where *n* ranges between 3 and 4 duplicates. Statistical significance of responses in the presence of overexpressed β-arrestins was compared to the response in the absence of β-arrestin, at $p < 0.05$, using a Two-Way ANOVA.

recruitment to the GPCR occurs at a 1:1 ratio. As a result, any loss of CLR-RAMP cell surface expression would be considered to have more of a deleterious effect upon β-arrestin recruitment than cAMP signalling. We therefore speculate that overexpression of GRKs might be leading to a reduction in CLR cell surface expression prior to agonist application.

To investigate this hypothesis, we investigated CLR cell surface expression in HEK293 cells using flow cytometry. CLR membrane expression was highest for CLR-RAMP1, and lowest for CLR-RAMP3, which showed only a small (~1.5 fold) increase above the expression in the absence of RAMP. For all three CLR-RAMP complexes, we observed reductions in CLR cell surface expression when each GRK was overexpressed (**Figure 5C**, **Supplementary Table S11**). Consistent with the signalling data, CLR expression was reduced the least when GRK2 and GRK3 were overexpressed for each RAMP complex. We next wondered if the GRK-induced agonist-independent CLR internalisation was dependent upon β-arrestins. CLR cell surface expression was still reduced when GRKs were overexpressed in the absence of β-arrestins although there was not difference between the GRKs, suggesting the more detrimental effects of GRK4/5/6 are in part dependent upon β-arrestins (**Figure 5D**, **Supplementary Table S11**). Overall, these data confirm that CLR is able to undergo GRK-mediated internalisation in an agonist independent manner, via a mechanism which is largely independent of β-arrestins.

DISCUSSION

While G protein mediated signalling at CLR has been extensively studied for the three endogenous peptide agonists at each CLR-RAMP complex (Weston et al., 2016; Clark et al., 2021), studies investigating G protein independent events, such as β-arrestin recruitment and internalisation have only been investigated for specific CLR-RAMP-peptide combinations (Chang and Hsu, 2019; Hendrikse et al., 2020). Here we provide the first global

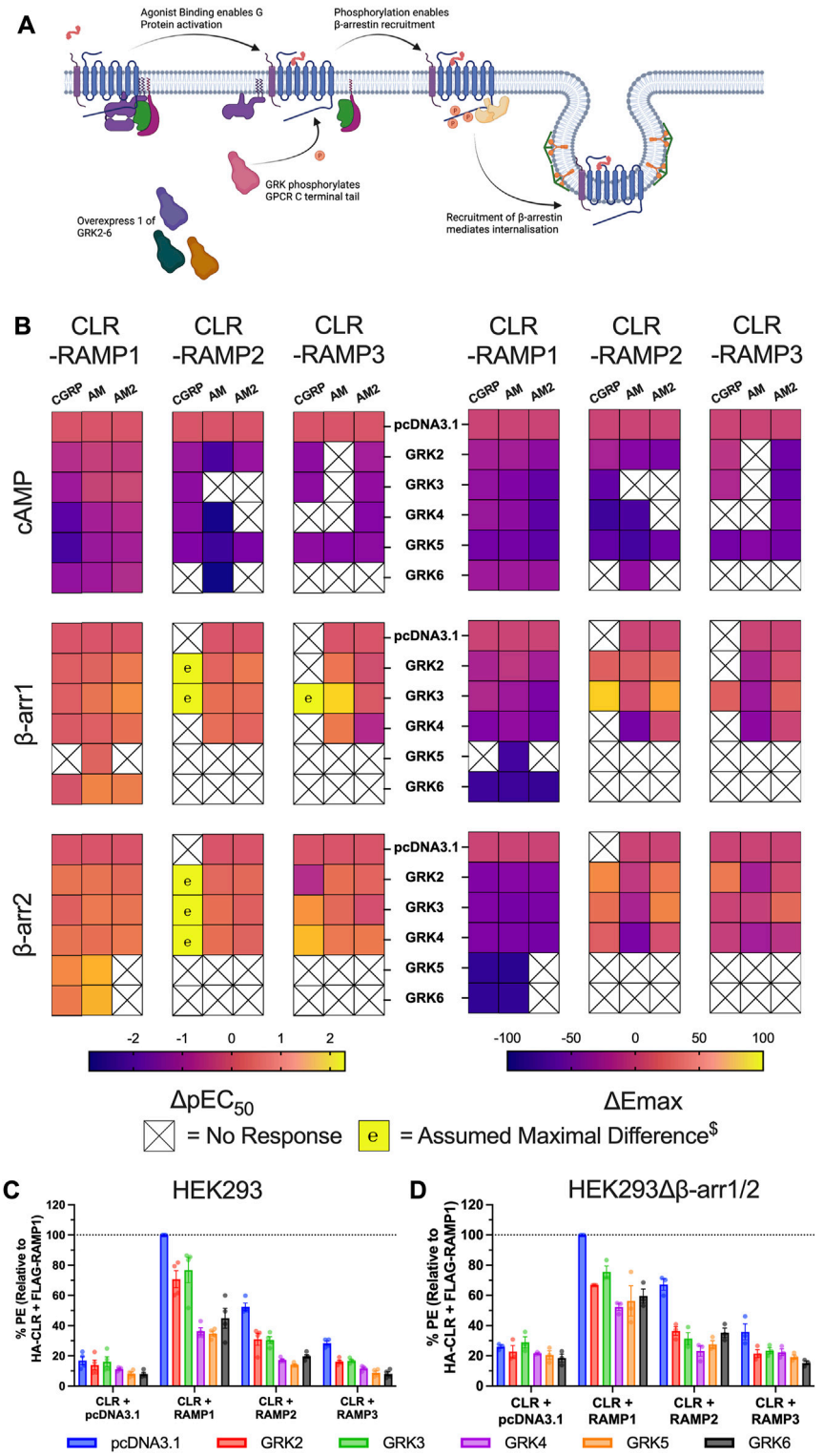

FIGURE 5 | GRK overexpression impairs CLR-RAMP complex signalling through constitutive receptor internalisation. **(A)** Schematic (created with BioRender.com) showing the role of GRKs in mediating GPCR desensitisation and internalisation, and highlighting how we manipulated this system for the experiment. **(B)** Heat maps illustrating the effects of overexpression of each GRK on the potency (left) and Emax (right) on cAMP accumulation, and β-arrestin1/2 recruitment at the three CLR-RAMP complexes when stimulated with CGRP, AM and AM2. $ Maximal change in pEC_{50} was assumed where no response was observed in the absence of GRK. **(C–D)** Cell surface expression of HA-CLR, measured using a phycoerythrin (PE)-conjugated anti-HA antibody, following overexpression of GRKs prior to addition of agonist in HEK293 cells **(C)** and HEK293Δβ-arrestin1/2 cells **(D)**. All data are mean ± SEM of *n* repeats where *n* ranges between 3 and 4 experiments.

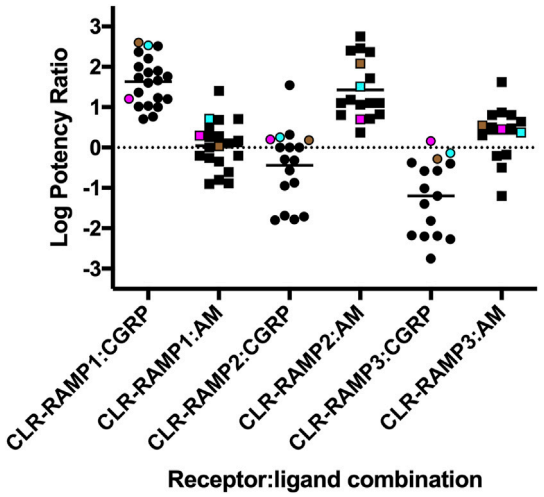

FIGURE 6 | Agonist potency rations for CGRP, AM and AM2 at the three different CLR-RAMP complexes. The log Potency ratios (as determined from cAMP accumulation assays) are defined as log(EC50 AM2/EC50 agonist). Data compiled from Weston et al., (2016), Garelja et al., (2020), Clark et al., (2021) and Harris et al., (2021). HEK293T cells expressing CLR-Nluc are shown in *cyan*, HEK293 expressing CLR-Nluc are shown as *magenta* and HEK293T cells expressing HA-CLR are shown in *brown*.

characterisation of β-arrestin recruitment, internalisation and endosomal sorting of the three CLR-RAMP complexes when stimulated with CGRP, AM and AM2. Our results for the β-arrestin recruitment to CLR-RAMP1 and -RAMP2 when stimulated with their cognate ligands are consistent with previous studies in terms of potency, despite the use of different cell backgrounds and CLR constructs (Chang and Hsu, 2019; Hendrikse et al., 2020). Furthermore, we suggest that CLR undergoes internalisation by context-dependent mechanisms, with our results providing important implications for other class B1 GPCRs. Finally, we highlight that GRK overexpression is deleterious to both G protein-dependent and independent signalling and thus caution must be applied when GRKs are routinely expressed to increase β-arrestin recruitment.

Initially, we determined that the rank order of potency for the CGRP-based peptides at each CLR-RAMP complex was consistent across the different assays e.g., the most potent peptide at cAMP was the most potent at β-arrestin recruitment and internalisation. Whilst all peptides were able to elicit maximal responses in the cAMP accumulation assay (with their relative potencies largely agreeing with previous observations (**Figure 6**), this was not the case for β-arrestin recruitment or receptor internalisation. Across all combinations, CLR-RAMP1 seemed the most capable of recruiting β-arrestins and internalising, followed by CLR-RAMP2 (~25%) and CLR-RAMP3 (~15%). The 1:1 nature of β-arrestin recruitment and internalisation means that the cell surface expression level of the different CLR-RAMP complexes may largely explain this observed difference between RAMPs; CLR-RAMP2, and -RAMP3 expression was 52 and 28% of CLR-RAMP1 respectively. When looking at the RAMP instead, each displayed comparable surface expression, which then correlated with comparable levels of internalisation. Furthermore, it is likely that all three agonists reached a maximal level of cAMP accumulation due to the substantial amplification in the pathway, indicating a receptor reserve. Correspondingly, only CLR-RAMP1 displayed substantial detectable subcellular trafficking, in agreement with previous studies (Yarwood et al., 2017), with the other complexes only displaying small colocalisation with each of the endosomal markers. It was found for CLR-RAMP1 and CLR-RAMP2 that there was an increase in Rab7 and Rab11 colocalisation after receptor internalisation, suggesting that both slow recycling and degradative pathways are employed. Little colocalization with these endosomal markers was observed for CLR-RAMP3, presumably due to its lack of significant internalisation.

The receptor internalisation was confirmed to be β-arrestin dependent through the use of a cell line where β-arrestin1 and 2 were genetically KO. However, inhibition of the β-arrestin-AP2 interaction, using barbadin, had no effect on receptor internalisation. Furthermore, it was determined that whilst barbadin was able to significantly reduce cAMP accumulation, a major part of its action was independent of the β-arrestin. This is supported by the observation that the effects of barbadin were similar in β-arrestin KO HEK293 cells and were agonist dependent, with no effect on CGRP at CLR-RAMP1, which undergoes the greatest internalisation. cAMP signalling was enhanced by the removal of β-arrestins (and therefore loss of internalisation), and correspondingly decreased by their overexpression, suggesting β-arrestin recruitment and internalisation is utilised by CLR as a traditional desensitisation pathway, as observed for many Class A GPCRs. This agrees with previous findings that reducing internalisation of CLR-RAMP2 through C terminal tail deletions increases cAMP accumulation by the receptor (Kuwasako et al., 2010).

The final part of this study has considered the effects of increasing GRK expression on CLR β-arrestin recruitment and cAMP accumulation. Here, we have shown that over-expression of GRKs had detrimental effects on receptor signalling. In particular, GRK5 and 6 significantly impaired β-arrestin recruitment and ablated the majority of the cAMP response. This was found to be agonist-independent, with reduced surface expression of CLR observed when GRKs were overexpressed, as had been previously observed for CLR-RAMP2 (Kuwasako et al., 2016). Interestingly, there was still a reduction in CLR surface expression in the HEK293Δβ-arrestin1/2 cells indicating β-arrestin independency. Some GPCRs have been shown to use endophilin to mediate dynamin-dependent internalisation that is independent of AP2, β-arrestin and clathrin (Boucrot and Ferreira, 2017). This mechanism is unlikely for CLR since it is mediated via a proline rich motif in the intracellular loop 3, which is absent from CLR. Other Class B1 GPCRs are known to internalise via caveolae (Thompson and Kanamarlapudi, 2015; Fletcher et al., 2018), and it has been noted that GRKs are able to interact with caveolin-1, indicating a potential role in caveolae-mediated endocytosis (Carman et al., 1999). Furthermore, CLR has been shown to co-immunoprecipitate with caveolin-1, with stimulation with CGRP reducing membrane localisation of caveolin-1 (Tang et al., 2013). Our analysis of the C-terminal tail identifies a potential motif ($I^{394/8.53b}$LRRNWNQY^{402}) which conforms to one of the consensus caveolin-1 interacting domains

(φXXXXφXXφ motif where X = any amino acid and φ = hydrophobic amino acids) (Couet et al., 1997). Thus, it is possible that the GRK-mediated agonist-independent internalisation of CLR could occur through caveolae. There is further precedent for context dependent mechanisms of internalisation as exemplified by the CB_1 cannabinoid receptor where agonist-induced internalisation is β-arrestin mediated, but agonist-independent internalisation is clathrin-dependent, but β-arrestin-independent (Gyombolai et al., 2013).

These studies have significance for other investigations into GPCR β-arrestin recruitment/internalisation. If the magnitude of β-arrestin recruitment is weak to the GPCR of choice, addition of GRKs is often used to increase the signal (Mackie et al., 2019; Harris et al., 2021). As demonstrated for CLR, this is not always appropriate as it can lead to the opposite effects if agonist-independent phosphorylation of GPCRs occurs.

The data we have obtained related to agonist-dependent internalisation of CLR in the presence of barbadin (**Figure 3C**) appear contradictory when compared to our results obtained with the HEK293Δβ-arr1/2 cell lines (**Figure 4A**). Barbadin has been suggested to block the interaction between β-arrestin and AP2 thereby inhibiting clathrin-dependent internalisation (Beautrait et al., 2017). It therefore seems unusual that barbadin did not block CLR internalisation. Barbadin has been shown to successfully block 5-HT2CR internalisation (He et al., 2021); β2-adrenergic (β2AR), V2-vasopressin (V2R), angiotensin-II type-1 (AT1R) receptors (Beautrait et al., 2017), free fatty acid receptor 2 (FFAR2) (Wang et al., 2020), protease-activated receptor 2 (PAR2) (Jung et al., 2021) and GLP-1R in our hands (**Figure 3A**). In the present study, we have used barbadin at the same concentrations as described previously and therefore we are unsure why it does not block agonist dependent CLR endocytosis. However, our observations that barbadin significantly attenuated cAMP signalling might provide some explanation. It is plausible that barbadin forces the β-arrestins to adopt a closed conformation on the agonist-occupied CLR which results in the G protein being unable to access the receptor, thus preventing signalling. This closed complex may then use an AP2 independent mechanism for internalisation, e.g. via caveolae. The studies from Yarwood et al., suggest that CLR-RAMP1 can also form a megaplex with both the G protein and the β-arrestins present to enable signalling from endosomal compartments (Yarwood et al., 2017). Presumably this complex uses AP2 for internalisation. As such our data suggests that the mechanism of CLR-RAMP internalisation may depend upon the conformation the β-arrestins adopt on the activated CLR. Further analysis will be required to determine the precise nature of barbadin's action, since it also displayed some activity in the HEK293Δβ-arrestin1/2 cells suggesting off target effects. Significantly, the differences observed between small molecular inhibitors and genetic manipulation highlighted here demonstrate the need for complimentary approaches to enable a more complete picture of the processes used for GPCR signal transduction.

Our study has highlighted the differences between the GRK subtypes in their ability to presumably phosphorylate non-agonist bound CLR. The effects, in the absence of agonist, were least detrimental to signalling with GRK2 and GRK3. This is probably not surprising since these two GRKs contain a pleckstrin homology (PH) domain which binds Gβγ domains (Pitcher et al., 1992; Koch et al., 1993). The interaction with Gβγ aids GRK2/3 recruitment to the plasma membrane. Thus, generally only active GPCRs will bring about recruitment of GRK2/3. The fact that GRK2/3 expression still results in a small attenuation to signalling may be explained by the endogenous expression of constitutively active GPCRs in the HEK293 cells used in this study. GRK4/5/6, on the other hand, do not contain the PH domain and associate with the plasma membrane through palmitoylation of C-terminal cysteine residues or through an amphipathic helix that interacts with the phospholipids found in the membrane (Gurevich et al., 2012; reviewed; Gurevich and Gurevich, 2019). As such, these GRKs have the potential to interact with and phosphorylate GPCRs independently of agonist binding. Prior to our study, GRK4 has been shown to constitutively phosphorylate the dopamine D1 receptor (Rankin et al., 2006), and both GRK5 and GRK6 have been reported to phosphorylate inactive GPCRs *in vitro* and *in vivo* (Tran et al., 2004; Baameur et al., 2010; Li et al., 2015). Our data directly aligns with these reports and appears to be the first example of agonist independent GRK phosphorylation for Class B1 GPCRs. Indeed, given these previously documented examples, it does seem unusual as to why only a limited set of inactive GPCRs are phosphorylated by GRK4/5/6.

It is important to highlight that when we investigated cell surface expression of CLR in the presence of the different RAMPs in our HEK293 and HEK293Δβ-arrestin1/2 cells, little cell surface expression above background was detected when RAMP3 was co-expressed with HA-CLR. Despite this low expression, it was sufficient to enable a full cAMP response to be detected upon agonist stimulation, which showed equivalent potency to other reports using equivalent HEK293 cells lines (Weston et al., 2016; Clark et al., 2021). However, the reduced expression of CLR with RAMP3, and to a lesser extent RAMP2 will almost certainly explain why it may have been hard to detect β-arrestin recruitment and internalisation, and why the overexpression of GRKs had such a dramatic effect on these two complexes. It will be interesting to determine if this low CLR expression in the presence of RAMP3 is observed in other cell lines and endogenous cells such as those found in the cardiovascular system.

To the best of our knowledge, the data described here is the first documented evidence of CLR-RAMP complexes undergoing agonist-independent internalisation. Early reports have demonstrated agonist-dependent internalisation for the CLR-RAMP1 complex (Kuwasako et al., 2000; Hilairet et al., 2001), but this was not observed in the absence of an agonist. Detailed reports related to AM1R and AM2R internalisation are rarer in the literature (Schönauer et al., 2015) and as such our study is the first comprehensive analysis of these events for all three CLR-RAMP complexes.

Overall, our study has highlighted that CLR internalisation is complex, being dependent on β-arrestins but apparently independent of AP2. Future work would need to investigate any potential AP2 interacting domain on CLR, or identify if RAMPs themselves are able to mediate internalisation in a β-arrestin dependent manner. Whilst C terminal phosphorylation by GRKs is important for receptor desensitisation, it appears the intense over expression, used to amplify β-arrestin recruitment, can result in agonist independent internalisation of the receptor, so caution must be exercised when overexpressing these proteins.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AP, TR-N, MH performed the experiments. DRP, MW, and GL conceived the idea and analysed data. AP and GL wrote the manuscript. All authors provided edits and comments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.840763/full#supplementary-material

REFERENCES

Aiyar, N., Disa, J., Dang, K., Pronin, A. N., Benovic, J. L., and Nambi, P. (2000). Involvement of G Protein-Coupled Receptor Kinase-6 in Desensitization of CGRP Receptors. *Eur. J. Pharmacol.* 403 (1-2), 1–7. doi:10.1016/s0014-2999(00)00419-2

Andresen, B. T. (2010). Characterization of G Protein-Coupled Receptor Kinase 4 and Measuring its Constitutive Activity *In Vivo*. *Meth Enzym* 484, 631–651. doi:10.1016/b978-0-12-381298-8.00031-9

Baameur, F., Morgan, D. H., Yao, H., Tran, T. M., Hammitt, R. A., Sabui, S., et al. (2010). Role for the Regulator of G-Protein Signaling Homology Domain of G Protein-Coupled Receptor Kinases 5 and 6 in β2-Adrenergic Receptor and Rhodopsin Phosphorylation. *Mol. Pharmacol.* 77, 405–415. doi:10.1124/mol.109.058115

Bailey, S., Harris, M., Barkan, K., Winfield, I., Harper, M. T., Simms, J., et al. (2019). Interactions between RAMP2 and CRF Receptors: The Effect of Receptor Subtypes, Splice Variants and Cell Context. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1861, 997–1003. doi:10.1016/j.bbamem.2019.02.008

Beautrait, A., Paradis, J. S., Zimmerman, B., Giubilaro, J., Nikolajev, L., Armando, S., et al. (2017). A New Inhibitor of the β-Arrestin/AP2 Endocytic Complex Reveals Interplay Between GPCR Internalization and Signalling. *Nat. Commun.* 8, 15054. doi:10.1038/ncomms15054

Bomberger, J. M., Parameswaran, N., Hall, C. S., Aiyar, N., and Spielman, W. S. (2005a2005). Novel Function for Receptor Activity-Modifying Proteins (RAMPs) in Post-endocytic Receptor Trafficking. *J. Biol. Chem.* 280, 9297–9307. doi:10.1074/jbc.m413786200

Bomberger, J. M., Spielman, W. S., Hall, C. S., Weinman, E. J., and Parameswaran, N. (2005b). Receptor Activity-Modifying Protein (RAMP) Isoform-specific Regulation of Adrenomedullin Receptor Trafficking by NHERF-1. *J. Biol. Chem.* 280, 23926–23935. doi:10.1074/jbc.M501751200

Boucrot, E., and Ferreira, A. P. A. (2017). Endophilin marks and Controls a Clathrin-independent Endocytic Pathway. *Nature* 517, 460. doi:10.1038/nature14067

Cahill, T. J., Thomsen, A. R. B., Tarrasch, J. T., Plouffe, B., Nguyen, A. H., Yang, F., et al. (2017). Distinct Conformations of GPCR-β-Arrestin Complexes Mediate Desensitization, Signaling, and Endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 114, 2562–2567. doi:10.1073/pnas.1701529114

Carman, C. V., Lisanti, M. P., and Benovic, J. L. (1999). Regulation of G Protein-Coupled Receptor Kinases by Caveolin. *J. Biol. Chem.* 274, 8858–8864. doi:10.1074/jbc.274.13.8858

Chang, C. L., and Hsu, S. Y. T. (2019). Development of Chimeric and Bifunctional Antagonists for CLR/RAMP Receptors. *PloS ONE* 14 (5), e0216996. doi:10.1371/journal.pone.0216996

Clark, A. J., Mullooly, N., Safitri, D., Harris, M., de Vries, T., MaassenVanDenBrink, A., et al. (2021). CGRP, Adrenomedullin and Adrenomedullin 2 Display Endogenous GPCR Agonist Bias in Primary Human Cardiovascular Cells. *Commun. Biol.* 4, 776. doi:10.1038/s42003-021-02293-w

Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997). Identification of Peptide and Protein Ligands for the Caveolin-Scaffolding Domain. *J. Biol. Chem.* 272, 6525–6533. doi:10.1074/jbc.272.10.6525

Fletcher, M. M., Halls, M. L., Zhao, P., Clydesdale, L., Christopoulos, A., Sexton, P. M., et al. (2018). Glucagon-like Peptide-1 Receptor Internalisation Controls Spatiotemporal Signalling Mediated by Biased Agonists. *Biochem. Pharmacol.* 156, 406–419. doi:10.1016/j.bcp.2018.09.003

Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996). Identification of the G Protein-Coupled Receptor Kinase Phosphorylation Sites in the Human β2-Adrenergic Receptor. *J. Biol. Chem.* 271, 13796–13803. doi:10.1074/jbc.271.23.13796

Garelja, M. L., Au, M., Brimble, M. A., Gingell, J. J., Hendrikse, E. R., Lovell, A., et al. (2020). Molecular Mechanisms of Class B GPCR Activation: Insights from Adrenomedullin Receptors. *ACS Pharmacol. Transl. Sci.* 3, 246–262. doi:10.1021/acsptsci.9b00083

Gingell, J. J., Rees, T. A., Hendrikse, E. R., Siow, A., Rennison, D., Scotter, J., et al. (2020). Distinct Patterns of Internalization of Different Calcitonin Gene-Related Peptide Receptors. *ACS Pharmacol. Transl. Sci.* 3, 296–304. doi:10.1021/acsptsci.9b00089

Gurevich, E. V., Tesmer, J. J. G., Mushegian, A., and Gurevich, V. V. (2012). G Protein-Coupled Receptor Kinases: More Than Just Kinases and Not Only for GPCRs. *Pharmacol. Ther.* 133, 40–69. doi:10.1016/j.pharmthera.2011.08.001

Gurevich, V. V., and Gurevich, E. V. (2019). GPCR Signaling Regulation: The Role of GRKs and Arrestins. *Front. Pharmacol.* 10, 125. doi:10.3389/fphar.2019.00125

Gyombolai, P., Boros, E., Hunyady, L., and Turu, G. (2013). Differential β-arrestin2 Requirements for Constitutive and Agonist-Induced Internalization of the CB1 Cannabinoid Receptor. *Mol. Cel. Endocrinol* 372, 116. doi:10.1016/j.mce.2013.03.013

Harris, M., Mackie, D. I., Pawlak, J. B., Carvalho, S., Troung, T. T., Safitri, D., et al. (2021). RAMPs Regulate Signalling Bias and Internalisation of the GIPR. *bioRxiv*, 436756. doi:10.1101/2021.04.08.436756

He, Y., Liu, H., Yin, N., Yang, Y., Wang, C., Yu, M., et al. (2021). Barbadin Potentiates Long-Term Effects of Lorcaserin on POMC Neurons and Weight Loss. *J. Neurosci.* 41, 5734–5746. doi:10.1523/JNEUROSCI.3210-20.2021

Hendrikse, E. R., Liew, L. P., Bower, R. L., Bonnet, M., Jamaluddin, M. A., Prodan, N., et al. (2020). Identification of Small-Molecule Positive Modulators of Calcitonin-like Receptor-Based Receptors. *ACS Pharmacol. Transl. Sci.* 3, 305–320. doi:10.1021/acsptsci.9b00108

Hilairet, S., Bélanger, C., Bertrand, J., Laperrière, A., Foord, S. M., and Bouvier, M. (2001). Agonist-promoted Internalization of a Ternary Complex between Calcitonin Receptor-like Receptor, Receptor Activity-Modifying Protein 1 (RAMP1), and β-Arrestin. *J. Biol. Chem.* 276, 42182–42190. doi:10.1074/jbc.m107323200

Jimenez-Vargas, N. N., Pattison, L. A., Zhao, P., Lieu, T., Latorre, R., Jensen, D. D., et al. (2018). Protease-activated Receptor-2 in Endosomes Signals Persistent Pain of Irritable Bowel Syndrome. *Proc. Natl. Acad. Sci. U S A.* 115, E7438–E7447. doi:10.1073/pnas.1721891115

Jimenez-Vargas, N. N., Gong, J., Wisdom, M. J., Jensen, D. D., Latorre, R., Hegron, A., et al. (2020). Endosomal Signaling of delta Opioid Receptors Is an Endogenous Mechanism and Therapeutic Target for Relief from Inflammatory Pain. *Proc. Natl. Acad. Sci. U.S.A.* 117, 15281–15292. doi:10.1073/pnas.2000500117

Jung, S. R., Jiang, Y., Seo, J. B., Chiu, D. T., Hille, B., and Koh, D. S. (2021). β-arrestin-dependent PI(4,5)P2 Synthesis Boosts GPCR Endocytosis. *Proc. Natl. Acad. Sci. U S A.* 118, e2011023118. doi:10.1073/pnas.2011023118

Kawakami, K., Yanagawa, M., Hiratsuka, S., Yoshida, M., Ono, Y., Hiroshima, M., et al. (2022). Heterotrimeric Gq Proteins Act as a Switch for GRK5/6 Selectivity Underlying β-arrestin Transducer Bias. *Nat. Commun.* 13, 487. doi:10.1038/s41467-022-28056-7

Knight, A., Hemmings, J. L., Winfield, I., Leuenberger, M., Frattini, E., Frenguelli, B. G., et al. (2016). Discovery of Novel Adenosine Receptor Agonists that Exhibit Subtype Selectivity. *J. Med. Chem.* 59, 947–964. doi:10.1021/acs.jmedchem.5b01402

Koch, W. J., Inglese, J., Stone, W. C., and Lefkowitz, R. J. (1993). The Binding Site for the Beta Gamma Subunits of Heterotrimeric G Proteins on the Beta-Adrenergic Receptor Kinase. *J. Biol. Chem.* 268, 8256–8260. doi:10.1016/s0021-9258(18)53090-8

Kuwasako, K., Kitamura, K., Nagata, S., Hikosaka, T., and Kato, J. (2010). Function of the Cytoplasmic Tail of Human Calcitonin Receptor-like Receptor in Complex with Receptor Activity-Modifying Protein 2. *Biochem. Biophysical Res. Commun.* 392, 380–385. doi:10.1016/j.bbrc.2010.01.030

Kuwasako, K., Sekiguchi, T., Nagata, S., Jiang, D., Hayashi, H., Murakami, M., et al. (2016). Inhibitory Effects of Two G Protein-Coupled Receptor Kinases on the Cell Surface Expression and Signaling of the Human Adrenomedullin Receptor. *Biochem. Biophysical Res. Commun.* 470, 894–899. doi:10.1016/j.bbrc.2016.01.138

Kuwasako, K., Shimekake, Y., Masuda, M., Nakahara, K., Yoshida, T., Kitaura, M., et al. (2000). Visualization of the Calcitonin Receptor-like Receptor and its Receptor Activity-Modifying Proteins during Internalization and Recycling. *J. Biol. Chem.* 275, 29602–29609. doi:10.1074/jbc.m004534200

Li, L., Homan, K. T., Vishnivetskiy, S. A., Manglik, A., Tesmer, J. J. G., Gurevich, V. V., et al. (2015). G Protein-Coupled Receptor Kinases of the GRK4 Protein Subfamily Phosphorylate Inactive G Protein-Coupled Receptors (GPCRs). *J. Biol. Chem.* 290, 10775–10790. doi:10.1074/jbc.M115.644773

Liggett, S. B. (2011). Phosphorylation Barcoding as a Mechanism of Directing GPCR Signaling. *Sci. Sig.* 4, pe36. doi:10.1126/scisignal.2002331

Mackie, D. I., Nielsen, N. R., Harris, M., Singh, S., Davis, R. B., Dy, D., et al. (2019). RAMP3 Determines Rapid Recycling of Atypical Chemokine Receptor-3 for Guided Angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 116, 24093–24099. doi:10.1073/pnas.1905561116

Marti-Solano, M., Crilly, S. E., Malinverni, D., Munk, C., Harris, M., Pearce, A., et al. (2020). Combinatorial Expression of GPCR Isoforms Affects Signalling and Drug Responses. *Nature* 587, 650–656. doi:10.1038/s41586-020-2888-2

Ménard, L., Ferguson, S. S. G., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A.-M., et al. (1996). Members of the G Protein-Coupled Receptor Kinase Family that Phosphorylate the β2-Adrenergic Receptor Facilitate Sequestration. *Biochemistry* 35, 4155–4160. doi:10.1021/bi952961+

Møller, T. C., Pedersen, M. F., van Senten, J. R., Seiersen, S. D., Mathiesen, J. M., Bouvier, M., et al. (2020). Dissecting the Roles of GRK2 and GRK3 in μ-opioid Receptor Internalization and β-arrestin2 Recruitment Using CRISPR/Cas9-edited HEK293 Cells. *Sci. Rep.* 10, 17395. doi:10.1038/s41598-020-73674-0

Nash, C. A., Nelson, C. P., Mistry, R., Moeller-Olsen, C., Christofidou, E., Challiss, R. A. J., et al. (2018). Differential Regulation of β2-adrenoceptor and Adenosine A2B Receptor Signalling by GRK and Arrestin Proteins in Arterial Smooth Muscle. *Cell Signal.* 51, 86–98. doi:10.1016/j.cellsig.2018.07.013

Nguyen, A. H., Thomsen, A. R. B., Cahill, T. J., Huang, R., Huang, L.-Y., Marcink, T., et al. (2019). Structure of an Endosomal Signaling GPCR-G Protein-β-Arrestin Megacomplex. *Nat. Struct. Mol. Biol.* 26, 1123–1131. doi:10.1038/s41594-019-0330-y

Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., et al. (2011). Distinct Phosphorylation Sites on the β(2)-adrenergic Receptor Establish a Barcode that Encodes Differential Functions of β-arrestin. *Sci. Signal.* 4, ra51. doi:10.1126/scisignal.2001707

O'Hayre, M., Eichel, K., Avino, S., Zhao, X., Steffen, D. J., Feng, X., et al. (2017). Genetic Evidence that β-arrestins Are Dispensable for the Initiation of β 2-adrenergic Receptor Signaling to ERK. *Sci. Signal.* 10, eaal3395. doi:10.1126/scisignal.aal3395

Palczewski, K., Ohguro, H., Premont, R. T., and Inglese, J. (1995). Rhodopsin Kinase Autophosphorylation. *J. Biol. Chem.* 270, 15294–15298. doi:10.1074/jbc.270.25.15294

Patel, M., Matti, C., Grimsey, N. L., Legler, D. F., Javitch, J. A., Finlay, D. B., et al. (2021). Delineating the Interactions between the Cannabinoid CB $_2$ Receptor and its Regulatory Effectors; β-arrestins and G Protein-coupled Receptor Kinases. *Br. J. Pharmacol.*

Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., et al. (2002). Targeting of Cyclic AMP Degradation to β 2 -Adrenergic Receptors by β-Arrestins. *Science* 298 (5594), 834–836. doi:10.1126/science.1074683

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., et al. (1992). Role of βγ Subunits of G Proteins in Targeting the β-Adrenergic Receptor Kinase to Membrane-Bound Receptors. *Science* 257, 1264–1267. doi:10.1126/science.1325672

Rankin, M. L., Marinec, P. S., Cabrera, D. M., Wang, Z., Jose, P. A., and Sibley, D. R. (2006). The D1Dopamine Receptor Is Constitutively Phosphorylated by G Protein-Coupled Receptor Kinase 4. *Mol. Pharmacol.* 69, 759–769. doi:10.1124/mol.105.019901

Safitri, D., Harris, M., Potter, H., Yan Yeung, H., Winfield, I., Kopanitsa, L., et al. (2020). Elevated Intracellular cAMP Concentration Mediates Growth Suppression in Glioma Cells. *Biochem. Pharmacol.* 174, 113823. doi:10.1016/j.bcp.2020.113823

Schönauer, R., Kaiser, A., Holze, C., Babilon, S., Köbberling, J., Riedl, B., et al. (2015). Fluorescently Labeled Adrenomedullin Allows Real-Time Monitoring of Adrenomedullin Receptor Trafficking in Living Cells. *J. Pept. Sci.* 21, 905–912. doi:10.1002/psc.2833

Slosky, L. M., Bai, Y., Toth, K., Ray, C., Rochelle, L. K., Badea, A., et al. (2020). β-Arrestin-Biased Allosteric Modulator of NTSR1 Selectively Attenuates Addictive Behaviors. *Cell* 181, 1364–1379. doi:10.1016/j.cell.2020.04.053

Tang, J. Q., Sun, F., Wang, Y. H., Chen, L. X., Yao, C. H., Fu, X. Q., et al. (2013). The Role of Caveolae in Regulating Calcitonin Receptor-like Receptor Subcellular Distribution in Vascular Smooth Muscle Cells. *Biochem. Cel Biol.* 91, 357–360. doi:10.1139/bcb-2013-0020

Thompson, A., and Kanamarlapudi, V. (2015). Agonist-induced Internalisation of the Glucagon-like Peptide-1 Receptor Is Mediated by the Gαq Pathway. *Biochem. Pharmacol.* 93, 72–84. doi:10.1016/j.bcp.2014.10.015

Thomsen, A. R. B., Plouffe, B., Cahill, T. J., Shukla, A. K., Tarrasch, J. T., Dosey, A. M., et al. (2016). GPCR-G Protein-β-Arrestin Super-complex Mediates Sustained G Protein Signaling. *Cell* 166, 907–919. doi:10.1016/j.cell.2016.07.004

Tran, T. M., Friedman, J., Qunaibi, E., Baameur, F., Moore, R. H., and Clark, R. B. (2004). Characterization of Agonist Stimulation of cAMP-dependent Protein Kinase and G Protein-Coupled Receptor Kinase Phosphorylation of the β2-Adrenergic Receptor Using Phosphoserine-specific Antibodies. *Mol. Pharmacol.* 65, 196–206. doi:10.1124/mol.65.1.196

Wang, G., Jiang, L., Wang, J., Zhang, J., Kong, F., Li, Q., et al. (2020). The G Protein-Coupled Receptor FFAR2 Promotes Internalization during Influenza A Virus Entry. *J. Virol.* 94, e01707–19. doi:10.1128/JVI.01707-19

Weston, C., Winfield, I., Harris, M., Hodgson, R., Shah, A., Dowell, S. J., et al. (2016). Receptor Activity-Modifying Protein-Directed G Protein Signaling Specificity for the Calcitonin Gene-Related Peptide Family of Receptors. *J. Biol. Chem.* 291, 21925–21944. doi:10.1074/jbc.M116.751362

Wisler, J. W., DeWire, S. M., Whalen, E. J., Violin, J. D., Drake, M. T., Ahn, S., et al. (2007). A Unique Mechanism of β-blocker Action: Carvedilol Stimulates β-arrestin Signaling. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16657–16662. doi:10.1073/pnas.0707936104

Yarwood, R. E., Imlach, W. L., Lieu, T., Veldhuis, N. A., Jensen, D. D., Klein Herenbrink, C., et al. (2017). Endosomal Signaling of the Receptor for Calcitonin Gene-Related Peptide Mediates Pain Transmission. *Proc. Natl. Acad. Sci. U.S.A.* 114, 12309–12314. doi:10.1073/pnas.1706656114

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REVIEW
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Dural Immune Cells, CGRP, and Migraine

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Migraine is the most common neurological disorder in the world, affecting 12% of the population. Migraine involves the central nervous system, trigeminal nerves and meninges. Recent advances have shown that targeting calcitonin gene-related peptide (CGRP) through either antibodies or small molecule receptor antagonists is effective at reducing episodic and chronic migraine episodes, but these therapeutics are not effective in all patients. This suggests that migraine does not have a singular molecular cause but is likely due to dysregulated physiology of multiple mechanisms. An often-overlooked part of migraine is the potential involvement of the immune system. Clinical studies have shown that migraine patients may have dysregulation in their immune system, with abnormal plasma cytokine levels either during the attack or at baseline. In addition, those who are immunocompromised appear to be at a higher risk of migraine-like disorders. A recent study showed that migraine caused changes to transcription of immune genes in the blood, even following treatment with sumatriptan. The dura mater is densely packed with macrophages, mast and dendritic cells, and they have been found to associate with meningeal blood vessels and trigeminal afferent endings. Recent work in mice shows activation and morphological changes of these cells in rodents following the migraine trigger cortical spreading depression. Importantly, each of these immune cell types can respond directly to CGRP. Since immune cells make up a large portion of the dura, have functional responses to CGRP, and interact with trigeminal afferents, CGRP actions on the dural immune system are likely to play key roles in migraine.

Keywords: meninges, immune, dura mater, migraine, CGRP, macrophage, perivascular

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INTRODUCTION

Migraine is a highly common neurological disorder characterized by intensive headaches. While almost everyone has headaches from time to time, what separates migraine is the duration, intensity and symptoms associated with the headache. It has been estimated that roughly 12% of the global population experiences migraine annually (1, 2). The International Headache Society (IHS) defines migraine as a recurring headache that lasts between 4 and 72 h. The headache occurs on one side of the head, and is accompanied by "pulsating quality, moderate or severe pain intensity, and aggravation by or causing avoidance of routine physical activity" (3). Additional diagnostic symptoms occur during the migraine attack, including vomiting, nausea, photophobia and phonophobia. Migraine patients experiencing an attack have a higher sensitivity to light

(photophobia) and sound (phonophobia) that can cause discomfort, and this is one quality of migraine that makes it much worse than normal headache. Another important fact is that migraine is 2 to 3 times more common in women than men, suggesting a sex difference impacting the neurological environment (4). Approximately 20–30% of migraine patients also experience a disconcerting sensory phenomena called an aura prior to the headache (5).

Migraine is a multifaceted disorder involving the peripheral and central nervous systems (CNS) and the meninges, particularly the dura mater (6). There are many potential triggers of migraine, so migraine shouldn't be seen as having a singular cause but rather, migraine represents a spectrum of triggers, symptoms, and potential treatments (7). Within this complexity, it is generally accepted that trigeminal neurons are responsible for the pain of migraine. These neurons sense modalities such as pain, touch, temperature and mechanoreception (8, 9). Trigeminal sensory neurons innervate the face, jaw, and anterior portion of the head and dura mater (9). The nerve endings also innervate the sutures of the skull and can even be found to traverse the sutures into extracranial tissue (10, 11). The interface of nerve endings with both extracerebral and extracranial targets add to complexity studying the TG.

One trigger associated with migraine with aura is cortical spreading depression (CSD) (12). CSD causes an increase in trigeminal ganglion (TG) firing following application in animal models (13). A recent study found that migraine with aura patients have increased uptake of a radiolabeled inflammation-associated protein into the meninges and occipital skull bone (14). This provides strong evidence that CSD-induced meningeal inflammation in rodent models also occurs in migraine patients. The role of CSD and meningeal immune cells will be further discussed below.

The afferent endings of the TG interface with the dura mater, and the complex microenvironment composed of nerve endings, blood vessels, fibroblasts, and immune cells (15, 16). Early hypotheses of migraine were that migraine was caused by a vascular component (17, 18). Proponents of such theories date back to the classical Greek physician Galen. In the 1950's, Wolff postulated that migraine was due to distention of cranial vessels and vasodilation. He found that treatment with ergotamine compounds reduced the pulsing and partially restored function. Ergotamine is a serotonergic vasoconstrictor that also inhibits trigeminal nerve transmission (19). This helped lead to development of more specific acute treatments. One of the main acute treatments of migraine, sumatriptan (a $5HT1_{B/D}$ agonist) alleviates migraine pain and causes vasoconstriction (20). Sumatriptan also reduces synaptic release of neurotransmitters of trigeminal neurons (21). A counter to the argument that migraine is a vascular disorder comes from the development of non-vasoactive serotonergic medication such as lasmiditan. Lasmiditan is a $5HT_F$ agonist with no vasoconstriction side effect, at least *in vitro* (22). The efficacy of lasmiditan with no significant vascular effects suggests that blood vessels are only partially involved. An additional argument was based on the observation that while some vasodilators like nitroglycerin induce headache in migraine patients, another dilator, vasoactive intestinal peptide failed (23, 24). However, this argument has lost weight since a recent study with prolonged infusion of vasoactive intestinal peptide showed that it can induce migraine in patients (25).

Recent advances in the field have involved the neuropeptide CGRP. It is a 37 amino acid peptide released from trigeminal ganglion neurons that can induce vasodilation, nociception, and neurogenic inflammation (26–28). CGRP is released both in the periphery and centrally (26, 29, 30). Researchers noted that CGRP can induce migraine-like headache in migraine patients following infusion, including symptoms such as photophobia (31). Jugular vein CGRP is higher during migraine onset than in control subjects (32). Chronic migraine patients also have elevated levels of plasma CGRP at baseline compared to healthy controls (33). Using the preclinical and clinical evidence, scientists developed treatments targeting either CGRP or its receptor complex. The FDA has approved both CGRP-blocking or receptor blocking antibodies, as well as small molecule antagonists against the CGRP receptor (34, 35). Monoclonal antibodies result in a 50% reduction in migraine days in 50% of episodic migraine patients (36). The antibodies can also be partially effective in drug resistant migraine by reducing migraine headache days (4.2 fewer headache days in a month compared to baseline) (37). CGRP antibodies have fewer adverse side effects compared to other prophylactics (38).

Despite the success of recent CGRP-targeting medications, CGRP is not the only component of migraine induction and pathogenesis. The failure to ablate migraine, and only provide a reduction in migraine headache days suggests that CGRP is merely one player in the complex physiology of migraine. The physiology of the meninges should be taken into consideration, due to the complexity of migraine and headache disorders. This review will focus on clinical evidence of immune dysfunction in migraine patients, the anatomical and physiological relationships of the immune cells in the dura mater and their potential regulation by CGRP, and pre-clinical evidence implicating the immune system in migraine-like symptoms.

CLINICAL EVIDENCE OF THE IMMUNE SYSTEM DYSREGULATION IN MIGRAINE

Over the years evidence has emerged that migraine patients may have immune system dysfunction. Peripheral cytokine levels have been used to show a shift in the general inflammatory state of the body. One study showed that migraine patients have higher levels of interleukin 1-beta (IL1-β) and interleukin-6 (IL6), and lower levels of interleukin-10 (IL10) compared to

Abbreviations: IHS, International Headache Society; CSD, Cortical spreading depression; CGRP, Calcitonin gene-related peptide; TG, Trigeminal ganglion; CNS, Central nervous system ; IL1-β, Interleukin 1-beta ; IL6, Interleukin-6; IL10, Interleukin 10; TNF-α, Tumor necrosis factor α; CSF, Cerebrospinal fluid; TGFβ-1, Transforming growth factor beta-1; IBS, Irritable bowel syndrome; GI, Gastrointestinal; SNP, Single nucleotide polymorphism; IL4, Interleukin 4; ECM, Extracellular matrix; ROS, Reactive oxygen species; LPS, Lipopolysaccharide; PAR, Proteinase-activated receptor; APC, Antigen presenting cell; NGF, Nerve growth factor.

healthy control patients (39). A 2015 study noted that migraine patients had higher IL6 levels compared to healthy controls (40). Tumor necrosis factor α (TNF-α) was elevated during attack in migraine patients with aura, and baseline levels were increased in general migraine patients. A 2021 study measured cytokine levels in the blood in migraine patients and healthy controls and discovered that TNF-α was elevated, but IL1-β was not compared to controls (41). Cerebrospinal fluid (CSF) protein measurements indicate that migraine patients have significantly different levels of transforming growth factor beta (TGF-β) 1, interleukin-1 receptor antagonist and monocyte chemoattractant protein 1 compared to controls (42). It is important to note that this is within the confines of the CNS, vs. peripheral blood levels that the previous studies have represented. Finally, while cytokine dysfunction in migraine is indicated by numerous studies, there are conflicting reports (39, 41).

Serum CGRP has been noted as either a potential biomarker or a poor marker due to variability in concentrations, patient conditions and diagnostic criterion. For example, Lee et al. found that healthy controls had a mean serum level of 75.7 picograms/mL CGRP, while episodic migraine patients showed 67.0 picograms/mL. In that study, chronic migraine patients had no increase in interictal levels of CGRP (43). But another study found that interictal CGRP levels were significantly elevated in chronic migraine patients (74.9 picograms/mL) compared to 46.37 picograms/mL in healthy patients (33). This discrepancy is highlighted in a Lancet review from 2021 (44). The lack of reproducibility could be explained in part due to different exclusion criteria, different ELISA sources, time of extraction, or methodology. The study by Cernuda-Mellon et al. and the one by Lee et al. did use similar timeframes, collection conditions, and measurement techniques (33, 43). More studies should be done to determine if CGRP is a valid biomarker of migraine.

Other evidence that doesn't rely on peripheral markers are comorbidities with immune disorders and migraine. Meta-analysis noted that headache had overlap with several autoimmune diseases like multiple sclerosis, rheumatoid arthritis, vasculitis, and allergic diseases (45). The authors hypothesize that headache or migraine phenotypes may be a "consequence of general inflammatory mechanisms involving meningeal vessels and activating trigeminal terminals, especially in individuals with a previous history of headache…" (45). A study investigating irritable bowel disorder (IBS) gave some evidence of a causal relationship between IBS and migraine (46). The potential dysfunction in cytokines in migraine patients, and abnormal CGRP activity may influence the gut-brain axis (47). It is important to note that CGRP doesn't just signal in the meninges, but all over the body, including the gastrointestinal (GI) tract. CGRP is postulated to influence the gut microbiome through interaction with resident GI tract immune cells as well as on gastroenteric motility (48, 49). This in turn may explain the correlation with migraine/headache in IBS disorders (47). There is also a significant association between celiac disease, an immune disease of the GI tract, and migraine (50).

Genome-wide association studies and single nucleotide polymorphism (SNP) studies provide additional evidence of the involvement of immune dysregulation in migraine. In a Jordanian population of 198 migraine patients and 200 controls, there was a significant association with two SNPs regarding TNF-α gene (51). There was also a decrease in circulating lymphocytes in the blood. Lymphocytes include cell types such as T and B cells (52). This is supported by a 2021 study showing that there is a significant decrease in peripheral regulatory T cells in migraine patients compared to controls (53). Changes to the immune system through different genes and SNPs may influence the environment of the dura mater, in parallel with the trigeminal afferent endings. In migraine patients, an RNA sequencing study in 2021 examined blood RNA levels during migraine attack or during the baseline period. Genes involved with the immune system, along with fat metabolism and signaling pathways were implicated in the study (54).

Measurement of peripheral gene expression through qPCR hints at immune dysfunction in migraine as well. A group of researchers examined migraine patients with or without aura compared to healthy controls and extracted jugular venous blood to measure gene expression of various cytokines (41). Migraine patients had elevated levels of interleukin-4 (IL4), TGF-β, TNF-α and interferon gamma. Some cytokines that were previously found to have higher circulating protein levels such as IL1-β were not significantly different from controls.

The evidence for immune system involvement is diverse, but not concrete enough to pinpoint a distinct role of these cells. But there does appear to be an involvement of this system, at least for part of the pathogenesis of migraine. The following section will highlight the immune cells present in the meninges, their actions, relation to trigeminal afferents and influence on neurons and blood vessels.

DURA MATER RELEVANCE AND OVERVIEW

The dura mater is the outermost layer of the meninges that contains extracerebral blood vessels, fibroblasts, trigeminal afferent endings, sympathetic and parasympathetic efferent endings, and numerous immune cells (55–60). The trigeminal afferents extensively innervate the blood vessels and other regions of the dura mater, and interface with the extracellular matrix (ECM) (61). The interplay between afferent endings, vessels, immune cells, and ECM is hypothesized to be one way in which migraine is induced, particularly by neuropeptides like CGRP as well as other small molecules. This is referred as the trigeminovascular hypothesis of migraine (62). It is thought that there is initial trigeminal activation, resulting in release of neuropeptides like CGRP and Substance P into the trigeminovascular space. The trigeminal afferents also release small molecules such as glutamate (63). CGRP directly binds to vascular smooth muscle cells and causes hyperpolarization through metabotropic signaling and downstream phosphorylation of K_{ATP} channels by protein kinase A (64). This results in relaxation and thus vasodilation. CGRP also acts on resident immune cells such as macrophages and mast cells. The macrophages may become activated, and the mast cells degranulate (65, 66). This degranulation involves

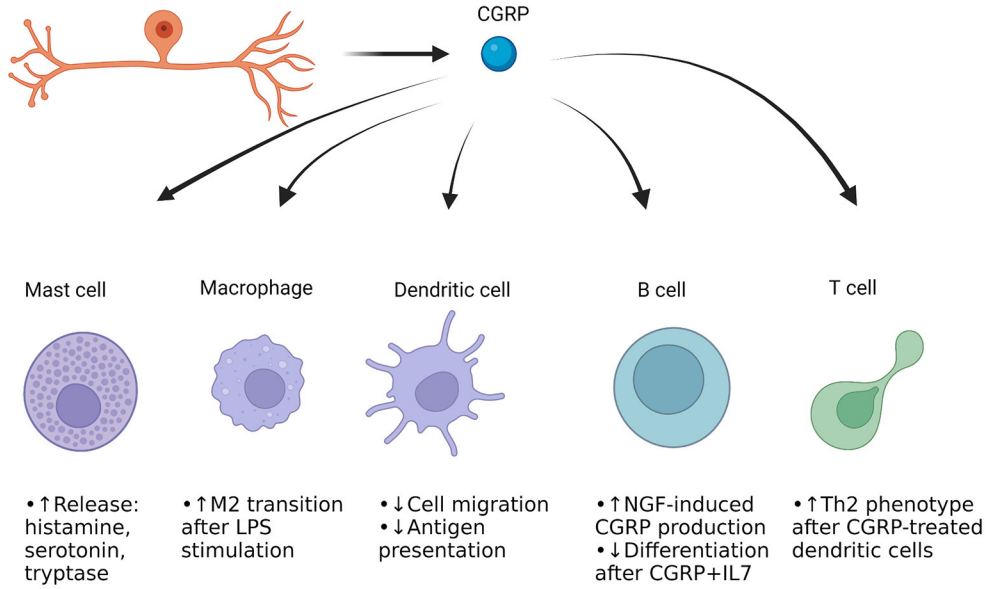

FIGURE 1 | CGRP actions on immune cells in the dura mater of the meninges. Cells of both the innate and adaptive immune systems are present. Mast cells and macrophages represent the immediate frontline defense against pathogens and harmful compounds, while dendritic cells help move parts of the harmful pathogen/protein to the adaptive immune cells (71–73). The B cells can produce antibodies against the antigens, and the T cells can differentiate to either kill cells or aid fellow B and T cells for enhanced immune response. Most of the work of CGRP's effect on these cells has been done in culture, but it appears that CGRP induces neurogenic inflammation through mast cell degranulation, while enhancing anti-inflammatory macrophage function when stimulated with an inflammatory compound (27, 65, 66, 74). CGRP appears to reduce dendritic cell presentation of antigens (75). CGRP's effect in B cells and T cells is not well-studied but may drive the adaptive immune system to undergo physiological changes upon presence of certain triggers.

release of inflammatory chemicals such as histamine, proteases and various cytokines that are implicated in headache (67). Many of the compounds released can sensitize trigeminal afferents, leading to an increase in firing (68). Macrophage activation may result in release of cytokines that could further sensitize afferents (69). It should be noted that CGRP itself can sensitize afferent endings to compounds such as ATP, so there could be a synergism with cytokines and CGRP regarding afferent sensitization (70). The following section will discuss each of the immune cells present in the dura mater, interactions with trigeminal afferents and CGRP, and how they could be involved in migraine. **Figure 1** provides a general overview of the immune cells present in the meninges as well as CGRP's actions on these cells.

IMMUNE CELLS IN THE MENINGES

Macrophages

Macrophages are monocytic lineage cells that act as frontline defenders through the innate immune system (71). In the dura mater, the macrophages are derived from bone marrow and replenish over time (76). They are highly mobile and engage in phagocytosis of pathogens or dying cells (77). Other functions of macrophages include tissue repair and remodeling and cytokine release (78). A 2017 study by McIlvried et al. found that in the rodent dura mater, over 17% of cells present are immune cells, and of these almost two-thirds are macrophages (79). The high proportion of cells in the dura being immune cells (roughly 1 in 10 dural cells being a macrophage) represents a dynamic and ever-changing portion of the dura. Research has shown that mouse macrophages interact with cultured trigeminal ganglion cells in either a genetic model or wild type mice (80). They found that macrophages underwent more phagocytosis of particles in co-culture with TGs of either genotype compared to no co-culture. This suggests that macrophages may have a physiological relationship with TG neurons.

Macrophage function is intertwined with its cell surface receptors, cytokines that are released and morphology (81). In the past macrophages have been thought as "undifferentiated," then respond to stimuli around them. Following activation, they were thought to shift to an inflammatory M1 phenotype or an anti-inflammatory M2 phenotype (82). The M1 inflammatory phenotype is typically characterized by anti-microbial and anti-tumor functions (**Figure 2**). An early study found that upon stimulation with interferon gamma, cultured human macrophages produced increased levels of hydrogen peroxide (84). This became known as the M1 phenotype. M1 macrophages produce other reactive oxygen species (ROS), release cytokines such as IL1-β to recruit other immune cells and change expression of genes to respond appropriately to the threat (85, 86). The release of ROS-related compounds can harm pathogens (86). When macrophages shift to the M1 phenotype,

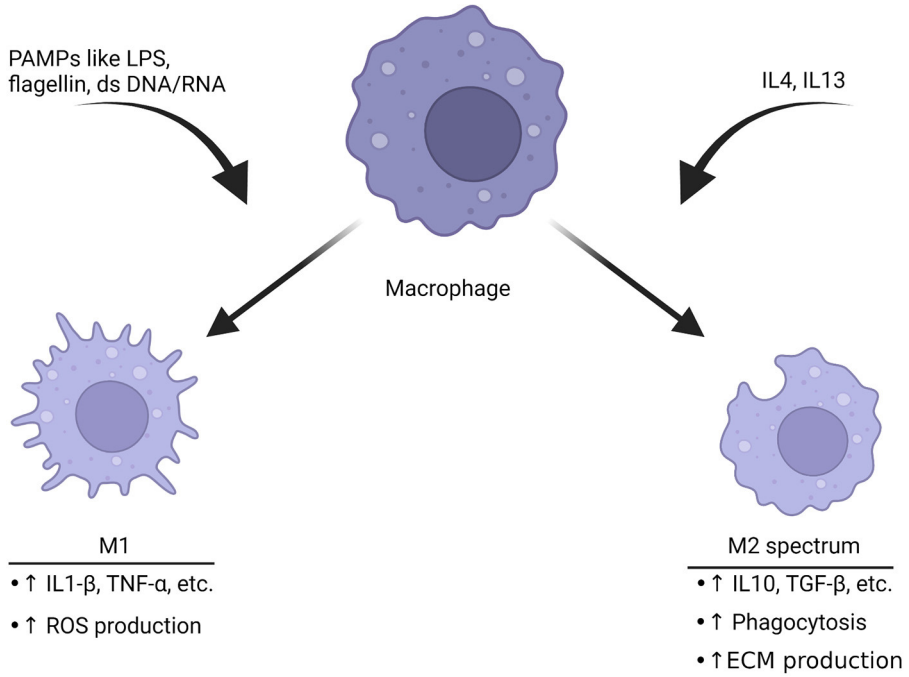

FIGURE 2 | Macrophages polarize toward different phenotypes depending on the stimuli. Triggers like pathogen-associated molecular patterns (PAMPs) including LPS, double-stranded DNA or RNA can induce M1 polarization. This phenotype is characterized by production of inflammatory cytokines and an increase in ROS production to help break down targets. The M2 spectrum of polarization is activated by cytokines such as IL4 or IL13. The result is a transformation into the M2 spectrum (M2a, b, c, and d) which is characterized by enhanced phagocytosis, anti-inflammatory cytokine release and increase in production of the ECM. These are associated with tissue repair (82, 83). The ability of macrophages to rapidly shift based upon of stimuli represents a potential target for migraine, especially since they have functional CGRP receptors.

they change to a more circular appearance and may have reduced mobility (87).

The M2 phenotype of macrophage activation is generally thought of as anti-inflammatory. These macrophages function to ensure wound repair, engulf debris through phagocytosis, promote neovascularization and interact with the ECM (83, 88). M2 macrophages also express cytokines that are thought to fight inflammation. Compounds released by M2 macrophages include IL10and TGF-β (89). There have been significant advancements in understanding the complexity of activation state. Macrophages have multiple M2 sub phenotypes such as M2a, M2b, M2c and M2d (81). In fact, it is possible to manipulate the cell shape and cause more characteristics of the phenotype associated with the shape (90).

The adaptability of macrophages and their constant change in location, morphology and cell processes might be one way in which the dysregulated trigeminovascular space is caused. A preclinical study sought to examine macrophage activity through *in-vivo* two photon imaging (91). This was done in anesthetized animals under a two-photon scope. The macrophages and other CX3CR1-expressing cells produced green fluorescent protein, allowing visualization of the cells and their morphology. The team found that macrophages adopted an increased circularity following CSD in the mouse brain. They also saw a reduction in movement of dendritic cells. Changes in morphology is a potential but not absolute sign that the macrophages are being activated into one of the phenotypes (92). The macrophages were closely associated with transient receptor potential vanilloid receptor 1-positive nerve endings, suggesting close association with the trigeminal afferents (92, 93). This corresponds with *in vitro* work showing functional communication between cultured trigeminal neurons and macrophages (80). Similar association was also found in immunohistochemical analysis of rat dura maters. Macrophages appeared near dural vessels, and a portion were associated with afferent nerve endings (94). All this points to macrophages having possible crosstalk with trigeminal afferents, especially given the high portion of dural cells being macrophages (79).

In the context of migraine, macrophages can respond to several compounds released by trigeminal afferent endings, such as CGRP. Macrophages in culture have been shown to express the CGRP receptor and treatment of cells with CGRP causes an increase in cAMP (indicative of CGRP receptor activation) (95). If there is an initial CGRP release from trigeminal afferents, then that could potentially activate the dural macrophages. Reciprocal release of cytokines from the now-activated macrophage could sensitize the nearby afferent endings. This is known as neurogenic inflammation (27). This type of inflammation via endogenous compounds such as cytokines or CGRP is also referred to as sterile inflammation since there is

a lack of an exogenous pathogenic trigger such as microbial membrane components (96).

The ultimate question is that even if CGRP binds to macrophages and increases cAMP levels, what is this doing to the macrophage itself regarding polarization? A recent pattern in studies observing macrophages is that CGRP appears to be preventing inflammation when cells are pretreated with a compound such as lipopolysaccharide (LPS) (97). In a study of mouse lung cells *in vitro*, lung macrophages were treated with LPS, and treatment with CGRP afterwards significantly reduced expression of inflammatory genes NLR family pyrin domain containing 3 and the pre-spliced IL1-β (97). Another study examined the role of CGRP-deficiency in bone marrow derived macrophages in the context of surgical implants (98). Cultured cells in the CGRP-knockout group had a far higher portion of CD86-expressing macrophages. This is generally considered a marker for M1 polarization (99). Supplementation in the knockout mice with CGRP reduced the M1 population and increased the M2 population, closer to those found in the wildtype controls. The results suggest that CGRP may be necessary for basal macrophage functioning, and a loss of CGRP results in a shift toward inflammation.

Another hint at CGRP's involvement in macrophages is with wound healing. In a recent study, CGRP's role in recovery was examined in corneal tissue. Cultured trigeminal ganglion neurons were co-cultured with macrophages in a rodent model with bacterial infection of the corneal nerves by *P. aeruginosa* (74). The research team found CGRP release from both macrophages and trigeminal ganglion cells when cultured separately following LPS administration. Together, they had a higher level of CGRP production following LPS than alone. CGRP prevented inflammation by promoting macrophages to express anti-inflammatory cytokines and markers. CGRP appears to be beneficial following infection of corneal afferents, and may help the local immune function, at least in the cornea. Another study found that CGRP levels were elevated in premolars from patients suffering from occlusion trauma in addition to orthodontic trauma (100). Other peptides were elevated, such as substance P and vascular endothelial growth factor. CGRP may be essential for tissue remodeling and wound healing acting as an activator or signal to macrophages in damaged tissue.

Mast Cells

Mast cells are another major portion of the innate immune system, and the dura mater hosts these cells (67, 101). These cells originate from bone marrow precursors, like macrophages, but are not of monocytic lineage (102, 103). They are referred to as granulocytes due to their ability to release granules containing compounds such as histamine, tryptase, heparin, substance P and numerous cytokines (72). These chemicals are thought to mediate part of the neurogenic inflammation portion of migraine (60, 104). These compounds can act on subtypes of trigeminal afferents to sensitize them directly (105, 106).

In the context of migraine neurobiology, both CGRP and substance P are capable of directly acting on mast cells to induce degranulation (107–109). However, CGRP didn't activate human mast cells in the study by Kulka in 2008 while substance P did (108). Treatment of single mast cells with immunoglobulin E potentiated CGRP-induced degranulation, but not substance P-induced degranulation in culture (66). It is possible that while CGRP is indeed capable of inducing degranulation in mast cells, a priming event is needed. Since substance P is released along with CGRP from trigeminal afferents, it could be that substance P and CGRP are working synergistically to cause sustained degranulation. More studies are needed to assess the role of CGRP-induced degranulation. Components of the CGRP receptor have been colocalized to mast cells, but the degree of functional receptor presence is not well-known.

Even if CGRP's role in degranulation is less clear than other neuropeptides, it still can interact and induce neurogenic inflammation. Preclinical work in rodents found that treatment of animals with the mast cell degranulating agent 48/80 given via intraperitoneal injection at 2 mg/kg increased firing of trigeminal afferent endings present in the dura mater (67). The C fibers, which are unmyelinated afferents that release CGRP, had a higher increase in firing than the A delta afferent fibers. Increased firing of C fibers could release more neuropeptides and thus have a bidirectional relationship with the local dural mast cells. Besides CGRP or substance P, mast cells respond to numerous compounds such as allergens, cytokines, LPS, chemokines and others (110). The diversity of agonists for mast cells is important for the innate immune system, but in the case of migraine patients and immune disorders it may negatively affect the trigeminovascular space. A common irritant acrolein is capable of degranulating mast cells and is a known headache trigger (111, 112).

Degranulation of mast cells releases a diverse number of chemicals and enzymes into the extracellular space (**Figure 3**). The most understood, histamine, is a known vasodilator (118). However, culture work on trigeminal afferents shows that treatment with histamine only resulted in a small increase in firing rate compared to compounds such as serotonin (113). Histamine was also found to promote an M2 phenotype like those seen in tumor associated macrophages, and prevention of histamine receptor H1 alleviates this shift in phenotype (119).

Other components released from mast cell degranulation include serotonin, heparin, tryptase, and numerous cytokines. Serotonin's effects on the dura and trigeminal neurons are complex, but can be summarized as increase in trigeminal firing, both vasoconstriction and dilation (depending on receptor subtype and concentration) and various immune actions, including secretion of specific cytokines (113, 115, 120, 121). For example, serotonin was found to dilate the feline middle meningeal artery, while triptans ($5HT1_{B/D}$ agonists) caused vasoconstriction (20, 114). Tryptase is of interest because it is a serine-threonine protease capable of inducing changes to the extracellular environment and inducing nociception (122, 123). Researchers found that tryptase can induce mechanical allodynia is a mouse paw, and it does this through cleavage of proteinase-activated receptor (PAR) 2 (123). Antibodies against PAR-2 have shown beneficial effects in rodent models of pain and will be discussed later (124). Cytokines found in mast cells are conflicting, in part because of differences of measuring mRNA vs. functional protein (117). Numerous pro- and anti-inflammatory

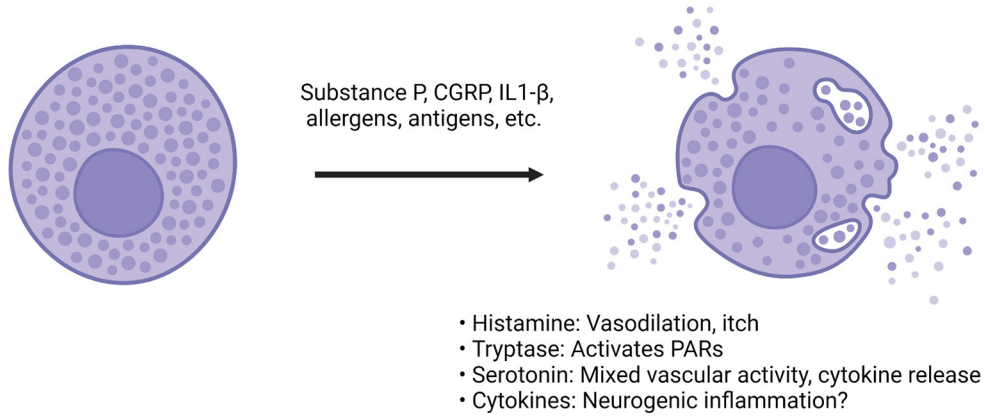

FIGURE 3 | CGRP actions on dural mast cells. Compounds such as CGRP, substance P or inflammatory cytokines can bind to mast cells via a diverse array of receptors (66, 108). Following activation, the mast cell releases granules that contain compounds such as histamine, heparin, enzymes like tryptase, many different cytokines and serotonin (68, 72, 113). The effect of these compounds is diverse. Among the most prominent are histamine and serotonin. Histamine can cause vasodilation and immune activation. Serotonin has mixed vascular activity depending on the site of action. It has been found to dilate middle meningeal arteries (114). It can also modulate cytokine release from monocytes (115). Tryptase is a protease that can cleave other proteins, such as protease-activated receptor (PAR) 2. PAR-2 activation is thought to sensitize transient receptor potential channels present on sensory afferents, so downstream protease activity could possibly result in TG sensitization (116). The cytokines found in mast cells are incredibly diverse, and both pro- and anti-inflammatory cytokines have been found but degranulation is believed to drive further inflammation and sensitization of afferents (117).

cytokines have been identified but more work needs to be done to parse out their relevance. Of the cytokines potentially released from mast cells, IL-1β and IL-6 have the potential to activate TG neurons or induce prolonged sensitization. In cultured rat TG cells, IL-1β increased prostaglandin 2 synthesis and subsequent CGRP release (125). In rodents, dural injection of IL-6 induced both facial and hind paw allodynia, demonstrating that local actions of cytokines in the dura can also apparently lead to central sensitization (126).

Dendritic Cells

Dendritic cells are another type of monocyte lineage cell found throughout the body, including the dura mater (127). While they are in far lower proportion to macrophages, at least in rodents, they still have a substantial presence (79). These cells are involved in the antigen presentation process for B and T cells for adaptive immunity (128). They are a type of antigen presenting cell (APC). APCs take fragments of viruses and bacteria and return them to T cells. The T cells recognize fragments of the epitope and can differentiate accordingly (73). Dendritic cells treated with CGRP have a change in cell surface receptors and a decrease in proliferation, suggesting a functional CGRP receptor on these cells (75). Cultured dendritic cells have reduced cell migration following application of CGRP (129). In the skin, aggregation of dendritic cells in the lymph nodes was inhibited by CGRP, consistent with previous studies (130). A recent review summarizes CGRP's inhibitory effect on dendritic cells, reduced migration and antigen presentation (131).

Dendritic cells may be a potential target for headache and migraine treatment. A group in 2019 found that when dendritic cells were cultured from rats and exposed to interferon gamma, the exosome extract reduced spreading depression in hippocampal slices *in vitro* (132). The reduced electrical activity suggests that mild exposure of dendritic cells to specific cytokines may suppress neuron firing downstream, and they suggest that this could be one way to help patients with migraine.

B Cells

B cells are another part of the adaptive immune system and can produce antibodies against specific antigens presented to them. Until recently, it was not known if the meninges had a substantial portion of B cells. In 2021, two papers reported a surprising abundance of B cells in the meninges, at least in rodents (133, 134).

CGRP's involvement with B cells is not well-studied, but CGRP was found to be expressed in these cells upon stimulation by nerve growth factor (NGF) (135). Levels were much lower in inactivated cells. B cells also appear to have CGRP receptors. B cells can respond to treatment with CGRP *in vitro*, and CGRP hinders development of B cell precursors. Blocking CGRP receptors with the peptide antagonist CGRP8-37 prevented this CGRP-driven inhibition (136).

T Cells

Part of the adaptive immune system, T cells are responsible for circulating throughout the body and attacking antigens based upon antibody recognition and absorbing fragments of the pathogen. These cells are found in the dura. Based upon cell surface receptors, they can be divided into CD4 and CD8 cells (137). The CD4 T cells can be further subdivided into Th1 or Th2 (137, 138). Th1 is seen as pro-inflammatory, primarily through interferon gamma. Th2 is anti-inflammatory. The baseline level of dural T cells remains relatively low, while in bacterial infection there is a significant increase (139). Immunohistochemical analysis found that T cells were present around dural sinuses,

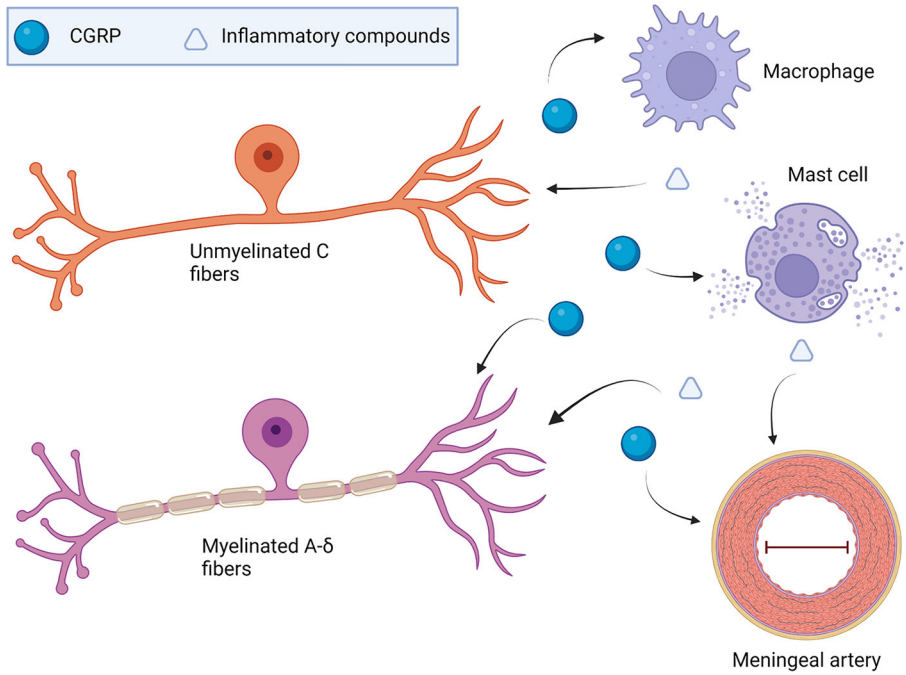

FIGURE 4 | Model of CGRP involvement with the dural immune system in migraine. TG-produced CGRP may drive dural mast cell degranulation that could sensitize nearby TG afferent endings. It could also result in vasodilation and activation of macrophages and other resident immune cells. Beyond the direct activation of TG afferents by inflammatory compounds, vasodilation via histamine or neuropeptides might activate mechanoreceptors present on TGs. Mechanoreceptor activation such as Piezo2 present on sensory neurons could be one way sensitization of TG neurons occurs. Macrophages could change the extracellular makeup on the dura following polarization from neuropeptides or cytokines. *in vitro* work shows that CGRP shifts either LPS-treated macrophages or tissue damage macrophages toward an M2 tissue-remodeling phenotype (65, 97). Changes to the interaction of the TG endings and the dura superstructure could potentially cause dysregulated firing via mechanoreceptors.

with multiple phenotypes, such as Th1 or Th2 (140). Dural T cells increased as mice aged and had differentially expressed transcripts, such as interferon gamma. Multi-photon imaging confirmed the accumulation of T cells in the dura mater in transgenic mice (140). The researchers postulate that the accumulation of T cells at sinuses may represent an avenue for peripheral meningeal immunity through interactions with the CNS.

Dural T cells recognize centrally derived antigens. APCs in the dura such as dendritic cells and macrophages are capable to transport proteins in the CSF to T cells (140). T cells appear to be able to respond to CGRP, suggesting presence of a functional receptor (141). CGRP-stimulated dendritic cells also can shift T cells to the Th2 phenotype (130). So CGRP may have multiple avenues to influence T cell functioning.

DISCUSSION

The immune system within the dura mater is extensive and dysregulation is likely to contribute to migraine pathophysiology. CGRP is a multifaceted neuropeptide that can modulate these dural immune cells. CGRP can induce neurogenic inflammation through mast cell degranulation and subsequent inflammatory chemical release. CGRP actions are likely to extend beyond mast cells, with recent articles hinting at CGRP being vital for shifting macrophages toward an anti-inflammatory phenotype following stimulation with either LPS or injury. CGRP also has a generally inhibitory effect on dendritic cells through reductions in migration and antigen presentation. Hence, CGRP cannot be pigeonholed as either a pro- or anti-inflammatory peptide since it is inflammatory via mast cells and anti-inflammatory through macrophages and dendritic cells. **Figure 4** presents a potential model for the role of CGRP and other neuropeptides on dural mast cells and macrophages. These are the two most well-studied immune cells in the context of CGRP. However, a caveat of this model is that most studies on CGRP and immune cells have been with either cell lines or bone marrow-derived immune cells, not immune cells in the meninges. Whether meningeal immune cells will respond in the same manner to CGRP remains to be determined.

CGRP-targeting medication is effective in 50% of chronic and episodic migraine patients, but some recent studies have brought on concerns about potential immune effects. A series of 8 case studies from Australia and Ireland found potential immune complications from the CGRP antibodies erenumab or galcanezumab (142). Patients had complications such as a man who suffered from rheumatoid arthritis. After taking the antibody treatment, he had hepatitis. Cessation of erenumab helped him recover, along with steroid treatment. Another patient had psoriasis upon treatment with CGRP

antibody. Another case study examined a 51-year-old woman who following erenumab treatment had skin infiltration of lymphocytes and thrombosis. While these are only case studies, this provides hints at CGRP's multifaceted, and nuanced role in human immune physiology.

Another aspect to consider is the response of the immune system to CGRP's blockage over time. We don't yet know the impact on the immune system if CGRP is blocked for years, but within a year timeframe of CGRP antibody usage there are apparently no major complications in most patients (143). A further complication of CGRP's role on dural immune cells is the ability of the immune cells to both respond to CGRP, and to also release it upon stimulation by certain chemicals. Studies have found that macrophages and B cells can produce CGRP (135, 144). So neural-produced CGRP might affect downstream CGRP production in these cell types, resulting in a potential feedback loop between neurons and immune cells. It is unknown if immune cell produced-CGRP has a physiological role but it does represent another layer of complexity of the system. In addition, other cell types may produce CGRP besides immune cells (145). Thus, both neural and locally expressed CGRP in the meninges is well-poised to modulate the activities many types of immune cells and possibly contribute to migraine pathogenesis.

CONCLUSION

The complexity of the neurobiology of migraine perplexed researchers for years, yielding the vascular vs. neuronal debate. While the neuronal side is favored as of late, it is only a part of the system. The neuropeptide CGRP is clearly involved in the genesis and continuation of migraine headache for many patients. Therapeutics targeting CGRP or the receptor are effective, but only in ∼50% of episodic or chronic migraine patients, which illustrates the need for a better understanding of how CGRP contributes to migraine pathogenesis. CGRP has primarily been viewed as a pro-inflammatory molecule in most migraine literature, but it also has anti-inflammatory and tissue repairing functions through macrophages, dendritic cells, B and T cells. CGRP is cardioprotective and helps healing following injury. There is evidence of immune dysfunction in migraine. More work should be done examining CGRP's role in not just migraine neurobiology, but also its function in the immune system. Targeting CGRP's role in migraine immune dysfunction could both augment CGRP therapeutics and possibly help patients who are non-responders to drugs that target only CGRP or its receptor.

AUTHOR CONTRIBUTIONS

LB wrote the article and made the figures, with thorough editing from AR and guidance on the layout of the figures. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Burch RC, Buse DC, Lipton RB. Migraine: epidemiology, burden, and comorbidity. *Neurol Clin.* (2019) 37:631–49. doi: 10.1016/j.ncl.2019.06.001
2. Yeh WZ, Blizzard L, Taylor BV. What is the actual prevalence of migraine? *Brain Behav.* (2018) 8:e00950. doi: 10.1002/brb3.950
3. Headache classification committee of the international headache society (IHS) the international classification of headache disorders, 3rd edition. *Cephalalgia.* (2018) 38:1–211. doi: 10.1177/0333102417738202
4. Lipton RB, Bigal ME, Diamond M, Freitag F, Reed ML, Stewart WF, et al. Migraine prevalence, disease burden, and the need for preventive therapy. *Neurology.* (2007) 68:343–9. doi: 10.1212/01.wnl.0000252808.97649.21
5. Eikermann-Haerter K, Kudo C, Moskowitz MA. Cortical spreading depression and estrogen. *Headache.* (2007) 47(Suppl 2):S79–85. doi: 10.1111/j.1526-4610.2007.00818.x
6. Panerai AE. Is migraine a disorder of the central nervous system? *Neurol Sci.* (2013) 34(Suppl 1):S33–5. doi: 10.1007/s10072-013-1363-3
7. Kelman L. The triggers or precipitants of the acute migraine attack. *Cephalalgia.* (2007) 27:394–402. doi: 10.1111/j.1468-2982.2007.01303.x
8. Walker H. *Clinical Methods: The History, Physical, and Laboratory*. Boston, MA: Butterworths. (1990).
9. Huff, Daly. Neuroanatomy, Cranial Nerve 52020.
10. Stovner LJ, Hagen K, Tronvik E, Bruvik Gravdahl G, Burstein R, Dodick DW. FollowTheSutures: piloting a new way to administer onabotulinumtoxinA for chronic migraine. *Cephalalgia.* (2022). doi: 10.1177/03331024211067775. [Epub ahead of print].
11. Burstein R, Blake P, Schain A, Perry C. Extracranial origin of headache. *Curr Opin Neurol.* (2017) 30:263–71. doi: 10.1097/WCO.0000000000000437
12. O'Hare L, Asher JM, Hibbard PB. Migraine visual aura and cortical spreading depression-linking mathematical models to empirical evidence. *Vision.* (2021) 5:30. doi: 10.3390/vision5020030
13. Brennan KC, Pietrobon D, A. Systems neuroscience approach to migraine. *Neuron.* (2018) 97:1004–21. doi: 10.1016/j.neuron.2018.01.029
14. Hadjikhani N, Albrecht DS, Mainero C, Ichijo E, Ward N, Granziera C, et al. Extra-axial inflammatory signal in parameninges in migraine with visual aura. *Ann Neurol.* (2020) 87:939–49. doi: 10.1002/ana.25731
15. Kekere, Alsayouri. Anatomy, Head and Neck, Dura Mater. Treasure Island, FL: StatPearls Publishing. (2021).
16. Rua R, McGavern DB. Advances in Meningeal Immunity. *Trends Mol Med.* (2018) 24:542–59. doi: 10.1016/j.molmed.2018.04.003
17. Isler H. The Galenic tradition and migraine. *J Hist Neurosci.* (1992) 1:227–33. doi: 10.1080/09647049209525535
18. Tunis MM, Wolff HG. Studies on headache; long-term observations of the reactivity of the cranial arteries in subjects with vascular headache of the migraine type. *AMA Arch Neurol Psychiatry.* (1953) 70:551–7. doi: 10.1001/archneurpsyc.1953.02320350003001
19. Silberstein SD. The pharmacology of ergotamine and dihydroergotamine. *Headache.* (1997) 37:S15-25.
20. Humphrey PP, Goadsby PJ. The mode of action of sumatriptan is vascular? A debate. *Cephalalgia.* (1994) 14:401–10. doi: 10.1046/j.1468-2982.1994.1406401.x

21. Levy D, Jakubowski M, Burstein R. Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists. *Proc Natl Acad Sci U S A.* (2004) 101:4274–9. doi: 10.1073/pnas.0306147101
22. Bouchelet I, Case B, Olivier A, Hamel E. No contractile effect for 5-HT1D and 5-HT1F receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br J Pharmacol.* (2000) 129:501–8. doi: 10.1038/sj.bjp.0703081
23. de Tommaso M, Libro G, Guido M, Difruscolo O, Losito L, Sardaro M, et al. Nitroglycerin induces migraine headache and central sensitization phenomena in patients with migraine without aura: a study of laser evoked potentials. *Neurosci Lett.* (2004) 363:272–5. doi: 10.1016/j.neulet.2004.04.029
24. Rahmann A, Wienecke T, Hansen JM, Fahrenkrug J, Olesen J, Ashina M. Vasoactive intestinal peptide causes marked cephalic vasodilation, but does not induce migraine. *Cephalalgia.* (2008) 28:226–36. doi: 10.1111/j.1468-2982.2007.01497.x
25. Pellesi L, Al-Karagholi MA, De Icco R, Coskun H, Elbahi FA, Lopez-Lopez C, et al. Effect of Vasoactive intestinal polypeptide on development of migraine headaches: a randomized clinical trial. *JAMA Netw Open.* (2021) 4:e2118543. doi: 10.1001/jamanetworkopen.2021.18543
26. Iyengar S, Ossipov MH, Johnson KW. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. *Pain.* (2017) 158:543–59. doi: 10.1097/j.pain.0000000000000831
27. Geppetti P, Capone JG, Trevisani M, Nicoletti P, Zagli G, Tola MR, et al. and migraine: neurogenic inflammation revisited. *J Headache Pain.* (2005) 6:61–70. doi: 10.1007/s10194-005-0153-6
28. Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. *Nature.* (1985) 313:54–6. doi: 10.1038/313054a0
29. Sexton PM. Central nervous system binding sites for calcitonin and calcitonin gene-related peptide. *Mol Neurobiol.* (1991) 5:251–73. doi: 10.1007/BF02935550
30. Messlinger K, Fischer MJ, Lennerz JK. Neuropeptide effects in the trigeminal system: pathophysiology and clinical relevance in migraine. *Keio J Med.* (2011) 60:82–9. doi: 10.2302/kjm.60.82
31. Younis S, Christensen CE, Toft NM, Soborg T, Amin FM, Hougaard A, et al. Investigation of distinct molecular pathways in migraine induction using calcitonin gene-related peptide and sildenafil. *Cephalalgia.* (2019) 39:1776–88. doi: 10.1177/0333102419882474
32. Goadsby PJ, Edvinsson L. Human in vivo evidence for trigeminovascular activation in cluster headache. Neuropeptide changes and effects of acute attacks therapies. *Brain.* (1994) 117(Pt 3):427–34. doi: 10.1093/brain/117.3.427
33. Cernuda-Morollon E, Larrosa D, Ramon C, Vega J, Martinez-Camblor P, Pascual J. Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine. *Neurology.* (2013) 81:1191–6. doi: 10.1212/WNL.0b013e3182a6cb72
34. FDA. New drug class employs novel mechanism for migraine treatment and prevention 2019. Available online at: https://www.fda.gov/drugs/news-events-human-drugs/new-drug-class-employs-novel-mechanism-migraine-treatment-and-prevention.
35. Antrim. FDA Approves Oral CGRP Receptor Antagonist Atogepant for Preventative Migraine Treatment 2021. Available online at: https://www.pharmacytimes.com/view/fda-approves-oral-cgrp-receptor-antagonist-atogepant-for-preventive-migraine-treatment.
36. de Vries Lentsch S, Verhagen IE, van den Hoek TC, MaassenVanDenBrink A, Terwindt GM. Treatment with the monoclonal calcitonin gene-related peptide receptor antibody erenumab: a real-life study. *Eur J Neurol.* (2021) 28:4194–203. doi: 10.1111/ene.15075
37. Scheffler A, Schenk H, Wurthmann S, Nsaka M, Kleinschnitz C, Glas M, et al. CGRP antibody therapy in patients with drug resistant migraine and chronic daily headache: a real-world experience. *J Headache Pain.* (2021) 22:111. doi: 10.1186/s10194-021-01323-6
38. Rivera-Mancilla E, Villalon CM, MaassenVanDenBrink A, CGRP. inhibitors for migraine prophylaxis: a safety review. *Expert Opin Drug Saf.* (2020) 19:1237–50. doi: 10.1080/14740338.2020.1811229
39. Uzar E, Evliyaoglu O, Yucel Y, Ugur Cevik M, Acar A, Guzel I, et al. Serum cytokine and pro-brain natriuretic peptide (BNP) levels in patients with migraine. *Eur Rev Med Pharmacol Sci.* (2011) 15:1111–6.
40. Arikanoglu A, Aydin M, Demir CF, Bulut S, Ilhan N. Plasma cytokine levels in migraineurs during and outside of attacks. *Elec J General Med.* (2015) 12:926–31. doi: 10.15197/ejgm.01255
41. Taheri M, Nicknafs F, Hesami O, Javadi A, Arsang-Jang S, Sayad A, et al. Differential Expression of cytokine-coding genes among migraine patients with and without aura and normal subjects. *J Mol Neurosci.* (2021) 71:1197–204. doi: 10.1007/s12031-020-01745-y
42. Bo SH, Davidsen EM, Gulbrandsen P, Dietrichs E, Bovim G, Stovner LJ, et al. Cerebrospinal fluid cytokine levels in migraine, tension-type headache and cervicogenic headache. *Cephalalgia.* (2009) 29:365–72. doi: 10.1111/j.1468-2982.2008.01727.x
43. Lee MJ, Lee SY, Cho S, Kang ES, Chung CS. Feasibility of serum CGRP measurement as a biomarker of chronic migraine: a critical reappraisal. *J Headache Pain.* (2018) 19:53. doi: 10.1186/s10194-018-0883-x
44. Ashina M, Terwindt GM, Al-Karagholi MA-M, de Boer I, Lee MJ, Hay DL, et al. Migraine: disease characterisation, biomarkers, and precision medicine. *Lancet.* (2021) 397:1496–504. doi: 10.1016/S0140-6736(20)32162-0
45. Biscetti L, De Vanna G, Cresta E, Corbelli I, Gaetani L, Cupini L, et al. Headache and immunological/autoimmune disorders: a comprehensive review of available epidemiological evidence with insights on potential underlying mechanisms. *J Neuroinflammation.* (2021) 18:259. doi: 10.1186/s12974-021-02229-5
46. Chen J, Chen X, Xie Y, Sun Y, Wang X, Hesketh T. Irritable bowel syndrome and migraine: evidence from Mendelian randomization analysis in the UK Biobank. *Expert Rev Gastroenterol Hepatol.* (2021) 15:1233–9. doi: 10.1080/17474124.2021.1949290
47. Arzani M, Jahromi SR, Ghorbani Z, Vahabizad F, Martelletti P, Ghaemi A, et al. Gut-brain Axis and migraine headache: a comprehensive review. *J Headache Pain.* (2020) 21:15. doi: 10.1186/s10194-020-1078-9
48. Kim JH, Yamamoto T, Lee J, Yashiro T, Hamada T, Hayashi S, et al. CGRP, a neurotransmitter of enteric sensory neurons, contributes to the development of food allergy due to the augmentation of microtubule reorganization in mucosal mast cells. *Biomed Res.* (2014) 35:285–93. doi: 10.2220/biomedres.35.285
49. Falkenberg K, Bjerg HR, Olesen J. 2-h CGRP infusion causes gastrointestinal hyperactivity: possible relevance for CGRP antibody treatment. *Headache.* (2020) 60:929–37. doi: 10.1111/head.13795
50. Fanaeian MM, Alibeik N, Ganji A, Fakheri H, Ekhlasi G, Shahbazkhani B. Prevalence of migraine in adults with celiac disease: a case control cross-sectional study. *PLoS ONE.* (2021) 16:e0259502. doi: 10.1371/journal.pone.0259502
51. Hamad N, Alzoubi KH, Swedan SF, Khabour OF, El-Salem K. Association between tumor necrosis factor alpha and lymphotoxin alpha gene polymorphisms and migraine occurrence among Jordanians. *Neurol Sci.* (2021) 42:3625–30. doi: 10.1007/s10072-020-04967-5
52. Olsen Saraiva Camara N, Lepique AP, Basso AS. Lymphocyte differentiation and effector functions. *Clin Dev Immunol.* (2012) 2012:510603. doi: 10.1155/2012/510603
53. Faraji F, Shojapour M, Farahani I, Ganji A, Mosayebi G. Reduced regulatory T lymphocytes in migraine patients. *Neurol Res.* (2021) 43:677–82. doi: 10.1080/01616412.2021.1915077
54. Kogelman LJA, Falkenberg K, Buil A, Erola P, Courraud J, Laursen SS, et al. Changes in the gene expression profile during spontaneous migraine attacks. *Sci Rep.* (2021) 11:8294. doi: 10.1038/s41598-021-87503-5
55. Bonasia S, Smajda S, Ciccio G, Robert T. Middle Meningeal Artery: Anatomy and Variations. *AJNR Am J Neuroradiol.* (2020) 41:1777–85. doi: 10.3174/ajnr.A6739
56. Wei X, Melemedjian OK, Ahn DD, Weinstein N, Dussor G. Dural fibroblasts play a potential role in headache pathophysiology. *Pain.* (2014) 155:1238–44. doi: 10.1016/j.pain.2014.03.013
57. Burgos-Vega C, Moy J, Dussor G. Meningeal afferent signaling and the pathophysiology of migraine. *Prog Mol Biol Transl Sci.* (2015) 131:537–64. doi: 10.1016/bs.pmbts.2015.01.001

58. Keller JT, Marfurt CF, Dimlich RV, Tierney BE. Sympathetic innervation of the supratentorial dura mater of the rat. *J Comp Neurol.* (1989) 290:310–21. doi: 10.1002/cne.902900210
59. Lv X, Wu Z, Li Y. Innervation of the cerebral dura mater. *Neuroradiol J.* (2014) 27:293–8. doi: 10.15274/NRJ-2014-10052
60. Erdener SE, Kaya Z, Dalkara T. Parenchymal neuroinflammatory signaling and dural neurogenic inflammation in migraine. *J Headache Pain.* (2021) 22:138. doi: 10.1186/s10194-021-01353-0
61. Moody SA, Quigg MS, Little CD. Extracellular matrix components of the peripheral pathway of chick trigeminal axons. *J Comp Neurol.* (1989) 283:38–53. doi: 10.1002/cne.902830105
62. Ashina M, Hansen JM, Do TP, Melo-Carrillo A, Burstein R, Moskowitz MA. Migraine and the trigeminovascular system-−40 years and counting. *Lancet Neurol.* (2019) 18:795–804. doi: 10.1016/S1474-4422(19)30185-1
63. Hegarty DM, Tonsfeldt K, Hermes SM, Helfand H, Aicher SA. Differential localization of vesicular glutamate transporters and peptides in corneal afferents to trigeminal nucleus caudalis. *J Comp Neurol.* (2010) 518:3557–69. doi: 10.1002/cne.22414
64. Norton CE, Boerman EM, Segal SS. Differential hyperpolarization to substance P and calcitonin gene-related peptide in smooth muscle versus endothelium of mouse mesenteric artery. *Microcirculation.* (2021) 28:e12733. doi: 10.1111/micc.12733
65. Zhou Y, Hua T, Weng X, Ma D, Li X. Calcitonin gene-related peptide alleviates hypertrophic scar formation by inhibiting the inflammation. *Arch Dermatol Res.* (2022) 314:53–60. doi: 10.1007/s00403-020-02179-7
66. Manning BM, Gruba SM, Meyer AF, Haynes CL. Neuropeptide-induced mast cell degranulation and characterization of signaling modulation in response to IgE conditioning. *ACS Chem Biol.* (2016) 11:3077–83. doi: 10.1021/acschembio.6b00616
67. Levy D, Burstein R, Kainz V, Jakubowski M, Strassman AM. Mast cell degranulation activates a pain pathway underlying migraine headache. *Pain.* (2007) 130:166–76. doi: 10.1016/j.pain.2007.03.012
68. Zhang XC, Strassman AM, Burstein R, Levy D. Sensitization and activation of intracranial meningeal nociceptors by mast cell mediators. *J Pharmacol Exp Ther.* (2007) 322:806–12. doi: 10.1124/jpet.107.123745
69. Khan S, Amin FM, Fliedner FP, Christensen CE, Tolnai D, Younis S, et al. Investigating macrophage-mediated inflammation in migraine using ultrasmall superparamagnetic iron oxide-enhanced 3T magnetic resonance imaging. *Cephalalgia.* (2019) 39:1407–20. doi: 10.1177/0333102419848122
70. Fabbretti E, D'Arco M, Fabbro A, Simonetti M, Nistri A, Giniatullin R. Delayed upregulation of ATP P2X3 receptors of trigeminal sensory neurons by calcitonin gene-related peptide. *J Neurosci.* (2006) 26:6163–71. doi: 10.1523/JNEUROSCI.0647-06.2006
71. Parihar A, Eubank TD, Doseff AI. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. *J Innate Immun.* (2010) 2:204–15. doi: 10.1159/000296507
72. da Silva EZ, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. *J Histochem Cytochem.* (2014) 62:698–738. doi: 10.1369/0022155414545334
73. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J.* (2014) 33:1104–16. doi: 10.1002/embj.201488027
74. Yuan K, Zheng J, Shen X, Wu Y, Han Y, Jin X, et al. Sensory nerves promote corneal inflammation resolution via CGRP mediated transformation of macrophages to the M2 phenotype through the PI3K/AKT signaling pathway. *Int Immunopharmacol.* (2022) 102:108426. doi: 10.1016/j.intimp.2021.108426
75. Carucci JA, Ignatius R, Wei Y, Cypess AM, Schaer DA, Pope M, et al. Calcitonin gene-related peptide decreases expression of HLA-DR and CD86 by human dendritic cells and dampens dendritic cell-driven T cell-proliferative responses via the type I calcitonin gene-related peptide receptor. *J Immunol.* (2000) 164:3494–9. doi: 10.4049/jimmunol.164.7.3494
76. Cugurra A, Mamuladze T, Rustenhoven J, Dykstra T, Beroshvili G, Greenberg ZJ, et al. Skull and vertebral bone marrow are myeloid cell reservoirs for the meninges and CNS parenchyma. *Science.* (2021) 73:7844. doi: 10.1126/science.abf7844
77. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol.* (1999) 17:593–623. doi: 10.1146/annurev.immunol.17.1.593
78. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med.* (2011) 13:e23. doi: 10.1017/S1462399411001943
79. McIlvried LA, Cruz JA, Borghesi LA, Gold MS. Sex-, stress-, and sympathetic post-ganglionic-dependent changes in identity and proportions of immune cells in the dura. *Cephalalgia.* (2017) 37:36–48. doi: 10.1177/0333102416637832
80. Franceschini A, Nair A, Bele T, van den Maagdenberg AM, Nistri A, Fabbretti E. Functional crosstalk in culture between macrophages and trigeminal sensory neurons of a mouse genetic model of migraine. *BMC Neurosci.* (2012) 13:143. doi: 10.1186/1471-2202-13-143
81. Chambers M, Rees A, Cronin JG, Nair M, Jones N, Thornton CA. Macrophage plasticity in reproduction and environmental influences on their function. *Front Immunol.* (2020) 11:607328. doi: 10.3389/fimmu.2020.607328
82. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* (2014) 6:13. doi: 10.12703/P6-13
83. Roszer T. Understanding the Mysterious M2 Macrophage through activation markers and effector mechanisms. *Mediators Inflamm.* (2015) 2015:816460. doi: 10.1155/2015/816460
84. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med.* (1983) 158:670–89. doi: 10.1084/jem.158.3.670
85. Li H, Jiang T, Li MQ, Zheng XL, Zhao GJ. Transcriptional regulation of macrophages polarization by MicroRNAs. *Front Immunol.* (2018) 9:1175. doi: 10.3389/fimmu.2018.01175
86. Canton M, Sanchez-Rodriguez R, Spera I, Venegas FC, Favia M, Viola A, et al. Reactive oxygen species in macrophages: sources and targets. *Front Immunol.* (2021) 12:734229. doi: 10.3389/fimmu.2021.734229
87. Zhang XM, Lund H, Mia S, Parsa R, Harris RA. Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice. *Glia.* (2014) 62:804–17. doi: 10.1002/glia.22643
88. Moore EM, Suresh V, Ying G, West JL. M0 and M2 Macrophages enhance vascularization of tissue engineering scaffolds. *Regenerative Engin Trans Med.* (2018) 4:51–61. doi: 10.1007/s40883-018-0048-0
89. Reeves AR, Spiller KL, Freytes DO, Vunjak-Novakovic G, Kaplan DL. Controlled release of cytokines using silk-biomaterials for macrophage polarization. *Biomaterials.* (2015) 73:272–83. doi: 10.1016/j.biomaterials.2015.09.027
90. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci U S A.* (2013) 110:17253–8. doi: 10.1073/pnas.1308887110
91. Schain AJ, Melo-Carrillo A, Borsook D, Grutzendler J, Strassman AM, Burstein R. Activation of pial and dural macrophages and dendritic cells by cortical spreading depression. *Ann Neurol.* (2018) 83:508–21. doi: 10.1002/ana.25169
92. Vogel DY, Glim JE, Stavenuiter AW, Breur M, Heijnen P, Amor S, et al. Human macrophage polarization *in vitro*: maturation and activation methods compared. *Immunobiology.* (2014) 219:695–703. doi: 10.1016/j.imbio.2014.05.002
93. Shimizu T, Toriumi H, Sato H, Shibata M, Nagata E, Gotoh K, et al. Distribution and origin of TRPV1 receptor-containing nerve fibers in the dura mater of rat. *Brain Res.* (2007) 1173:84–91. doi: 10.1016/j.brainres.2007.07.068
94. Sato T, Konishi H, Tamada H, Nishiwaki K, Kiyama H. Morphology, localization, and postnatal development of dural macrophages. *Cell Tissue Res.* (2021) 384:49–58. doi: 10.1007/s00441-020-03346-y
95. Asahina A, Moro O, Hosoi J, Lerner EA, Xu S, Takashima A, et al. Specific induction of cAMP in Langerhans cells by calcitonin gene-related peptide: relevance to functional effects. *Proc Natl Acad Sci U S A.* (1995) 92:8323–7. doi: 10.1073/pnas.92.18.8323

96. Moskowitz MA. The neurobiology of vascular head pain. *Ann Neurol.* (1984) 16:157–68. doi: 10.1002/ana.410160202
97. Duan JX, Zhou Y, Zhou AY, Guan XX, Liu T, Yang HH, et al. Calcitonin gene-related peptide exerts anti-inflammatory property through regulating murine macrophages polarization *in vitro*. *Mol Immunol.* (2017) 91:105–13. doi: 10.1016/j.molimm.2017.08.020
98. Yuan Y, Jiang Y, Wang B, Guo Y, Gong P, Xiang L. Deficiency of calcitonin gene-related peptide affects macrophage polarization in osseointegration. *Front Physiol.* (2020) 11:733. doi: 10.3389/fphys.2020.00733
99. Zhou Y, Yoshida S, Kubo Y, Yoshimura T, Kobayashi Y, Nakama T, et al. Different distributions of M1 and M2 macrophages in a mouse model of laser-induced choroidal neovascularization. *Mol Med Rep.* (2017) 15:3949–56. doi: 10.3892/mmr.2017.6491
100. Caviedes-Bucheli J, Lopez-Moncayo LF, Munoz-Alvear HD, Gomez-Sosa JF, Diaz-Barrera LE, Curtidor H, et al. Expression of substance P, calcitonin gene-related peptide and vascular endothelial growth factor in human dental pulp under different clinical stimuli. *BMC Oral Health.* (2021) 21:152. doi: 10.1186/s12903-021-01519-x
101. Levy D, Edut S, Baraz-Goldstein R, Rubovitch V, Defrin R, Bree D, et al. Responses of dural mast cells in concussive and blast models of mild traumatic brain injury in mice: Potential implications for post-traumatic headache. *Cephalalgia.* (2016) 36:915–23. doi: 10.1177/0333102415617412
102. Wan SW, Wu-Hsieh BA, Lin YS, Chen WY, Huang Y, Anderson R. The monocyte-macrophage-mast cell axis in dengue pathogenesis. *J Biomed Sci.* (2018) 25:77. doi: 10.1186/s12929-018-0482-9
103. Dahlin JS, Hallgren J. Mast cell progenitors: origin, development and migration to tissues. *Mol Immunol.* (2015) 63:9–17. doi: 10.1016/j.molimm.2014.01.018
104. Theoharides TC, Donelan J, Kandere-Grzybowska K, Konstantinidou A. The role of mast cells in migraine pathophysiology. *Brain Res Brain Res Rev.* (2005) 49:65–76. doi: 10.1016/j.brainresrev.2004.11.006
105. Greco R, Tassorelli C, Sandrini G, Di Bella P, Buscone S, Nappi G. Role of calcitonin gene-related peptide and substance P in different models of pain. *Cephalalgia.* (2008) 28:114–26.
106. Cornelison LE. Calcitonin Gene-Related Ponin Gene-Related Peptide Promotes Peripheral and Central and Central Trigeminal Sensitization Missouri State University, Springfield, MO. (2015).
107. Ferry X, Brehin S, Kamel R, Landry Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. *Peptides.* (2002) 23:1507–15. doi: 10.1016/S0196-9781(02)00090-6
108. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology.* (2008) 123:398–410. doi: 10.1111/j.1365-2567.2007.02705.x
109. Xu H, Shi X, Li X, Zou J, Zhou C, Liu W, et al. Neurotransmitter and neuropeptide regulation of mast cell function: a systematic review. *J Neuroinflammation.* (2020) 17:356. doi: 10.1186/s12974-020-02029-3
110. Yu Y, Blokhuis BR, Garssen J, Redegeld FA. Non-IgE mediated mast cell activation. *Eur J Pharmacol.* (2016) 778:33–43. doi: 10.1016/j.ejphar.2015.07.017
111. Hochman DJ, Collaco CR, Brooks EG. Acrolein induction of oxidative stress and degranulation in mast cells. *Environ Toxicol.* (2014) 29:908–15. doi: 10.1002/tox.21818
112. Leishman E, Kunkler PE, Manchanda M, Sangani K, Stuart JM, Oxford GS, et al. Environmental toxin acrolein alters levels of endogenous lipids, including TRP agonists: a potential mechanism for headache driven by TRPA1 activation. *Neurobiol Pain.* (2017) 1:28–36. doi: 10.1016/j.ynpai.2017.03.001
113. Nurkhametova DF, Koroleva KS, Gafurov OS, Giniatullina RR, Sitdikova GF, Giniatullin RA. Mast cell mediators as pain triggers in migraine: comparison of histamine and serotonin in the activation of primary afferents in the meninges in rats. *Neurosci Behav Physiol.* (2020) 50:900–6. doi: 10.1007/s11055-020-00983-2
114. Michalicek J, Gordon V, Lambert G. Reactions of the middle meningeal artery of the cat to neural and humoral stimulation. *Cephalalgia.* (1996) 16:27–36. doi: 10.1046/j.1468-2982.1996.1601027.x
115. Herr N, Bode C, Duerschmied D. The Effects of Serotonin in Immune Cells. *Front Cardiovasc Med.* (2017) 4:48. doi: 10.3389/fcvm.2017.00048
116. Dai Y, Wang S, Tominaga M, Yamamoto S, Fukuoka T, Higashi T, et al. Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J Clin Invest.* (2007) 117:1979–87. doi: 10.1172/JCI30951
117. Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. *Immunol Rev.* (2018) 282:121–50. doi: 10.1111/imr.12634
118. Jin H, Koyama T, Hatanaka Y, Akiyama S, Takayama F, Kawasaki H. Histamine-induced vasodilation and vasoconstriction in the mesenteric resistance artery of the rat. *Eur J Pharmacol.* (2006) 529:136–44. doi: 10.1016/j.ejphar.2005.10.060
119. Li H, Xiao Y, Li Q, Yao J, Yuan X, Zhang Y, et al. The allergy mediator histamine confers resistance to immunotherapy in cancer patients via activation of the macrophage histamine receptor H1. *Cancer Cell.* (2022) 40:36–52. doi: 10.1016/j.ccell.2021.11.002
120. Suleimanova A, Talanov M, Gafurov O, Gafarov F, Koroleva K, Virenque A, et al. Modeling a nociceptive neuro-immune synapse activated by ATP and 5-HT in meninges: novel clues on transduction of chemical signals into persistent or rhythmic neuronal firing. *Front Cell Neurosci.* (2020) 14:135. doi: 10.3389/fncel.2020.00135
121. Van Nueten JM, Janssens WJ, Vanhoutte PM. Serotonin and vascular reactivity. *Pharmacol Res Commun.* (1985) 17:585–608. doi: 10.1016/0031-6989(85)90067-0
122. Grant GA, Goldberg GI, Wilhelm SM, He C, Eisen AZ. Activation of extracellular matrix metalloproteases by proteases and organomercurials. *Matrix Suppl.* (1992) 1:217–23.
123. Paszcuk AF, Quintao NL, Fernandes ES, Juliano L, Chapman K, Andrade-Gordon P, et al. Mechanisms underlying the nociceptive and inflammatory responses induced by trypsin in the mouse paw. *Eur J Pharmacol.* (2008) 581:204–15. doi: 10.1016/j.ejphar.2007.11.025
124. Kopruszinski CM, Thornton P, Arnold J, Newton P, Lowne D, Navratilova E, et al. Characterization and preclinical evaluation of a protease activated receptor 2 (PAR2) monoclonal antibody as a preventive therapy for migraine. *Cephalalgia.* (2020) 40:1535–50. doi: 10.1177/0333102420966581
125. Neeb L, Hellen P, Boehnke C, Hoffmann J, Schuh-Hofer S, Dirnagl U, et al. IL-1beta stimulates COX-2 dependent PGE(2) synthesis and CGRP release in rat trigeminal ganglia cells. *PLoS ONE.* (2011) 6:e17360. doi: 10.1371/journal.pone.0017360
126. Avona A, Price TJ, Dussor G. Interleukin-6 induces spatially dependent whole-body hypersensitivity in rats: implications for extracephalic hypersensitivity in migraine. *J Headache Pain.* (2021) 22:70. doi: 10.1186/s10194-021-01286-8
127. Chinnery HR, Ruitenberg MJ, McMenamin PG. Novel characterization of monocyte-derived cell populations in the meninges and choroid plexus and their rates of replenishment in bone marrow chimeric mice. *J Neuropathol Exp Neurol.* (2010) 69:896–909. doi: 10.1097/NEN.0b013e3181edbc1a
128. Tiberio L, Del Prete A, Schioppa T, Sozio F, Bosisio D, Sozzani S. Chemokine and chemotactic signals in dendritic cell migration. *Cell Mol Immunol.* (2018) 15:346–52. doi: 10.1038/s41423-018-0005-3
129. Dunzendorfer S, Kaser A, Meierhofer C, Tilg H, Wiedermann CJ. Cutting edge: peripheral neuropeptides attract immature and arrest mature blood-derived dendritic cells. *J Immunol.* (2001) 166:2167–72. doi: 10.4049/jimmunol.166.4.2167
130. Mikami N, Matsushita H, Kato T, Kawasaki R, Sawazaki T, Kishimoto T, et al. Calcitonin gene-related peptide is an important regulator of cutaneous immunity: effect on dendritic cell and T cell functions. *J Immunol.* (2011) 186:6886–93. doi: 10.4049/jimmunol.1100028
131. Assas BM, Pennock JI, Miyan JA. Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. *Front Neurosci.* (2014) 8:23. doi: 10.3389/fnins.2014.00023
132. Pusic KM, Won L, Kraig RP, Pusic AD. IFNgamma-stimulated dendritic cell exosomes for treatment of migraine modeled using spreading depression. *Front Neurosci.* (2019) 13:942. doi: 10.3389/fnins.2019.00942
133. Schafflick D, Wolbert J, Heming M, Thomas C, Hartlehnert M, Borsch AL, et al. Single-cell profiling of CNS border compartment leukocytes reveals that B cells and their progenitors reside in non-diseased meninges. *Nat Neurosci.* (2021) 24:1225–34. doi: 10.1038/s41593-021-00880-y

134. Brioschi S, Wang WL, Peng V, Wang M, Shchukina I, Greenberg ZJ, et al. Heterogeneity of meningeal B cells reveals a lymphopoietic niche at the CNS borders. *Science*. (2021) 373:9277. doi: 10.1126/science.abf9277
135. Bracci-Laudiero L, Aloe L, Buanne P, Finn A, Stenfors C, Vigneti E, et al. NGF modulates CGRP synthesis in human B-lymphocytes: a possible anti-inflammatory action of NGF? *J Neuroimmunol.* (2002) 123:58–65. doi: 10.1016/S0165-5728(01)00475-1
136. Schlomer JJ, Storey BB, Ciornei RT, McGillis JP. Calcitonin gene-related peptide inhibits early B cell development *in vivo*. *J Leukoc Biol.* (2007) 81:802–8. doi: 10.1189/jlb.0306229
137. Berger A. Th1 and Th2 responses: what are they? *BMJ.* (2000) 321:424. doi: 10.1136/bmj.321.7258.424
138. Constant SL, Bottomly K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol.* (1997) 15:297–322. doi: 10.1146/annurev.immunol.15.1.297
139. Divan A, Casselli T, Narayanan SA, Mukherjee S, Zawieja DC, Watt JA, et al. Borrelia burgdorferi adhere to blood vessels in the dura mater and are associated with increased meningeal T cells during murine disseminated borreliosis. *PLoS One.* (2018) 13:e0196893. doi: 10.1371/journal.pone.0196893
140. Rustenhoven J, Drieu A, Mamuladze T, de Lima KA, Dykstra T, Wall M, et al. Functional characterization of the dural sinuses as a neuroimmune interface. *Cell.* (2021) 184:1000–16. doi: 10.1016/j.cell.2020.12.040
141. Levite M. Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr Opin Pharmacol.* (2008) 8:460–71. doi: 10.1016/j.coph.2008.05.001
142. Ray JC, Allen P, Bacsi A, Bosco JJ, Chen L, Eller M, et al. Inflammatory complications of CGRP monoclonal antibodies: a case series. *J Headache Pain.* (2021) 22:121. doi: 10.1186/s10194-021-01330-7
143. Schoenen J, Timmermans G, Nonis R, Manise M, Fumal A, Gerard P. Erenumab for migraine prevention in a 1-year compassionate use program: efficacy, tolerability, and differences between clinical phenotypes. *Front Neurol.* (2021) 12:805334. doi: 10.3389/fneur.2021.805334
144. Ma W, Dumont Y, Vercauteren F, Quirion R. Lipopolysaccharide induces calcitonin gene-related peptide in the RAW264. 7 macrophage cell line. *Immunology.* (2010) 130:399–409. doi: 10.1111/j.1365-2567.2009.03239.x
145. Hu R, Li YJ Li XH. An Overview of Non-Neural Sources of Calcitonin Gene-Related Peptide. *Curr Med Chem.* (2016) 23:763–73. doi: 10.2174/0929867323666160210125416

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The Neuropeptide α-Calcitonin Gene-Related Peptide as the Mediator of Beneficial Effects of Exercise in the Cardiovascular System

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Regular physical activity exerts cardiovascular protective effects in healthy individuals and those with chronic cardiovascular diseases. Exercise is accompanied by an increased plasma concentration of α-calcitonin gene-related peptide (αCGRP), a 37-amino acid peptide with vasodilatory effects and causative roles in migraine. Moreover, mouse models revealed that loss of αCGRP disrupts physiological adaptation of the cardiovascular system to exercise in normotension and aggravates cardiovascular impairment in primary chronic hypertension, both can be reversed by αCGRP administration. This suggests that αCGRP agonists could be a therapeutic option to mediate the cardiovascular protective effects of exercise in clinical setting where exercise is not possible or contraindicated. Of note, FDA has recently approved αCGRP antagonists for migraine prophylaxis therapy, however, the cardiovascular safety of long-term anti-CGRP therapy in individuals with cardiovascular diseases has yet to be established. Current evidence from preclinical models suggests that chronic αCGRP antagonism may abolish the cardiovascular protective effects of exercise in both normotension and chronic hypertension.

Keywords: exercise, cardiovascular health, αCGRP, myokines, migraine

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INTRODUCTION

Cardiovascular diseases (CVD) including hypertensive heart disease, heart failure (HF), peripheral arterial disease, and stroke represent the main cause of morbidity and mortality. During the past decades, the number of deaths due to CVD increased steadily and it is estimated that CVD may cause more than 230 million deaths across the globe in 2030. Moreover, more than 10% of world's annual total healthcare expenditure is spent for CVD (Roth et al., 2020; Amini et al., 2021).

Cardiovascular diseases is attributed to factors such as hypertension, unhealthy diet, heavy alcohol drinking, overweight, diabetes mellitus and physical inactivity (Amini et al., 2021). Numerous studies reveal that physical activity (PA) or reversal of sedentary lifestyle reduces the risk of cardiovascular events including HF and stroke (Virani et al., 2021). PA or exercise not only reduces the risk of CVD in healthy subjects but is also a central part of cardiac rehabilitation program recommended for subjects with CVD to reduce secondary events, hospital admissions and mortality (Virani et al., 2021). In fact, the ability of regular PA to increase cardiorespiratory fitness (CRF), an indicator of metabolic health, and thus to decrease CVD is well demonstrated (Kodama et al., 2009).

Additionally, regular PA lowers the incidence of typical CVD risk factors such as systemic hypertension, hyperlipidemia, and diabetes mellitus. It also exerts direct beneficial effects on the structure of blood vessels and myocardium, improves autonomic balance and attenuates tissue damaging chronic proinflammatory responses (Fiuza-Luces et al., 2018). Thus, there is no doubt that exercise is beneficial in reducing CVD risk in healthy subjects and improving cardiovascular function in cardiovascular-compromised patients.

α-CALCITONIN GENE-RELATED PEPTIDE: A DRUGGABLE MOLECULAR MEDIATOR OF CARDIOVASCULAR PROTECTIVE EFFECTS OF EXERCISE

Myokines, the peptides secreted by exercising skeletal muscles or nerve fibers innervating the contracting muscles, play important roles in mediating some of the structural and functional cardiovascular benefits of exercise (Pinckard et al., 2019). Myokines with prominent effects on cardiovascular system include C1q/TNF-related protein-1, neuron-derived neurotrophic factor, follistatin-like 1, IL-6 (Pinckard et al., 2019) and α-Calcitonin Gene-Related Peptide (αCGRP). Whether C1q/TNF-related protein-1, neuron-derived neurotrophic factor, follistatin-like 1 and IL-6 are indispensable for cardiovascular protective effects of exercise, i.e., whether their deficiency adversely affect the cardiovascular system during exercise, and their druggability still need to be defined by preclinical studies. αCGRP is a 37-amino acid druggable peptide released by nerve fibers of exercising skeletal muscles, trigeminal and perivascular nerves, endothelium, adipocytes, activated B lymphocytes, macrophages and keratinocytes. It differs from its isoform βCGRP by only three amino acids in humans. Sensory neurons preferentially express αCGRP (3–6-times higher than βCGRP) while in enteric neurons, βCGRP is upto 7-times more abundant than αCGRP [extensively reviewed by Russell et al. (2014) and Mulderry et al. (1988)]. αCGRP interacts with the receptor formed of calcitonin receptor-like receptor (CLR), receptor activity modifying protein (RAMP) and receptor component protein. Association of RAMP1 with CLR forms the specific receptor for CGRP whereas that of RAMP2 with CLR constitute the receptor for adrenomedullin [extensively discussed by Russell et al. (2014) and Liang et al. (2018)]. αCGRP, recently shown to mediate physiological cardiovascular adaptations to exercise in normotension and cardiovascular protective effects of exercise in chronic hypertension in preclinical models, is the focus of this review.

Plasma and Muscle Tissue Concentrations of α-Calcitonin Gene-Related Peptide Increase During Exercise

A previous study observed highly increased plasma αCGRP concentrations in response to exercise in human runners before a training break, following 3 weeks of physical inactivity, and 2 and 4 weeks after recommencement of training, which showed positive correlation with heart rate (Schifter et al., 1995). Others found progressive increase in plasma αCGRP concentration during exercise in normal humans, hypertensives and diabetes patients and highest αCGRP concentration was observed at maximum exercise (Lind et al., 1996). A recent study has shown increased circulating αCGRP concentration in response to maximal exercise in 2/3 of study subjects who were healthy adults (Aracil-Marco et al., 2021). In an another study on subjects suffering from headache and headache-free subjects, endurance exercise increased plasma αCGRP concentration, and the post-run variation of circulating αCGRP was inversely related to running time (Tarperi et al., 2020). Moreover, graded exercise was shown to increase plasma αCGRP concentration in normal healthy subjects and those with coronary heart disease (CHD) with an additional, interesting finding that lower workload results in highest αCGRP concentrations in subjects with CHD. Increased oxygen demand in CHD might cause an earlier αCGRP release from sensory neurons during exercise (Lechleitner et al., 1994). Another study found increase in circulating αCGRP concentrations in close linear correlation to lactate in response to exercise in healthy sea level natives at sea level and subsequently after 24 h and 5 days in high altitude hypoxia. αCGRP release was not associated with increase in catecholamines or sympathetic vasoconstrictors like noradrenaline, rather it was strongly correlated with increased lactate levels only (Hasbak et al., 2002).

The major fraction of circulating immunoreactive αCGRP comprises the intact neuropeptide, suggesting exercise-induced αCGRP production and excluding the possibility that the increase in concentrations of immunoreactive αCGRP detected during exercise is caused by molecule degradation products (Schifter, 1991; Schifter et al., 1995; Hasbak et al., 2002). Both endurance and resistance training are capable of increasing αCGRP content in skeletal muscle tissues (Parnow et al., 2012) and increased αCGRP content is detectable in motor neurons and skeletal muscle tissues even 2 days after exercise (Homonko and Theriault, 1997).

Putative Release Mechanism and Source of α-Calcitonin Gene-Related Peptide During Exercise

During exercise, group-III and group-IV afferent neurons containing metaboreceptors and mechanoreceptors within the contracting skeletal muscles are activated. This leads to activation of sympathetic nervous system causing exercise pressor reflex that regulates blood pressure (BP) and heart rate changes during exercise (Cooper et al., 2016). The vanilloid receptor transient receptor potential vanilloid subtype-1 (TRPV1), a ligand-gated non-selective cation channel expressed abundantly in group III afferent neurons, is a metaboreceptor mediating exercise pressor reflex and is activated by capsaicin, noxious heat (43°C), and protons. Lactic acid formation and consequent drop in pH activates TRPV1, leading to both neuronal exocytosis of αCGRP and activation of the nociceptive transcription factor 'cAMP response element-binding protein' which in turn enhances αCGRP expression (**Figure 1**). On the other

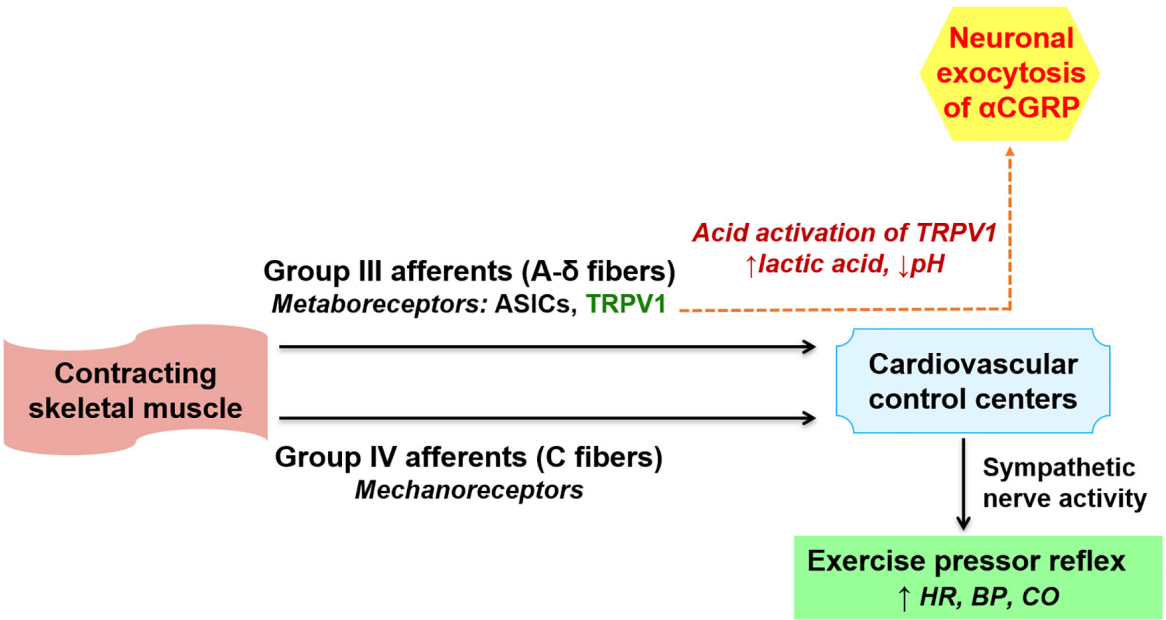

FIGURE 1 | Mechanism of αCGRP release associated with exercise pressor reflex. During exercise, group III and IV afferent neurons within skeletal muscles are activated. This activates the sympathetic nervous system resulting in exercise pressor reflex (lower part of the figure). During exercise, tissue acidification (lactate production and drop in pH) induces αCGRP release from group III afferents through activation of transient receptor potential vanilloid subtype 1 (TRPV1) channels (dashed arrow, upper part of the figure). Post release, αCGRP entering the circulation may trigger physiological cardiac hypertrophy in normotension and mediate cardio-protection during pressure overload. ASICs, acid sensing ion channels, HR, heart rate, BP, blood pressure, CO, cardiac output.

hand, αCGRP expression is inhibited by an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase (CaMK), thereby suggesting the involvement of CaMK in the downstream signaling associated with TRPV1-mediated αCGRP expression (Nakanishi et al., 2010). Lowering the pH or lactic acid alone triggered the release of αCGRP in rat spinal cord slices, and a combination of lactic acid with low pH, a condition mimicking strenuous exercise, caused more than additive stimulation of αCGRP release, suggesting lactic acid potentiates low pH-triggered αCGRP release (Wang and Fiscus, 1997). However, the underlying molecular mechanism involved in lactic acid-potentiated αCGRP release remain still undefined.

Cardiovascular Protective Effects of Exercise-Activated α-Calcitonin Gene-Related Peptide Signaling in Physiological States

α-calcitonin gene-related peptide enhances the synthesis of acetyl choline receptors and activates Na^+/K^+ pump to counteract exercise-induced K^+ depletion in myocytes. Therefore, an increase in circulating αCGRP concentrations may enhance both these processes at neuromuscular junctions during exercise (Schifter et al., 1995). Increase in αCGRP during exercise may also counteract the vasoconstrictor responses mediated by increased noradrenaline and neuropeptide-Y concentrations during sympathetic activation (Lind et al., 1996). αCGRP induces vasorelaxation through both endothelium- and NO-dependent and -independent pathways but does not regulate systemic BP in normal individuals (Russell et al., 2014). αCGRP may activate cAMP-dependent signaling pathways to mediate the cardiovascular protective effects of exercise. αCGRP protects cardiomyocytes from stress-induced apoptosis (Sueur et al., 2005) and triggers physiological cardiomyocyte growth *in vitro*, and produces positive inotropy and chronotropy (Bell et al., 1995; Al-Rubaiee et al., 2013; Schuler et al., 2014) which are the main cardiac adaptations to exercise. Exercise training caused fetal gene reactivation in the heart of adult, normotensive αCGRP knock out mice, resembling the pathological cardiac phenotype typically seen in hypertension. Moreover, treatment with the αCGRP receptor antagonist CGRP8-37 blunted exercise-induced physiological cardiac hypertrophy in normal mice. Furthermore, exercise performance was attenuated in normotensive, αCGRP knock out or CGRP8-37-treated wild-type mice but enhanced in transgenic mice overexpressing calcitonin receptor-like receptor (**Table 1**). This suggests that αCGRP augments maximum exercise capacity not only by acutely triggering positive chronotropy and inotropy but also by exerting direct hormonal protective effects on the heart to drive physiological cardiac hypertrophy (Schuler et al., 2014).

In accordance with lipolytic effects observed in muscles after CGRP administration (Danaher et al., 2008), an acute increase in plasma αCGRP concentration enhanced adipose tissue lipolysis during exercise in rats, and this effect was inhibited by pretreatment with CGRP8-37 (Aveseh et al., 2018). This evidence supports the notion that exercise-induced

TABLE 1 | Cardiovascular effects of exercise-activated endogenous, aCGRP signaling.

Subjects	Study setting/exercise intervention	Post exercise outcomes/findings on cardiovascular health
Humans	Normal, physically active healthy volunteers subjected to a graded exercise test up to exhaustion	Increased plasma CGRP concentration accompanied with enhanced cardiorespiratory fitness as indicated by increase in VO_2, VCO_2, carbohydrate oxidation rate and relative power in 2/3 of subjects (Aracil-Marco et al., 2021).
Mice	Adult mice globally deficient for aCGRP or CGRP receptor antagonist-treated WT mice with baseline hemodynamic variables including normal systemic blood pressure, subjected to treadmill exercise	Attenuated exercise performance, reactivation of myocardial foetal gene expression program (Schuler et al., 2014).
	Adult mice overexpressing the calcitonin receptor-like receptor with baseline hemodynamic variables including normal systemic blood pressure, subjected to treadmill exercise	Enhanced exercise performance (Schuler et al., 2014).
	Adult mice globally deficient for aCGRP or CGRP receptor antagonist-treated WT mice subjected to one-kidney one-clip model of chronic hypertension, subjected to 4 weeks of voluntary wheel running	Impaired survival, reduced voluntary wheel running activity, loss of beneficial effects of exercise on chronic hypertension-induced myocardial foetal gene reprogramming, pathological hypertrophic growth, fibrosis and function (Skaria et al., 2019).
Rats	6 weeks old rats underwent training protocol for 12 weeks and then treated with CGRP receptor antagonist prior subjected to single session endurance training	Attenuation of phospholipase C (PIPLC/IP3) pathway-mediated adipose tissue lipolysis during exercise (Aveseh et al., 2018).

αCGRP may exert hormonal effects (**Table 1**). An association of endogenous αCGRP with exercise-induced physiological cardiac hypertrophic growth is yet to be demonstrated in human subjects. However, a recent study has shown that healthy humans exhibiting an increase in circulating CGRP concentration also show a higher CRF, carbohydrate oxidation and work performance compared with those showing unaltered plasma CGRP concentrations post-exercise, suggesting CGRP release may be associated with physiological responses related to exercise (Aracil-Marco et al., 2021; **Table 1**).

Cardiovascular Protective Effects of Exercise-Activated α-Calcitonin Gene-Related Peptide Signaling in Hypertension

A recent study has shown markedly reduced survival of αCGRP knock out mice following induction of chronic hypertension. Moreover, it was shown that inhibiting endogenous αCGRP signaling by gene knock out or treatment with CGRP8-37 does not further increase the systemic BP in mice with stable chronic hypertension. However, chronic hypertensive αCGRP knock out mice exhibited reduced voluntary running activity and cardiac function compared with chronic hypertensive wild type (wt) mice. Four weeks of voluntary exercise or systemic administration of αCGRP peptide increased cardiac function and suppressed myocardial fetal gene reactivation as well as cardiac fibrosis in chronic hypertensive wt mice. However, the cardioprotective effects of voluntary exercise in chronic hypertensive wt mice was abolished by CGRP8-37 treatment. Moreover, only systemic administration of αCGRP peptide but not voluntary running could suppress adverse myocardial remodeling and improve cardiac function in chronic hypertensive αCGRP knock out mice (Skaria et al., 2019; **Table 1**).

The aforesaid studies that investigated the role of endogenous αCGRP signaling in normotension (Schuler et al., 2014) and chronic hypertension (Skaria et al., 2019) used αCGRP knock out mice with unaltered, baseline systemic BP and calcitonin expression (Lu et al., 1999). Other αCGRP knock out mice with combined deletion of αCGRP and calcitonin, and altered baseline cardiovascular variables apparently due to calcitonin deficiency also exhibit impaired survival and cardiac function upon pressure stress (Supowit et al., 2005). Collectively, these findings from mice models suggest that even basal αCGRP concentration is important for survival and maintaining cardiac function in chronic hypertension, and myocardial protective effects of voluntary exercise in hypertension is mediated by endogenous αCGRP signaling. Moreover, these findings also suggest that αCGRP agonism may be a potential alternative or supplemental therapeutic strategy to mimic some of the therapeutically relevant cardioprotective effects of exercise in clinical conditions where patients are mobility impaired or exercise is otherwise contraindicated, i.e., αCGRP agonists could be used as an exercise mimetic.

Despite several independent previous studies reporting increased plasma αCGRP concentrations in response to exercise in humans (Schifter et al., 1995; Lind et al., 1996; Hasbak et al., 2003; Aracil-Marco et al., 2021), evidence for a direct effect of increased plasma αCGRP in mediating cardiovascular benefits of exercise and the molecular pathway(s) involved in these protective effects in humans is still scant and should be the focus of further studies. The intensity of exercise required to increase plasma αCGRP concentration, and whether there could be a correlation between circulating αCGRP concentrations with improvements in cardiovascular function in human subjects with chronic hypertension should be addressed.

A recent study employing endogenous αCGRP inhibition by gene knock out or by the αCGRP receptor antagonist BIBN4096 BS found that endogenous αCGRP can protect against elevated BP when nitric oxide synthase is inhibited. Moreover, it was shown that systemic αCGRP administration can reduce systemic BP and suppress pathological cardiovascular remodeling during states where vascular endothelial/nitric oxide system is dysfunctional. It suggests that αCGRP agonism may exert protective effects in pathological states associated with

impaired nitric oxide production such as systemic hypertension (Argunhan et al., 2021). αCGRP exerts direct antifibrotic action by suppressing myofibroblast differentiation of cardiac fibroblasts through the activation of cAMP signaling pathway in the presence of hypertensive peptides such as Angiotensin-II (Skaria et al., 2019). αCGRP's direct effects on inhibiting pathological collagen synthesis in cardiac fibroblasts (Skaria et al., 2019; Li et al., 2020) is in accordance with BP-independent protective effects of αCGRP in various cell types including cardiomyocytes (Bell et al., 1995; Sueur et al., 2005; Schuler et al., 2014).

Efficiency and druggability were the two major issues encountered while translating the previously discovered different, cardioprotective exercise signaling pathways to therapeutic regimen in the clinical setting (Vega et al., 2017). Intravenous administration of αCGRP improves myocardial contractility in patients with congestive HF (Gennari et al., 1990; Shekhar et al., 1991), thereby confirming αCGRP's cardioprotective efficiency in clinical setting. The problem of the short plasma half-life ($T_{1/2}$) of naive αCGRP peptide (<6 min) may be circumvented by using a new CGRP-analog (α-Analog) with extended $T_{1/2}$ (>7 h) (Nilsson et al., 2016). The systemic administration of this acylated α-Analog with improved pharmacokinetics prevented end organ damage, and was well-tolerated in murine model of hypertension and HF (Aubdool et al., 2017).

Potential Cardiovascular Adverse Effects of Anti-α-Calcitonin Gene-Related Peptide-Based Migraine Prophylaxis

It is well-established that αCGRP, through vasodilation and regulation of cerebrovascular nociception, plays a critical role in the pathophysiology of migraine (Russell et al., 2014), a highly devastating neurovascular disorder affecting upto 16% of the population worldwide. Therefore, there is unwaning interest in recently approved, αCGRP antagonism-based migraine prophylaxis. The monoclonal antibodies developed against αCGRP or its receptor (with long biological half-life of >45 days) are presumed to have no side effects due to toxic metabolites formation (Vollbracht and Rapoport, 2013; Bigal and Walter, 2014). Several phase-II and phase-III trials conducted to date could not find an increase in cardiovascular adverse effects following treatment with CGRP monoclonal antibodies compared with placebo (Sun et al., 2016; Skljarevski et al., 2018; Ashina et al., 2019; Ferrari et al., 2019; Silberstein et al., 2019; Xu et al., 2019). However, the safety of long-term CGRP antagonist therapy in cardiovascular-compromised patients (e.g., chronic primary hypertension) or those with major cardiovascular risk is not established yet.

A single iv. infusion of the anti-CGRP-receptor monoclonal antibody Erenumab did not affect exercise time in patients with stable angina due to coronary artery disease, prompting the authors to conclude that CGRP receptor blockade may not worsen myocardial ischemia (Depre et al., 2018). However, a serious concern about this study is that it does not provide insights to long-term effects of αCGRP antagonism. Such studies should observe patients for many years after administering antibodies to block αCGRP pathways. To the best of our knowledge, studies observing patients longer than 5 years (Ashina et al., 2019) haven't been reported yet. Moreover, the study population of Depre et al. (2018) consisted of subjects with stable angina pectoris, often caused by stenosis of the epicardial conducting portions of the coronary artery. Actions of αCGRP are known to be limited in proximal, epicardial parts of the coronary artery bed whereas it is well-known that αCGRP is a potent vasodilator in the intramyocardial, smaller (distal) parts of the coronary artery bed. Another limitation of the study population is that 78% of the subjects were male despite the fact that majority of migraine sufferers are females. In female patients with angina pectoris, coronary artery disease is typically diffuse atherosclerosis with coronary microvascular dysfunction but without arteriographically-detectable stenosis. Therefore, it is likely that compared with males, females may respond differently to inhibition of αCGRP signaling. Furthermore, exercise treadmill test was conducted approximately 30 min after intravenous administration of the Erenumab, which may be a too short time period for the large molecular size Erenumab to reach the smooth muscle cells inside the vessels and bind to the CGRP receptor to effectively block it [extensively reviewed by Maassen van den Brink et al. (2018) and Rivera-Mancilla et al. (2020)].

α-calcitonin gene-related peptide deficient mice are resistant to develop diet-induced obesity and exhibit improved glucose handling and insulin sensitivity (Liu et al., 2017) whereas it was shown that treatment with CGRP receptor antagonist olcegepant does not affect systemic glucose and lipid metabolism but impair bone formation in mice model of diet-induced obesity (Köhli et al., 2021). This warrants further investigations on clarifying the role of endogenous CGRP signaling in regulating lipid and glucose metabolism in humans in physiological and pathological conditions and assessing bone status in patients treated with CGRP antagonists.

Strong evidence from preclinical models suggest that αCGRP, through nitric oxide- and vasodilation- dependent and independent mechanisms, protects against hypertension and hypertension-induced end organ damage including HF, and ischemic stroke (Aubdool et al., 2017; Skaria et al., 2019; Mulder et al., 2020). A recent single case study reported the development of a right thalamic infarction following the first dose of Erenumab in a 41-year-old woman with migraine without aura and with a history of long-term use of oral contraceptives (Aradi et al., 2019). As suggested by independent groups across the world, long term inhibition of αCGRP (e.g., for several years in humans) may severely impair cardiovascular function [Maassen van den Brink et al., 2016; Aubdool et al., 2017; Danser and Maassen van den Brink, 2017; extensively reviewed by Rivera-Mancilla et al. (2020)] and may invalidate therapeutically important beneficial cardiac effects of exercise in hypertensive subjects (Skaria et al., 2019).

CONCLUSION

Exercise or regular PA undoubtedly reduces the risk of CVD and improves cardiovascular function in individuals with clinically

diagnosed CVD. However, prevalence of PA did not increase considerably over the last decade, and physical inactivity was responsible for more than 800000 deaths in the year 2019. Moreover, subjects with contraindications for exercise or those with disabilities may fail to meet the PA requirements and thus may not derive the cardiovascular benefits of exercise which necessitates the need for an exercise mimetic. αCGRP could potentially be such an exercise mimetic because findings from preclinical models suggest that activation of endogenous αCGRP signaling may be one of the mechanisms by which exercise improves cardiovascular function in physiological conditions and cardiovascular diseases such as chronic hypertension. On the other hand, αCGRP's cardiovascular protective effects raise concerns about the potential cardiovascular risks associated with chronic αCGRP antagonism, which has been recently approved by FDA for migraine prophylaxis.

AUTHOR CONTRIBUTIONS

TS and JV designed and wrote the manuscript. Both authors read and approved the final manuscript.

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REFERENCES

Al-Rubaiee, M., Gangula, P. R., Millis, R. M., Walker, R. K., Umoh, N. A., Cousins, V. M., et al. (2013). Inotropic and lusitropic effects of calcitonin gene-related peptide in the heart. *Am. J. Physiol. Heart Circ. Physiol.* 304, H1525–H1537. doi: 10.1152/ajpheart.00874.2012

Amini, M., Zayeri, F., and Salehi, M. (2021). Trend analysis of cardiovascular disease mortality, incidence, and mortality-to-incidence ratio: results from global burden of disease study 2017. *BMC Public Health* 21:401. doi: 10.1186/s12889-021-10429-0

Aracil-Marco, A., Sarabia, J. M., Pastor, D., Guillén, S., López-Grueso, R., Gallar, J., et al. (2021). Acute Increase in Blood αCGRP at Maximal Exercise and Its Association to Cardiorespiratory Fitness, Carbohydrate Oxidation and Work Performed: an Exploratory Study in Young Men. *Biology* 10:783. doi: 10.3390/biology10080783

Aradi, S., Kaiser, E., and Cucchiara, B. (2019). Ischemic Stroke Associated With Calcitonin Gene-Related Peptide Inhibitor Therapy for Migraine: a Case Report. *J. Stroke Cerebrovasc. Dis.* 28:104286. doi: 10.1016/j.jstrokecerebrovasdis.2019.07.002

Argunhan, F., Thapa, D., Aubdool, A. A., Carlini, E., Arkless, K., Hendrikse, E. R., et al. (2021). Calcitonin Gene-Related Peptide Protects Against Cardiovascular Dysfunction Independently of Nitric Oxide In Vivo. *Hypertension* 77, 1178–1190. doi: 10.1161/hypertensionaha.120.14851

Ashina, M., Goadsby, P. J., Reuter, U., Silberstein, S., Dodick, D., Rippon, G. A., et al. (2019). Long-term safety and tolerability of erenumab: three-plus year results from a five-year open-label extension study in episodic migraine. *Cephalalgia* 39, 1455–1464. doi: 10.1177/0333102419854082

Aubdool, A. A., Thakore, P., Argunhan, F., Smillie, S. J., Schnelle, M., Srivastava, S., et al. (2017). A Novel alpha-Calcitonin Gene-Related Peptide Analogue Protects Against End-Organ Damage in Experimental Hypertension, Cardiac Hypertrophy, and Heart Failure. *Circulation* 136, 367–383. doi: 10.1161/circulationaha.117.028388

Aveseh, M., Koushkie-Jahromi, M., Nemati, J., and Esmaeili-Mahani, S. (2018). Serum calcitonin gene-related peptide facilitates adipose tissue lipolysis during exercise via PIPLC/IP3 pathways. *Endocrine* 61, 462–472. doi: 10.1007/s12020-018-1640-2

Bell, D., Schluter, K. D., Zhou, X. J., Mcdermott, B. J., and Piper, H. M. (1995). Hypertrophic effects of calcitonin gene-related peptide (CGRP) and amylin on adult mammalian ventricular cardiomyocytes. *J. Mol. Cell Cardiol.* 27, 2433–2443. doi: 10.1006/jmcc.1995.0231

Bigal, M. E., and Walter, S. (2014). Monoclonal antibodies for migraine: preventing calcitonin gene-related peptide activity. *CNS Drugs* 28, 389–399. doi: 10.1007/s40263-014-0156-4

Cooper, M. A., Kluding, P. M., and Wright, D. E. (2016). Emerging Relationships between Exercise, Sensory Nerves, and Neuropathic Pain. *Front. Neurosci.* 10, 372. doi: 10.3389/fnins.2016.00372

Danaher, R. N., Loomes, K. M., Leonard, B. L., Whiting, L., Hay, D. L., Xu, L. Y., et al. (2008). Evidence that alpha-calcitonin gene-related peptide is a neurohormone that controls systemic lipid availability and utilization. *Endocrinology* 149, 154–160. doi: 10.1210/en.2007-0583

Danser, A. H. J., and Maassen van den Brink, A. (2017). Calcitonin Gene-Related Peptide Receptor Agonism: a Double-Edged Sword? *Circulation* 136, 384–387. doi: 10.1161/CIRCULATIONAHA.117.028955

Depre, C., Antalik, L., Starling, A., Koren, M., Eisele, O., Lenz, R. A., et al. (2018). A Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Effect of Erenumab on Exercise Time During a Treadmill Test in Patients With Stable Angina. *Headache* 58, 715–723. doi: 10.1111/head.13316

Ferrari, M. D., Diener, H. C., Ning, X., Galic, M., Cohen, J. M., Yang, R., et al. (2019). Fremanezumab versus placebo for migraine prevention in patients with documented failure to up to four migraine preventive medication classes (FOCUS): a randomised, double-blind, placebo-controlled, phase 3b trial. *Lancet* 394, 1030–1040. doi: 10.1016/S0140-6736(19)31946-4

Fiuza-Luces, C., Santos-Lozano, A., Joyner, M., Carrera-Bastos, P., Picazo, O., and Zugaza, J. L. (2018). Exercise benefits in cardiovascular disease: beyond attenuation of traditional risk factors. *Nat. Rev. Cardiol.* 15, 731–743. doi: 10.1038/s41569-018-0065-1

Gennari, C., Nami, R., Agnusdei, D., and Fischer, J. A. (1990). Improved cardiac performance with human calcitonin gene related peptide in patients with congestive heart failure. *Cardiovasc. Res.* 24, 239–241. doi: 10.1093/cvr/24.3.239

Hasbak, P., Lundby, C., Olsen, N. V., Schifter, S., and Kanstrup, I. L. (2002). Calcitonin gene-related peptide and adrenomedullin release in humans: effects of exercise and hypoxia. *Regul. Pept.* 108, 89–95. doi: 10.1016/s0167-0115(02)00129-5

Hasbak, P., Saetrum Opgaard, O., Eskesen, K., Schifter, S., Arendrup, H., Longmore, J., et al. (2003). Investigation of CGRP receptors and peptide pharmacology in human coronary arteries. *J. Pharmacol. Exp. Ther.* 304, 326–333. doi: 10.1124/jpet.102.037754

Homonko, D. A., and Theriault, E. (1997). Calcitonin gene-related peptide is increased in hindlimb motoneurons after exercise. *Int. J. Sports Med.* 18, 503–509. doi: 10.1055/s-2007-972672

Kodama, S., Saito, K., Tanaka, S., Maki, M., Yachi, Y., Asumi, M., et al. (2009). Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. *JAMA* 301, 2024–2035. doi: 10.1001/jama.2009.681

Köhli, P., Appelt, J., Otto, E., Jahn, D., Baranowsky, A., Bahn, A., et al. (2021). Effects of CGRP receptor antagonism on glucose and bone metabolism in mice with diet-induced obesity. *Bone* 143, 115646. doi: 10.1016/j.bone.2020.115646

Lechleitner, P., Haring, C., Mair, J., Genser, N., Wiedermann, C. J., Dienstl, F., et al. (1994). Exercise-induced increase in plasma concentrations of calcitonin gene-related peptide in patients with coronary heart disease and healthy controls. *Int. J. Angiol.* 3, 16–19. doi: 10.1007/bf02014907

Li, W., Zhang, Z., Li, X., Cai, J., Li, D., Du, J., et al. (2020). CGRP derived from cardiac fibroblasts is an endogenous suppressor of cardiac fibrosis. *Cardiovasc. Res.* 116, 1335–1348. doi: 10.1093/cvr/cvz234

Liang, Y.-L., Khoshouei, M., Deganutti, G., Glukhova, A., Koole, C., Peat, T. S., et al. (2018). Cryo-EM structure of the active, Gs-protein complexed, human CGRP receptor. *Nature* 561, 492–497. doi: 10.1038/s41586-018-0535-y

Lind, H., Brudin, L., Lindholm, L., and Edvinsson, L. (1996). Different levels of sensory neuropeptides (calcitonin gene-related peptide and substance P) during and after exercise in man. *Clin. Physiol.* 16, 73–82. doi: 10.1111/j.1475-097x.1996.tb00557.x

Liu, T., Kamiyoshi, A., Sakurai, T., Ichikawa-Shindo, Y., Kawate, H., Yang, L., et al. (2017). Endogenous Calcitonin Gene-Related Peptide Regulates Lipid Metabolism and Energy Homeostasis in Male Mice. *Endocrinology* 158, 1194–1206. doi: 10.1210/en.2016-1510

Lu, J. T., Son, Y. J., Lee, J., Jetton, T. L., Shiota, M., Moscoso, L., et al. (1999). Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. *Mol. Cell Neurosci.* 14, 99–120. doi: 10.1006/mcne.1999.0767

Maassen van den Brink, A., Rubio-Beltrán, E., Duncker, D., and Villalón, C. M. (2018). Is CGRP Receptor Blockade Cardiovascularly Safe? Appropriate Studies Are Needed. *Headache:. J. Head Face Pain* 58, 1257–1258.

Maassen van den Brink, A., Meijer, J., Villalon, C. M., and Ferrari, M. D. (2016). Wiping Out CGRP: potential Cardiovascular Risks. *Trends Pharmacol. Sci.* 37, 779–788. doi: 10.1016/j.tips.2016.06.002

Mulder, I. A., Li, M., De Vries, T., Qin, T., Yanagisawa, T., Sugimoto, K., et al. (2020). Anti-migraine Calcitonin Gene-Related Peptide Receptor Antagonists Worsen Cerebral Ischemic Outcome in Mice. *Ann. Neurol.* 88, 771–784. doi: 10.1002/ana.25831

Mulderry, P. K., Ghatki, M. A., Spokks, R. A., et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 25, 195–205. doi: 10.1016/0306-4522(88)90018-8

Nakanishi, M., Hata, K., Nagayama, T., Sakurai, T., Nishisho, T., Wakabayashi, H., et al. (2010). Acid activation of Trpv1 leads to an up-regulation of calcitonin gene-related peptide expression in dorsal root ganglion neurons via the CaMK-CREB cascade: a potential mechanism of inflammatory pain. *Mol. Biol. Cell* 21, 2568–2577. doi: 10.1091/mbc.E10-01-0049

Nilsson, C., Hansen, T. K., Rosenquist, C., Hartmann, B., Kodra, J. T., Lau, J. F., et al. (2016). Long acting analogue of the calcitonin gene-related peptide induces positive metabolic effects and secretion of the glucagon-like peptide-1. *Eur. J. Pharmacol.* 773, 24–31. doi: 10.1016/j.ejphar.2016.01.003

Parnow, A., Gharakhanlou, R., Gorginkaraji, Z., Rajabi, S., Eslami, R., Hedayati, M., et al. (2012). Effects of endurance and resistance training on calcitonin gene-related Peptide and acetylcholine receptor at slow and fast twitch skeletal muscles and sciatic nerve in male wistar rats. *Int. J. Pept.* 2012:962651. doi: 10.1155/2012/962651

Pinckard, K., Baskin, K. K., and Stanford, K. I. (2019). Effects of Exercise to Improve Cardiovascular Health. *Front. Cardiovasc. Med.* 6:69. doi: 10.3389/fcvm.2019.00069

Rivera-Mancilla, E., Villalón, C. M., and Maassenvandenbrink, A. (2020). CGRP inhibitors for migraine prophylaxis: a safety review. *Expert Opin. Drug Saf.* 19, 1237–1250. doi: 10.1080/14740338.2020.1811229

Roth, G. A., Mensah, G. A., Johnson, C. O., Addolorato, G., Ammirati, E., Baddour, L. M., et al. (2020). Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019: update From the GBD 2019 Study. *J. Am. Coll. Cardiol.* 76, 2982–3021. doi: 10.1016/j.jacc.2020.11.010

Russell, F. A., King, R., Smillie, S. J., Kodji, X., and Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* 94, 1099–1142. doi: 10.1152/physrev.00034.2013

Schifter, S. (1991). Circulating concentrations of calcitonin gene-related peptide (CGRP) in normal man determined with a new, highly sensitive radioimmunoassay. *Peptides* 12, 365–369. doi: 10.1016/0196-9781(91)90027-m

Schifter, S., Breum, L., Niclasen, B., Vollmer-Larsen, A., Rasmussen, H. S., and Graff-Larsen, O. (1995). Calcitonin gene-related peptide during exercise and training. *Horm. Metab. Res.* 27, 473–475. doi: 10.1055/s-2007-980005

Schuler, B., Rieger, G., Gubser, M., Arras, M., Gianella, M., Vogel, O., et al. (2014). Endogenous alpha-calcitonin-gene-related peptide promotes exercise-induced, physiological heart hypertrophy in mice. *Acta Physiol.* 211, 107–121. doi: 10.1111/apha.12244

Shekhar, Y. C., Anand, I. S., Sarma, R., Ferrari, R., Wahi, P. L., and Poole-Wilson, P. A. (1991). Effects of prolonged infusion of human alpha calcitonin gene-related peptide on hemodynamics, renal blood flow and hormone levels in congestive heart failure. *Am. J. Cardiol.* 67, 732–736. doi: 10.1016/0002-9149(91)90531-o

Silberstein, S. D., Mcallister, P., Ning, X., Faulhaber, N., Lang, N., Yeung, P., et al. (2019). Safety and Tolerability of Fremanezumab for the Prevention of Migraine: a Pooled Analysis of Phases 2b and 3 Clinical Trials. *Headache* 59, 880–890. doi: 10.1111/head.13534

Skaria, T., Mitchell, K., Vogel, O., Walchli, T., Gassmann, M., and Vogel, J. (2019). Blood Pressure Normalization-Independent Cardioprotective Effects of Endogenous, Physical Activity-Induced Alpha Calcitonin Gene-Related Peptide (alphaCGRP) in Chronically Hypertensive Mice. *Circ. Res.* 125, 1124–1140 doi: 10.1161/CIRCRESAHA.119.315429

Skljarevski, V., Matharu, M., Millen, B. A., Ossipov, M. H., Kim, B. K., and Yang, J. Y. (2018). Efficacy and safety of galcanezumab for the prevention of episodic migraine: results of the EVOLVE-2 Phase 3 randomized controlled clinical trial. *Cephalalgia* 38, 1442–1454. doi: 10.1177/0333102418779543

Sueur, S., Pesant, M., Rochette, L., and Connat, J. L. (2005). Antiapoptotic effect of calcitonin gene-related peptide on oxidative stress-induced injury in H9c2 cardiomyocytes via the RAMP1/CRLR complex. *J. Mol. Cell Cardiol.* 39, 955–963. doi: 10.1016/j.yjmcc.2005.09.008

Sun, H., Dodick, D. W., Silberstein, S., Goadsby, P. J., Reuter, U., Ashina, M., et al. (2016). Safety and efficacy of AMG 334 for prevention of episodic migraine: a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* 15, 382–390. doi: 10.1016/S1474-4422(16)00019-3

Supowit, S. C., Rao, A., Bowers, M. C., Zhao, H., Fink, G., Steficek, B., et al. (2005). Calcitonin gene-related peptide protects against hypertension-induced heart and kidney damage. *Hypertension* 45, 109–114. doi: 10.1161/01.HYP.0000151130.34874.fa

Tarperi, C., Sanchis-Gomar, F., Montagnana, M., Danese, E., Salvagno, G. L., Gelati, M., et al. (2020). Effects of endurance exercise on serum concentration of calcitonin gene-related peptide (CGRP): a potential link between exercise intensity and headache. *Clin. Chem. Lab. Med.* 58, 1707–1712. doi: 10.1515/cclm-2019-1337

Vega, R. B., Konhilas, J. P., Kelly, D. P., and Leinwand, L. A. (2017). Molecular Mechanisms Underlying Cardiac Adaptation to Exercise. *Cell Metab.* 25, 1012–1026. doi: 10.1016/j.cmet.2017.04.025

Virani, S. S., Alonso, A., Aparicio, H. J., Benjamin, E. J., Bittencourt, M. S., and Callaway, C. W. (2021). Heart Disease and Stroke Statistics—2021 Update. *Circulation* 143, e254–e743.

Vollbracht, S., and Rapoport, A. M. (2013). The pipeline in headache therapy. *CNS Drugs* 27, 717–729. doi: 10.1007/s40263-013-0090-x

Wang, X., and Fiscus, R. R. (1997). Lactic acid potentiates bradykinin- and low-pH-induced release of CGRP from rat spinal cord slices. *Am. J. Physiol.* 273, E92–E98. doi: 10.1152/ajpendo.1997.273.1.E92

Xu, D., Chen, D., Zhu, L. N., Tan, G., Wang, H. J., Zhang, Y., et al. (2019). Safety and tolerability of calcitonin-gene-related peptide binding monoclonal antibodies for the prevention of episodic migraine - a meta-analysis of randomized controlled trials. *Cephalalgia* 39, 1164–1179. doi: 10.1177/0333102419829007

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CGRP and the Calcitonin Receptor are Co-Expressed in Mouse, Rat and Human Trigeminal Ganglia Neurons

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The neuropeptide calcitonin gene-related peptide (CGRP) is expressed in the trigeminal ganglia, a key site in craniofacial pain and migraine. CGRP potently activates two receptors: the CGRP receptor and the AMY_1 receptor. These receptors are heterodimers consisting of receptor activity-modifying protein 1 (RAMP1) with either the calcitonin receptor-like receptor (CLR) to form the CGRP receptor or the calcitonin receptor (CTR) to form the AMY_1 receptor. The expression of the CGRP receptor in trigeminal ganglia has been described in several studies; however, there is comparatively limited data available describing AMY_1 receptor expression and in which cellular subtypes it is found. This research aimed to determine the relative distributions of the AMY_1 receptor subunit, CTR, and CGRP in neurons or glia in rat, mouse and human trigeminal ganglia. Antibodies against CTR, CGRP and neuronal/glial cell markers were applied to trigeminal ganglia sections to investigate their distribution. CTR-like and CGRP-like immunoreactivity were observed in both discrete and overlapping populations of neurons. In rats and mice, 30–40% of trigeminal ganglia neurons displayed CTR-like immunoreactivity in their cell bodies, with approximately 78–80% of these also containing CGRP-like immunoreactivity. Although human cases were more variable, a similar overall pattern of CTR-like immunoreactivity to rodents was observed in the human trigeminal ganglia. CTR and CGRP appeared to be primarily colocalized in small to medium sized neurons, suggesting that colocalization of CTR and CGRP may occur in C-fiber neurons. CGRP-like or CTR-like immunoreactivity were not typically observed in glial cells. Western blotting confirmed that CTR was expressed in the trigeminal ganglia of all three species. These results confirm that CTR is expressed in trigeminal ganglia neurons. The identification of populations of neurons that express both CGRP and CTR suggests that CGRP could act in an autocrine manner through a CTR-based receptor, such as the AMY_1 receptor. Overall, this suggests that a trigeminal ganglia CTR-based receptor may be activated during migraine and could therefore represent a potential target to develop treatments for craniofacial pain and migraine.

Keywords: CGRP—calcitonin gene-related peptide, migraine, headache, G protein-coupled receptor, trigeminal ganglia, amylin, amylin 1 (AMY_1)

1 INTRODUCTION

Migraine is one of the most disabling neurological conditions and is estimated to affect 15–20% of people worldwide (Agosti, 2018). The discovery of calcitonin gene-related peptide (CGRP), a neuropeptide with potent vasodilatory and neuromodulatory activity, and its role in migraine pathogenesis led to the development of several breakthrough therapeutics (Edvinsson et al., 2018). These include humanized antibodies and small molecules which target CGRP and its canonical receptor, the CGRP receptor, to reduce receptor activation and signaling in migraine relevant structures (Dubowchik et al., 2020).

The precise pathophysiology of migraine is unclear, however, the trigeminovascular system appears to play a major role in the generation, processing, and modulation of migraine pain, particularly the trigeminal ganglia (TG) and the trigeminal nerves (TN) (May and Goadsby, 1999; Buzzi et al., 2003; Edvinsson J. C. A. et al., 2020). The trigeminovascular system is part of the peripheral nervous system, located outside the blood-brain barrier, and can be modulated by circulating or locally released molecules. CGRP and the molecular subunits of the canonical CGRP receptor, the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1), are all expressed within the TG where they may contribute to migraine pathogenesis (Eftekhari et al., 2010; Eftekhari et al., 2013; Eftekhari et al., 2015; Hay et al., 2018; Rees et al., 2021b).

The AMY_1 receptor, which comprises RAMP1 together with the calcitonin receptor (CTR), is another CGRP-responsive receptor that can be potently activated by CGRP and amylin (Bower et al., 2018; Hay et al., 2018). AMY_2 and AMY_3 receptors use RAMP2 or RAMP3 with CTR to form other amylin receptor subtypes (Bower et al., 2018; Hay et al., 2018). Amylin, a peptide hormone secreted from pancreatic islet β-cells in response to food intake, is closely related to CGRP (Hay et al., 2015). A recent provocation study demonstrated that infusion of an amylin analogue, pramlintide, was sufficient to induce headache and migraine-like attacks in migraineurs (Ghanizada et al., 2021). This highlights a possible role for amylin-responsive receptors, like the AMY_1 receptor, in headache and migraine pathogenesis, through peripheral migraine-relevant structures such as the TG.

Pharmacological and protein data suggest that CTR and RAMP1 are expressed in the TG. Both subunits are co-expressed in some neurons, indicating potential expression of the AMY_1 receptor (Walker et al., 2015; Bohn et al., 2017; Edvinsson L. et al., 2020). However, mRNA studies of CTR expression are conflicting, with little mRNA detected (Manteniotis et al., 2013; Flegel et al., 2015; Walker et al., 2015; LaPaglia et al., 2018; Edvinsson L. et al., 2020). This lack of correlation between protein and mRNA may be because mRNA abundance is not necessarily proportionally linked to protein expression (Vogel et al., 2010; Vogel and Marcotte, 2012; Gingell et al., 2020). Transcriptome and proteomic studies propose that translation efficiency and the rate of protein degradation make a significant contribution to overall protein expression levels (Vogel et al., 2010; Vogel and Marcotte, 2012). Additionally, G protein-coupled receptors (GPCR) like the CTR have highly amplified and carefully regulated signal transduction cascades and do not need to be expressed at high levels to produce functional effects. Measures of protein expression with validated antibodies are therefore important. Previous immunohistochemical studies localizing CTR in the TG used antibodies that were uncharacterized or later found to be unable to detect CTR in a particular species (Walker et al., 2015; Edvinsson L. et al., 2020; Hendrikse et al., 2022). Therefore, it is necessary to conduct additional studies with well-validated, species-appropriate antibodies to substantiate these prior reports.

Several studies report that CGRP can promote its own expression in an autocrine manner. In the TG, this autoregulatory mechanism has been linked to migraine chronification (Zhang et al., 2007; Guo et al., 2020). However, the underlying mechanism by which CGRP exerts this effect is unknown because CLR and CGRP tend to be expressed in distinct neuronal subpopulations (Lennerz et al., 2008; Eftekhari et al., 2013; Edvinsson et al., 2019). This suggests that other CGRP-responsive receptors, such as the AMY_1 receptor, could instead act as an autoregulatory CGRP-receptor. Of note, there are species differences in the number of potential CGRP-responsive receptors. More rat and mouse CLR or CTR-based receptors are responsive to CGRP, as compared to their human counterparts (Hay et al., 2003; Bailey et al., 2012; Bohn et al., 2017; Garelja et al., 2021a). Therefore, it is important to understand where CTR might be found in relation to CGRP-expressing structures in pre-clinical model species, and in humans.

The present study was therefore designed to investigate the protein expression of the CTR, and its spatial relationship to CGRP expression in the TG of rats, mice and humans using well-validated antibodies. Histology was complimented with immunoblotting to provide an orthogonal method for determining the presence of the CTR in the TG.

2 MATERIALS AND METHODS

2.1 Antibodies

All primary and secondary antibodies are detailed in **Supplementary Table S1**.

2.2 Plasmids

The N-terminally HA-tagged human CLR and CTR ($CT_{(a)}$ splice variant, leucine polymorphic variant), myc- or untagged human RAMP1 constructs in pcDNA3.1 were as previously described (Qi et al., 2013). Untagged rat CLR, CTR and RAMP1 constructs in pCMV6 were from Origene (Rockville, United States). The untagged mouse CLR, CTR and RAMP1 in pCMV6 plasmids were as previously described (Garelja et al., 2021a). Rat and mouse CTR were the $CT_{(a)}$ variant.

2.3 Cell Culture and Transfection

HEK293S cells were cultured and transfected as previously described (Gingell et al., 2020). Cells were plated into poly-D-lysine coated Cell-Carrier Ultra plates (PerkinElmer, Waltham, MA) at 10,000 cells per well and transfected 36 h after plating with 0.25 μg of DNA, using polyethylenimine as previously described (Gingell et al., 2020). Immunocytochemistry was

performed 24–36 h after transfection. For western blotting, HEK293S cells were grown in 15 cm^2 dishes and transfected with 60 μg of DNA when ~70% confluent. Cells were harvested for whole cell lysate preparations 48 h after transfection. In all cases, CLR or CTR were transfected in a 1:1 ratio with either RAMP1 or pcDNA3.1. Rat, mouse and human $CT_{(a)}$ splice variants were used as controls for immunocytochemistry and immunoblotting. However, the antigenic sequences for mAb8B9, pAb188 and mAb31-01 are present in both the $CT_{(a)}$ and $CT_{(b)}$ variants. Therefore, these antibodies are expected to detect both variants.

2.4 Immunocytochemistry

Transfected HEK293S cells were fixed with 4% paraformaldehyde (PFA) and washed with Tris-buffered saline (TBS) containing 0.1% Tween20 (TBS-T). Cells were blocked with 10% donkey serum (Abcam, Cambridge, United Kingdom) in TBS-T for 1 h at room temperature (RT). Cells were then incubated with primary antibody (**Supplementary Table S1**) in 1% serum/TBS-T overnight at 4°C. Cells were washed twice with TBS-T then incubated with secondary antibody (1:200, **Supplementary Table S1**) and DAPI (ThermoFisher Scientific, Waltham, MA) in 1% serum/TBS-T for 1 h at RT. Cells were then washed twice in TBS-T and imaged.

2.5 Peptides

All peptides were synthesized as previously described (Bower et al., 2018; Ghanizada et al., 2021). Human αCGRP (hαCGRP) and rat αCGRP (rαCGRP) were made as 1 mM stocks in water. Human amylin (hAmy) and rat amylin (rAmy) were made up as previously described as 13 mM stocks in 100% DMSO or water, respectively (Bower et al., 2018; Ghanizada et al., 2021). All peptides were stored as aliquots in protein LoBind tubes (022431081, Eppendorf, Hamburg, DE) at−30°C.

2.6 Immunoblotting (Dot Blotting)

Dot blotting was performed as previously described (Rees et al., 2021a). Briefly, stock solutions of hAmy, rAmy, hαCGRP, and rαCGRP peptides were serially diluted in sterile water to give the required concentrations. The species and peptide order were randomized. Two microliters containing the total amount of each peptide required was loaded as a single spot on 0.45 μm nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were then incubated for 1 h at RT in TBS-T with 5% (w/v) low-fat milk (assay buffer). This buffer was removed, and the membranes were then incubated with primary anti-CGRP antibodies (**Supplementary Table S1**) diluted in assay buffer for 1 h at RT. Membranes were then washed twice for 5 min in TBS-T and incubated with secondary antibodies diluted 1:1,000 in assay buffer for 1 h at RT. Membranes were then washed twice before the blots were developed with SuperSignal West Pico PLUS (34577, ThermoFisher Scientific) for ~5 min. Blots were imaged using an Amersham A600 imager (GE Healthcare, Chicago, IL). Image acquisition was performed using the automated exposure function with the high dynamic range setting. All blots presented are representative of consistent results from at least three independent experiments.

As the 100 μg hAmy stock was dissolved in 100% DMSO, comparisons were made to 100% DMSO alone to determine whether it affected immunoreactivity. This was performed on both nitrocellulose and polyvinylidene fluoride (PVDF) membranes (Cat# LC 2,005 Life Technologies, Carlsbad, CA). For nitrocellulose membranes, the addition of 2 μl of 100 μg hAmy (in 100% DMSO) or 100% DMSO alone was performed as above. For the PVDF membranes, 2 μl of hAmy (in 100% DMSO) or 100% DMSO alone was added to membranes pre-wet with methanol. Fifty ng of rαCGRP was included as a positive control. Membranes were then incubated at RT for approximately 5 min while being kept moist with TBS-T. Membranes were then incubated with assay buffer and immunoblotting was performed as above.

2.7 Tissue Collection—Mouse and Rat

All procedures involving the use of animals were conducted in accordance with the New Zealand Animal Welfare Act (1999) and approved by the University of Auckland Animal Ethics Committee. Rodents of the same sex were housed with littermates in Tecniplast Greenline IVC with Sealsafe Plus GM500 cages (mice) or as pairs in Teciplast Conventional 1500U cages (rats) in a controlled environment (12-h light-dark cycle; room temperature, 22 ± 2°C) with ad libitum access to standard chow (Teklad TB 2018; Harlan, Madison, WI) and water. Cages also contained an additional enrichment item (house or toy). Tissue was collected from available animals culled as part of routine colony maintenance. The estrous cycle phase was not assessed or recorded for female rats or mice. Animal details are provided in **Supplementary Table S2**.

Anesthesia was induced with 5% isoflurane in 2 L/min O_2, and the animals euthanized by cervical dislocation. Tissues were dissected quickly from male and female Sprague-Dawley (SD) rats and C57BL/6J mice. Tissues collected for western blotting were snap-frozen in liquid nitrogen and stored at −80°C. Tissues collected for immunohistochemistry were washed with phosphate-buffered saline (PBS) and placed in 4% PFA for 24 h at 4°C. After fixation, tissues were cryoprotected with 10%, then 20% sucrose (w/v) in PBS and embedded in optimal cutting temperature compound (Sakura Tissue-Tek, 4583). Pancreata and TG were cryo-sectioned transversely or sagittally, respectively, at a thickness of 12 μm using a Leica CM1850 microtome (Leica Biosystems, Wetzlar, Germany). Sections were mounted onto slides and then stored at −80°C.

2.8 Tissue Collection—Human

For immunohistochemistry, postmortem human TG were obtained from the University of Auckland Human Anatomy Laboratory, with informed consent by the donor before death and next of kin after death as part of the University of Auckland Human Body Bequest Program for teaching and research. This program and its procedures operate under the Human Tissue Act of 2008 and are overseen by the New Zealand Police Inspector of Anatomy. For western blotting, fresh-frozen postmortem human TG and trigeminal nerve (TN) was obtained from the NIH NeuroBioBank. Case details are provided in **Supplementary Table S3**.

After dissection from the cadaver, TG specimens were fixed with 15% formaldehyde in 0.1 M phosphate buffer for 24 h at 4°C. Specimens were then dehydrated in sequential incubations of 70%, 80%, 95%, and 100% ethanol, followed by clearing with xylene as per a standard, pre-set 'biopsy' cycle in a tissue processor (ASP6025, Leica Biosystems) at RT under vacuum. Specimens were then embedded in paraffin wax and sectioned sagittally (10 μm) on a rotary microtome (Leica Biosystems, HI 2235). Sections were floated in a water bath set at 38°C (Leica Biosystems, HI1210), mounted individually on SuperFrost slides, and allowed to dry at RT for at least 18 h before storage at RT indefinitely.

2.9 Histology—Mouse and Rat

Rat and mouse histology was performed as previously described (Rees et al., 2021a; Ghanizada et al., 2021). Briefly, sections were thawed at RT, washed twice with TBS-T, then blocked with TBS-T containing 10% normal donkey serum (v/v) for 1 h at RT. Sections were then incubated with primary antibodies, diluted in TBS-T with 1% (v/v) normal donkey serum (immunobuffer), and incubated overnight at 4°C. For TG histology, sections were co-incubated with primary antibodies (pAb188, 1:100 (mouse) or 1: 200 (rat); pAb36001, 1:500; β tubulin III, 1:500 or NF200, 1:200). Sections were then washed with TBS-T twice and incubated with secondary antibodies (1:200) in immunobuffer with DAPI for 1 h at RT. After secondary antibody incubation, sections were washed twice with TBS-T and coverslips mounted with ProLong Diamond Antifade (P36965, ThermoFisher Scientific).

Primary and secondary antibody details are outlined and compared in **Supplementary Table S1**. For all experiments, sections from each individual mouse or rat were processed separately in independent experiments. TG sections were obtained from the middle third of the ganglia in the sagittal plane. Sections were observed under a light microscope to check for morphology, tissue quality and neuron numbers. Sections displaying good tissue condition and sufficient neuron numbers (visually estimated to be > 200 neurons) were selected for staining. Triple staining of CGRP and CTR with β tubulin III or NF200 was performed in parallel on serial sections, generating two technical replicates of CGRP and CTR co-staining per animal. Sections were imaged using an Operetta high-content imaging system in non-confocal mode (rat pancreas) or confocal mode (mouse and rat TG) using a 20x high-numerical-aperture (0.75) objective (Perkin Elmer Life and Analytical Sciences, Waltham, MA). Some rat TG sections were also imaged with a 20x (0.8) lens on an LSM 710 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

2.10 Histology—Human

Postmortem human TG histology was performed as previously described (Ghanizada et al., 2021). Briefly, sections were heated at 60°C for 1 h, then dewaxed and rehydrated in xylene for 2 × 20 min, followed by 2 × 10 min of 100% ethanol, and 5 min each of 95%, 80%, and 75% ethanol. Sections were then washed for 3 × 5 min in water. Rehydration of tissue sections was performed at RT. Antigen retrieval was performed using 10 mM sodium citrate buffer, pH 6.0, at 121°C for 20 min. Sections were permeabilized with PBS + 0.2% Triton X-100 (v/v) (PBS-T) for 5 min, washed twice with PBS, then incubated with PBS containing 10% normal donkey serum for 1 h at RT. After blocking, sections were co-incubated with primary antibodies (mAb31-01, 1:250; pAb36001, 1:500; β tubulin III, 1:500 or S100, 1:200), diluted in PBS-T + 1% normal donkey serum (v/v) (human immunobuffer) overnight at 4°C. After primary antibody incubation, sections were washed once with PBS-T, twice with PBS, then incubated with secondary antibodies in human immunobuffer (1:250, **Supplementary Table S1**) for 3 h at RT. Sections were then washed with PBS, incubated with Hoechst (1:10,000) for 10 min at RT, washed again with PBS and the coverslips mounted with ProLong Diamond Antifade.

For all experiments, sections from each human case were processed separately as independent experiments. Sections were observed under a light microscope to check for morphology, tissue quality and neuron numbers. Sections displaying good tissue condition and sufficient neuron numbers (visually estimated to be > 150 neurons) were selected for staining. Triple staining of CGRP and CTR with β tubulin III or S100 was performed in parallel on serial sections, generating two technical replicates of CGRP and CTR co-staining per human case. Sections were imaged using an Operetta high-content imaging system in confocal mode using a 20x high-numerical-aperture (0.75) objective.

2.11 Immunoblotting (Western Blotting)

Preparation of whole cell or whole tissue lysates and western blotting were performed as previously described (Hendrikse et al., 2022). Transfected HEK293S cells in 15 cm^2 dishes were washed with ice-cold PBS. The cells were harvested on ice in 10 ml of ice-cold PBS using a cell scraper. Cells were pelleted by centrifugation (1,000 x g, 10 min, 4°C) and the supernatant removed. One 15 cm^2 dish corresponded to one whole cell lysate preparation. Transfected HEK293S cell pellets, fresh-frozen mouse (C57BL/6J), rat (SD) or human tissue were homogenized using a 1 ml Dounce glass homogenizer in immunoprecipitation buffer (Tris-NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 8.0) containing a complete mini EDTA-free protease inhibitor cocktail tablet (Cat #4693159001, 1:10,000; Roche Applied Science). The homogenized samples were then left to solubilize for 2 h at 4°C. Samples were centrifuged (16,000 x g, 20 min, 4°C), and the supernatant was aliquoted into protein LoBind tubes and stored at -80°C. The protein concentration of a sample was quantified using a bicinchoninic acid protein assay kit (ThermoFisher Scientific).

Protein samples were incubated for 1 h at 37°C in 4x loading dye (2.5 ml 1 M Tris-HCL, 4 ml 20% sodium dodecyl sulfate, 4 ml 100% glycerol, 0.04 mg bromophenol blue) and 0.1 M DTT. Protein samples (0.1–20 μg, **Supplementary Table S4**) were loaded alongside the ab116027 (Abcam) or PrecisionPlus (BIORAD, 1610373) protein ladders onto 4–12% SurePage SDS gels (GenScript, Piscataway, NJ) and run at 180 V in MOPS buffer. Due to availability issues multiple protein ladders were used. The Abcam and PrecisionPlus ladders displayed comparable apparent molecular weights and were in line with each other (**Supplementary Figure S1**). Proteins were transferred to 0.45 μm PVDF (mouse/rat TG) or nitrocellulose

membranes (human TG/TN) (Life Technologies and BIORAD) and were blocked with 5% low-fat milk in TBS-T for 1 h at RT. Blots were incubated with primary antibody (mAb8B9, 1:500; pAb188, 1:500; mAb31-01; 1:500) overnight at 4°C, washed twice with TBS-T, then incubated with secondary antibody (1:2,000, **Supplementary Table S1**) for 1 h at RT. Blots were washed twice with TBS-T, developed with Supersignal West Pico Plus ECL (ThermoFisher Scientific) and imaged using an Amersham Imager A600 (GE Healthcare). Image acquisition was performed using the automated exposure function with the high dynamic range setting. All blots presented are representative results from at least three independent experiments.

2.12 Image Preparation and Processing

Representative immunocytochemistry, western blotting and immunohistochemistry images are presented from at least three independent experiments performed using separate antibody dilutions. Independent immunocytochemistry experiments are defined as the immunoreactivity detected in cells from independent transfection and staining experiments performed with two technical replicates. Western blotting experiments are defined as independent experiments generated using one transfected cell lysate preparation or different tissue lysates prepared from three individual rodents and two human cases. Independent immunohistochemistry images are defined as the immunoreactivity detected in tissue from individual rodents or human cases.

Images were minimally processed using the FIJI open-source imaging platform to adjust color and brightness for presentation purposes (Schindelin et al., 2012). Any processing was uniformly applied across each image and all conditions for an antibody. Brightness and contrast, unless otherwise stated, were adjusted to the top and bottom of the histogram to allow visualization of staining across all intensities and prevent loss of data (Johnson, 2012). Therefore, minimally processed images are presented for most figures. However, for some figures, as noted in their legends, adjustment of contrast and brightness was made via the histogram (contrast stretching) to enhance visualization of positively stained cells with varying intensities. This did not affect the study conclusions.

2.13 Image Analysis and Statistical Analysis

To determine the size of neuronal cell bodies and quantify the proportion of stained neuronal cell bodies, image analysis was performed on multiple fields of view (20x high NA lens, Operetta) on each section from individual animals; three fields of view for mice and five for rats. Each field of view contained between ~50–150 neurons. In total 1967 and 2205 neurons were analyzed for mouse and rat, respectively. All images were analyzed as unedited 16-bit TIFFs in greyscale. Image analysis was performed using FIJI and was partially automated using the macro function to generate counts and the mean diameter (mean of Feret diameter and minimum diameter for each neuron) of neurons demonstrating immunostaining for β tubulin III, CGRP, CTR and CTR/CGRP (see **Supplementary Material**: Supplementary method and **Supplementary Figure S2A**).

Graphing and statistical analysis was performed using Prism GraphPad 8.0.2 (GraphPad Software, La Jolla, CA). For each section/animal, neuronal cell body diameter data from all three or five fields of view were center binned in 2.5 μm increments for the four different neuron subsets (β tubulin III, CGRP, CTR and CTR/CGRP). These data were then plotted to generate histograms to visualize the combined data from each species, presented as mean ± standard error of the mean (s.e.m.) from six individual animals. A cubic spline was applied to help visualize the distribution.

The validity of the image and data analysis procedures were confirmed using staining of the pan neuronal marker β tubulin III (**Supplementary Figure S2B**). The neuronal cell body size distribution histograms generated for rat and mouse β tubulin III were consistent with the literature (Ambalavanar and Morris, 1992; Ruscheweyh et al., 2007; Lennerz et al., 2008). This indicated that the analysis was robust and analysis of the CGRP and CTR sub-populations within the total β tubulin III population was appropriate. NF200 immunoreactivity was not amenable to this analysis approach and therefore image quantification was restricted to β tubulin III.

For each section/animal, the proportion (percentage) of neurons expressing CGRP or CTR were determined for each of the fields of view. These were combined to give a mean percentage value for each animal. The mean percentage values were combined for each sex and species as appropriate and presented as mean ± s.e.m from three (sex) or six (species) individual animals. The same approach was used to assess the proportion of CTR + neurons which co-expressed CGRP. For statistical analysis, the combined mean values from three (sex) or six (species) individual animals were compared using Student's t-tests. Statistical significance was defined as $p < 0.05$. Image analysis and quantification was not performed for human TG due to the lower number of human cases and variability in staining patterns between the different cases.

3 RESULTS

3.1 Distribution of CTR and CGRP in Rat and Mouse TG

To examine the spatial relationships between CTR and CGRP, we first needed to identify and characterize an anti-CGRP antibody. Four anti-CGRP antibodies were tested (**Supplementary Figure S3**). In immunoblotting, all four anti-CGRP antibodies detected rat and human CGRP. Interestingly, immunoreactivity was more intense for rat, than for human CGRP (**Supplementary Figure S3A**). There was no cross-reactivity in immunoblotting with high amounts of amylin and no immunoreactivity in rat pancreatic islets for three of the CGRP antibodies, pAb36001, mAb81887 and pAbC8198 (**Supplementary Figure S3A, C**). However, mAbABS 026–05-02 displayed cross-reactivity with 100 μg of rat amylin in dot blots and immunoreactivity in rat pancreatic islets (**Supplementary Figure S3A, C**). All four anti-CGRP antibodies displayed similar patterns of immunoreactivity in rat TG neuronal cell bodies (**Supplementary Figure S3D**).

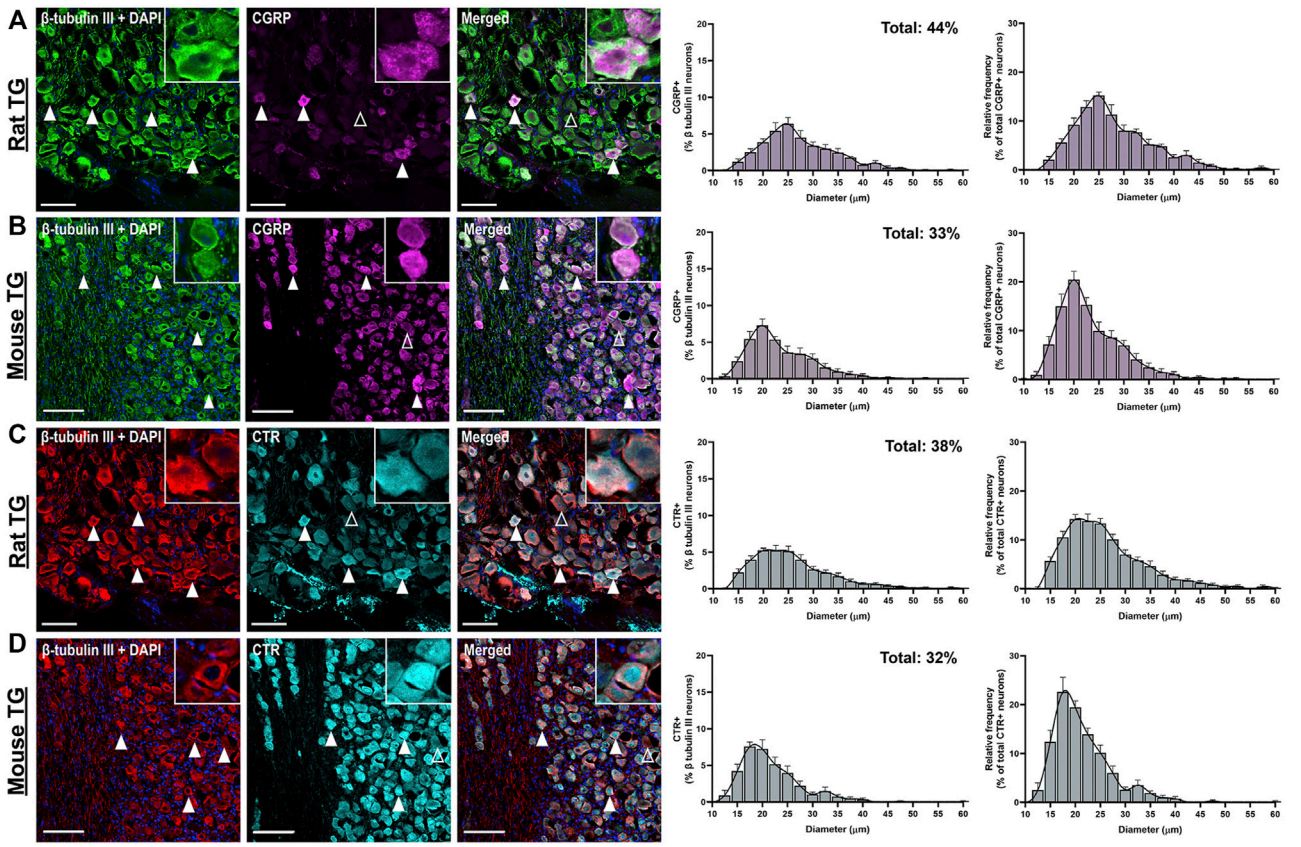

FIGURE 1 | Immunohistochemical localization and quantification of CGRP (pAb36001, 10 μg/ml) or CTR (pAb188, 10 or 20 μg/ml) individually with β tubulin III (2 μg/ml) in adult rat and mouse TG. **(A)** CGRP and β tubulin III in rat **(B)** CGRP and β tubulin III in mouse **(C)** CTR and β tubulin III in rat and **(D)** CTR and β tubulin III in mouse. Filled white arrowheads indicate examples of positive staining; empty arrowheads indicate examples of an absence of staining. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Scale bar = 100 μm. Images are representative of six rats and six mice (three male and three female). The size distribution (diameter) of neuronal cell bodies expressing CGRP or CTR for each species are displayed as histograms. The distribution of neuron size was quantified relative to the total β tubulin III (pan-neuronal marker) expressing neuron population and then relative to either CTR or CGRP expression. Data are the mean ± s.e.m, combined from six individual rats or mice (three male and three female).

The primary anti-CGRP antibody pAb36001 was selected for further studies based on a combination of factors. It was able to detect CGRP with sufficient sensitivity in immunofluorescence and immunoblotting and did not cross-react with amylin under the conditions used (**Supplementary Figure S3**, **Supplementary Table S5**). Additionally, as pAb36001 was raised in goat it enabled colocalization with antibodies against CTR and other cellular markers, which were raised in rabbit or mouse. For CTR, we used pAb188 for these experiments. pAb188 has been knockout validated in several studies and displays robust immunoreactivity in several regions in rodent nervous tissue (Goda et al., 2018; Coester et al., 2020; Hendrikse et al., 2022).

To localize the CTR-like and CGRP-like immunoreactivity (LI) in the TG, sections were co-incubated with anti-CGRP, anti-CTR and primary antibodies for neuronal cell markers, β tubulin III (pan-neuronal) and NF200 (rodent A-fiber neuronal marker) (Shiers et al., 2020; von Buchholtz et al., 2020). For the purposes of stepwise description of the data, CGRP and CTR results are first presented individually with cellular markers, and then they are presented together to examine their spatial relationship.

3.1.1 CGRP-like Immunoreactivity

CGRP-LI was present in the cell bodies of 44 ± 3.9% of rat and 33 ± 3.1% of mouse TG neurons. The size distribution was consistent with that of small to medium-sized neurons (Rat: 15–35 μm; Mouse: 10–30 μm), as indicated by β tubulin III staining (**Figures 1A,B**) (Messlinger and Russo, 2019). Immunoreactivity was usually observed as puncta in medium-sized neurons, indicating the expression of CGRP in vesicles, or dense/intense staining in smaller neurons (**Figures 1A,B**, **Supplementary Figures S4–S5**). No notable CGRP-LI was observed in satellite glia surrounding the neurons, nor the myelinating Schwann cells (**Figures 1A,B**). Visually, CGRP-LI appeared to be more frequent in mice than rats. However, this is likely due to lower signal intensity above background in mice, in combination with the limited histogram adjustment during image processing.

CGRP-LI did not notably overlap with NF200 (**Figures 2A,B**). "Pearl-like" or varicose CGRP-LI was observed in neuronal fibers, as indicated by β tubulin III, but not those expressing NF200, suggesting that CGRP is more commonly expressed in

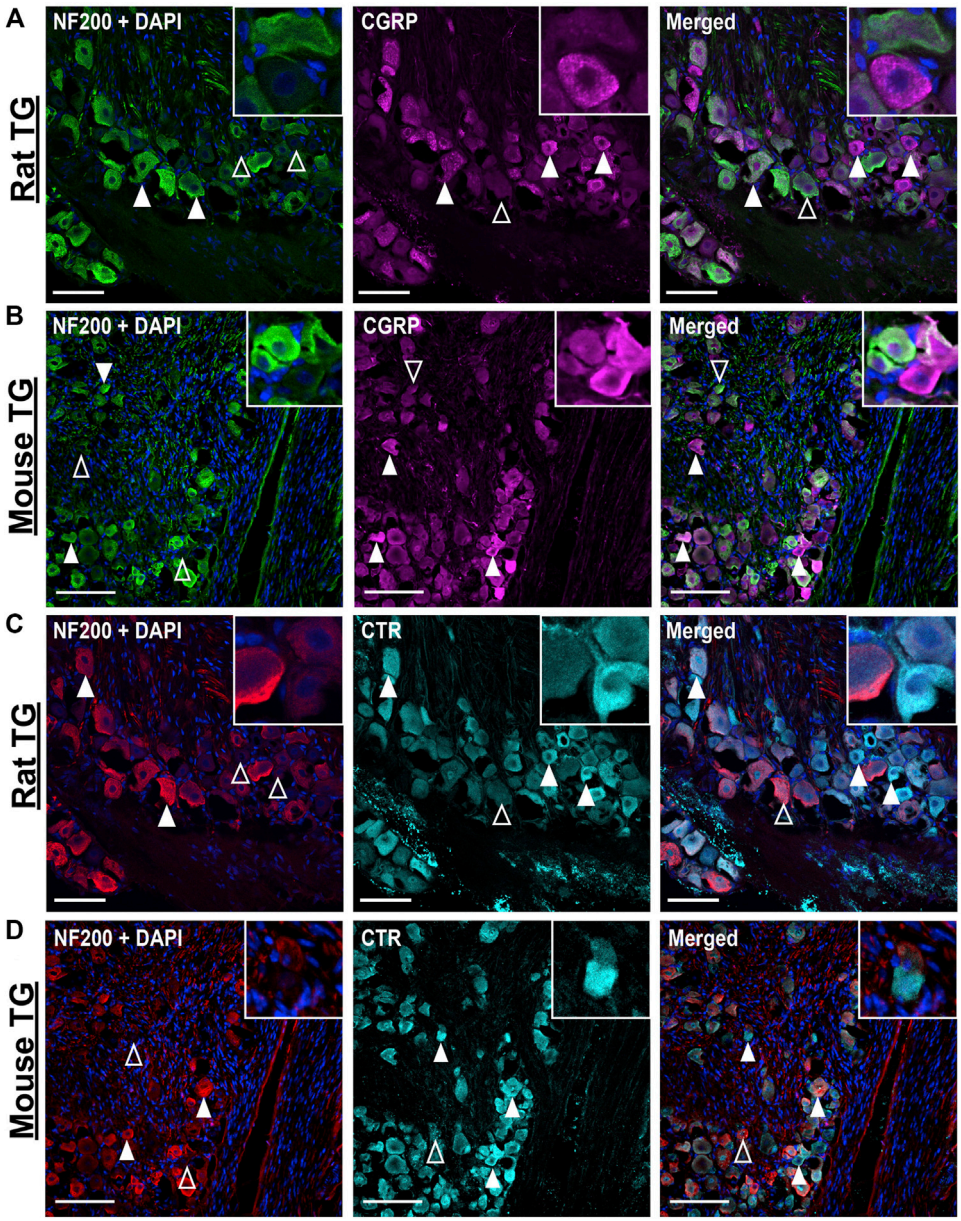

FIGURE 2 | Immunohistochemical localization of CGRP (pAb36001, 10 µg/ml) or CTR (pAb188, 10 or 20 µg/ml) individually with NF200 (3 µg/ml) in adult rat and mouse TG. **(A)** CGRP and NF200 in rat **(B)** CGRP and NF200 in mouse **(C)** CTR and NF200 in rat and **(D)** CTR and NF200 in mouse. Filled white arrowheads indicate examples of positive staining; empty arrowheads indicate examples of an absence of staining. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Scale bar = 100 µm. Images are representative of six rats and six mice (three male and three female).

unmyelinated C-fibers (**Supplementary Figures S4–S5**). These data are strongly in agreement with previous publications, which indicate that CGRP is expressed in approximately 30–50% of neurons (Lennerz et al., 2008; Eftekhari et al., 2010; Eftekhari et al., 2013; Edvinsson et al., 2019; Edvinsson L. et al., 2020; Guo et al., 2020).

3.1.2 CTR-like Immunoreactivity

CTR-LI was observed in the cell bodies of 38 ± 4.1% of rat and 32 ± 3.8% of mouse TG neurons (**Figures 1C,D**). These CTR-positive neurons tended to be small to medium in size. The intensity of the CTR staining was variable, with some occasional bright cells, whereas other positively stained neurons were more moderate in intensity. CTR staining was often diffuse and present throughout the cytoplasm of the neurons, rather than clearly localized to the cell surface. CTR staining did not appear to commonly overlap with NF200 staining (**Figures 2C,D**). CTR-LI did not appear to overlap with either β tubulin III or NF200 stained neuronal fibers, nor was any CTR-LI observed in satellite glia or Schwann cells (**Figures 1C,D**). Like CGPR-LI, CTR-LI visually appeared to be more intense

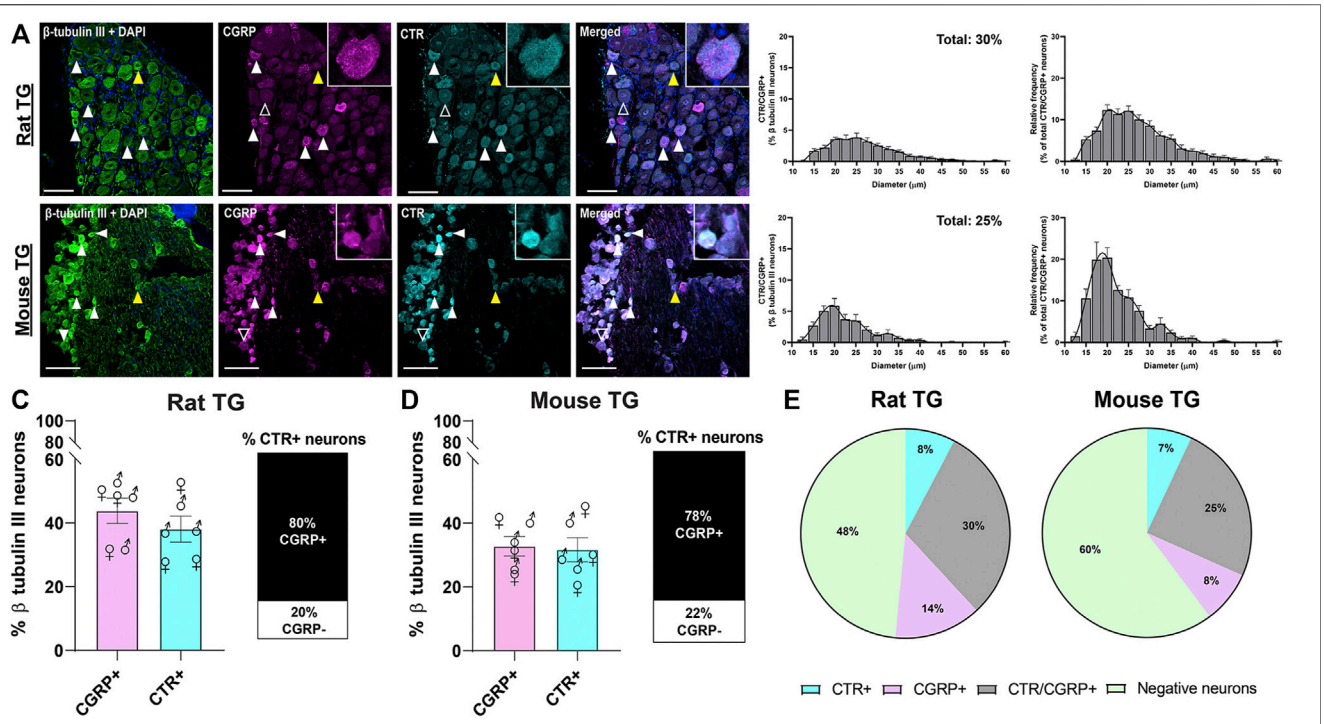

FIGURE 3 | Immunohistochemical localization and quantification of CGRP (pAb36001, 10 μg/ml) and of CTR (pAb188, 10 or 20 μg/ml) together with β tubulin III (2 μg/ml) in adult rat and mouse TG. **(A)** CGRP and CTR in rat and **(B)** CGRP and CTR in mouse. β tubulin III is shown for reference. Filled white arrowheads indicate examples of positive staining; empty arrowheads indicate examples of an absence of staining; yellow arrowheads indicate expression in adjacent neurons. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Scale bar = 100 μm. Images are representative of six rats or mice (three male and three female). The size distribution (diameter) of neuronal cell bodies expressing CGRP and CTR for each species are displayed as histograms. The distribution of neuron size was quantified relative to the total β tubulin III (pan-neuronal marker) expressing neuron population and then relative to CTR/CGRP expression. The percentage of **(C)** rat or **(D)** mouse β tubulin III TG neurons expressing CGRP or CTR and the proportion of CTR expressing neurons which also express CGRP TG. Data are the mean ± s.e.m, combined from six individual rats or mice (three male and three female). Percentage of the **(E)** total neuronal population (β tubulin III) which express CGRP alone without overlapping with CTR, CTR alone without overlapping with CGRP, or co-express CTR and CGRP in rat or mouse TG. Negative neurons refers to the neuronal population (β tubulin III) which do not express CGRP or CTR. Data are the mean, combined from six individual rats or mice (three male and three female).

in mice than rats but this is again due to lower signal to background and limited image processing.

3.1.3 Relative Distribution of CGRP-like and CTR-like Immunoreactivity

The distributions of CTR and CGRP-LI relative to one-another are shown in **Figures 3**, **4**. CGRP-LI and CTR-LI was observed both in distinct and in overlapping populations of β tubulin III-positive neuronal cell bodies, indicating that there are neurons which express CGRP alone, CTR alone and co-express CGRP and CTR together. No notable co-staining of CTR and CGRP was observed in neuronal fibers, satellite glia or Schwann cells (**Figures 3A,B**).

Interestingly, 30 ± 4.3% and 25 ± 3.9% of all rat and mouse TG neurons, respectively, exhibited overlapping CGRP-LI and CTR-LI (white filled arrowheads), suggesting co-expression in these neurons. These neurons were typically small to medium in size (**Figures 3A,B**). The proportion of CTR-stained neurons that also exhibited CGRP-LI was 80 ± 4.6% of rat and 78 ± 1.7% of mouse TG neurons (**Figures 3C,D**).

Some neurons were positive for only CGRP or CTR (**Figure 3E**). In rat TG, the presence of neurons which exclusively expressed CGRP was more common than those which exclusively expressed CTR, with 13% of β tubulin III neurons expressing CGRP alone, compared to 8% for CTR alone. In mouse TG, exclusive expression of CGRP or CTR was 8 and 7%, respectively (**Figure 3E**). Neurons which displayed CTR-LI alone were often located next to neurons that expressed CGRP (yellow filled arrowheads). Co-staining of CTR and CGRP did not notably occur in NF200-expressing neurons (**Figure 4**).

There were no significant differences in the proportion of CGRP-LI or CTR-LI between rats and mice, nor the proportion of CTR-positive neurons which co-expressed CGRP (**Table 1**; **Figures 3C,D**). Similarly, there were no significant differences in CGRP-LI and CTR-LI, or CTR and CGRP co-expression between the female and male mice or rats (**Table 2**). The individual data points for each male or female rat or mouse are plotted in **Figures 3C,D**, to show the range and scatter of the data for each sex.

3.2 Immunoblotting - CTR Expression in Rat and Mouse TG

An orthogonal method, western blotting, was employed to gain additional molecular insight into CTR expression in rodent

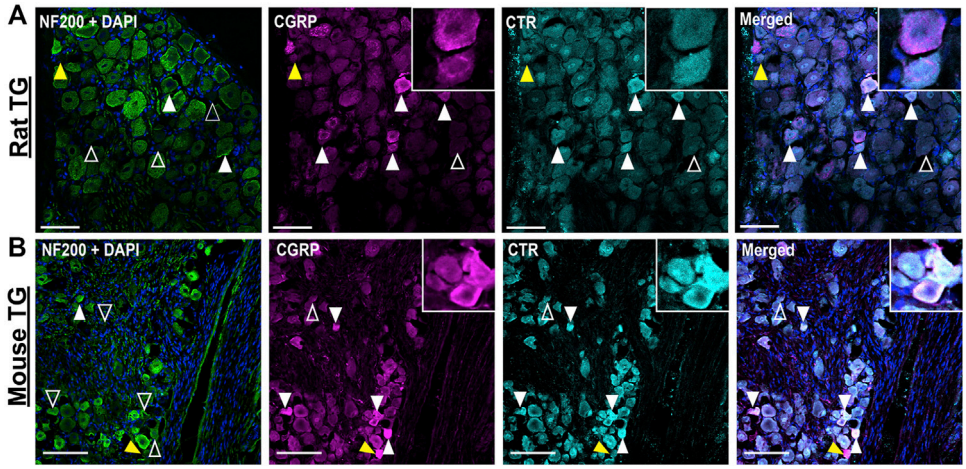

FIGURE 4 | Immunohistochemical localization of CGRP (pAb36001, 10 µg/ml) and CTR (pAb188, 10 or 20 µg/ml) together with NF200 (3 µg/ml) in adult rat and mouse TG. **(A)** CGRP and CTR in rat and **(B)** CGRP and CTR in mouse. NF200 is shown for reference. Filled white arrowheads indicate examples of positive staining; empty arrowheads indicate examples of an absence of staining; yellow arrowheads indicate expression in adjacent neurons. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Scale bar = 100 µm. Images and quantification are representative of six rats and six mice (three male and three female).

TABLE 1 | Comparison of CGRP and CTR expression and CTR/CGRP co-expression between rat and mouse.

	CGRP+ (% β tubulin III neurons)	CTR+ (% β tubulin III neurons)	CGRP+ (% CTR+ neurons)
Rat	44 ± 3.9	38 ± 4.1	80 ± 4.6
Mouse	33 ± 3.1	32 ± 3.8	78 ± 1.7

Data are the mean ± s.e.m, combined from six individual rats or mice (three male and three female). Comparisons of the percentage of CGRP+ neurons, CTR+ neurons and the percentage of CTR+ neurons that co-express CGRP between rat and mouse were made by unpaired Student's t*-test. There were no significant differences.*

TG. The predicted molecular weight of the rat and mouse $CT_{(a)}$ splice variant is approximately 52–53 kDa, although the CTR can also exist as other molecular forms due to alternative splicing, dimerization and post-translational modifications (Albrandt et al., 1995; Quiza et al., 1997; Harikumar et al., 2010). Rodent CTR protein expression was profiled using the monoclonal anti-rat CTR antibody mAb8B9, which has been well-validated and is suitable for western blotting (Hendrikse et al., 2022). In addition, the polyclonal anti-rat CTR antibody pAb188 was also used to allow a direct parallel to the immunohistochemistry data, although this antibody has been previously shown to be less suitable for western blotting (Hendrikse et al., 2022).

The mAb8B9 and pAb188 antibodies were both tested in the same tissue lysates from a mixture of male and female rodents. All blots for the mAb8B9 antibody are shown in **Figure 5**. In $mCT_{(a)}$ and $rCT_{(a)}$ transfected HEK293S cell lysates, mAb8B9 detected a clear immunoreactive band at ~53 kDa and a weaker band at ~100 kDa (**Figure 5**). Additionally, an immunoreactive smear from ~65-70 kDa was also commonly observed. In rat and mouse TG lysate, a strong immunoreactive band was observed at ~53 and ~120 kDa with an additional band at ~100 kDa present in mouse TG lysate, but not rat. Three lower molecular weight bands of less than 30 kDa were also observed in both species. Interestingly, a band at ~56 kDa was observed in male but not in female rat and mouse TG lysate (**Figure 5**). Too few animals of each sex were used for any definitive conclusions to be made but this observation is reported so that future studies could be designed to formally test this. Additionally, the effect of estrous cycle on expression could be considered.

For pAb188, the band pattern was consistent between sexes and blots are representative of both sexes (**Figure 6**). In the $mCT_{(a)}$ and $rCT_{(a)}$ transfected HEK293S cell lysates, pAb188 detected an immunoreactive band at ~52 kDa and a weaker band at ~110 kDa.

TABLE 2 | Comparison of CGRP and CTR expression and CTR/CGRP co-expression between female and male rat and mouse.

Species	Sex	CGRP+ (% β tubulin III neurons)	CTR+ (% β tubulin III neurons)	CGRP+ (% CTR+ neurons)
Rat	Female	43 ± 5.9	35 ± 8.2	74 ± 4.7
	Male	45 ± 6.4	41 ± 2.8	86 ± 7.0
Mouse	Female	31 ± 5.3	31 ± 7.2	79 ± 0.4
	Male	34 ± 4.1	33 ± 4.4	78 ± 3.8

Data are the mean ± s.e.m, combined from three individual female or male rats or mice. Comparisons between females and males of the percentage of CGRP+ neurons, CTR+ neurons and percentage of CTR+ neurons that co-express CGRP in rats or mice were made by unpaired Student's t*-test. There were no significant differences.*

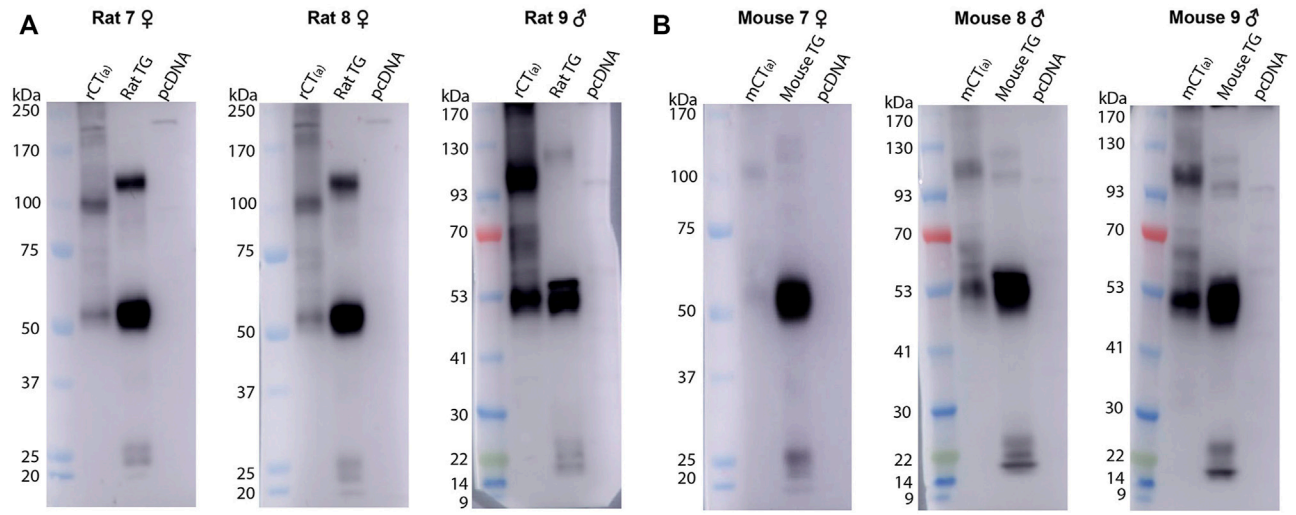

FIGURE 5 | CTR-like immunoreactivity in rat and mouse TG lysate using immunoblotting with mAb8B9. **(A)** Immunoblots using lysate preparations from adult rat TG (20 µg) and HEK293S cells (10 µg) transfected with rat $CT_{(a)}$ or vector alone (pcDNA). **(B)** Immunoblots using lysate preparations from adult mouse TG (20 µg) and HEK293S cells (10 µg) transfected with mouse $CT_{(a)}$ or vector alone. Blots were probed with mAb8B9 (2 µg/ml). MW markers are shown on the left of each blot, with apparent MW in kDa. This image shows western blots of samples from individual mouse or rat TG. Images were adjusted uniformly for brightness and contrast for presentation purposes.

Additionally, an immunoreactive smear from 52–70 kDa was also commonly observed (**Figure 6**). Generally, pAb188 displayed similar immunoreactivity patterns to mAb8B9 in transfected cells, rat TG and mouse TG lysates. However, a greater number of non-specific bands were observed for pAb188, likely due to its polyclonal nature (**Figure 6**). The bands between 100 and 120 kDa seen for mAb8B9 were absent in rat and mouse TG lysates (**Figure 6**). Similarly, the wide, strong band at ~52 kDa was absent in mouse TG lysate. Several additional strongly immunoreactive bands were observed at approximately 60 and 230 kDa. However, the ~60 kDa band was also present in the vector-transfected cell lysate when a higher amount of protein was loaded, suggesting that it could be non-specific (**Supplementary Figure S6**, **Supplementary Table S4**).

Collectively, the data with pAb188 and mAb8B9 indicate that protein is present in rat and mouse TG at molecular weights consistent with that expected for CTR. Interestingly, the band sizes were not always directly comparable to the transfected cell samples.

3.3 Validation of Anti-Human CTR Antibodies

We next proceeded to test human samples. To examine the expression of CTR in human TG the anti-human CTR antibody mAb31-01 was selected as it has been reported to detect human CTR in immunohistochemistry and immunoblotting, whereas our recent results suggest that pAb188 is not suitable for detecting human CTR (Wookey et al., 2008; Wookey et al., 2012; Ostrovskaya et al., 2019; Hendrikse et al., 2022). The mAb31-01 antibody was validated using immunocytochemistry and immunoblotting (**Figure 7**). The immunocytochemistry controls are provided in **Supplementary Figure S7**. The predicted molecular weight of non-glycosylated human $CT_{(a)}$ is approximately 52 kDa (Nygaard et al., 1997; Quiza et al., 1997; UniProt, 2021).

In immunocytochemistry, mAb31-01 displayed strong immunoreactivity in cells transfected with human CTR, in the presence and absence of RAMP1 but little to no immunoreactivity in the vector control (**Figure 7A**). There was no visible detection of rat or mouse CTR or apparent cross-reactivity with CLR for any of the species tested. In immunoblotting, mAb31-01 displayed immunoreactivity at ~50 kDa and a wider intense band at ~53–63 kDa, similar to the HA control (**Figure 7B**). This is consistent with its ability to detect human CTR using immunoblotting in the existing literature (Wookey et al., 2012; Furness S. et al., 2016;Furness S. G. B. et al., 2016). Two bands at ~100 and 120 kDa were also observed.

An additional two antibodies reported in the literature or by their commercial supplier to detect human CTR were also tested in immunocytochemistry in transfected cells to determine their potential suitability for studying CTR expression (Fu et al., 2017). pAbPA1-84457 displayed diffuse background staining and was unable to robustly detect human CTR, whereas pAb230500 could detect human CTR, but had a lower signal compared to background than mAb31-01 (**Supplementary Figures S8–S9**). Therefore, based on these results in transfected cells, we used mAb31-01 for subsequent experiments due to its strong detection of human CTR, low background staining, and its previous use in human tissue or primary cell lines.

3.4 Expression of CTR and CGRP in Human TG

To localize CTR-LI and CGRP-LI in human TG, sections were co-incubated with anti-CGRP pAb36001 (as rat and mouse studies), anti-CTR mAb31-01 and anti-β tubulin III antibodies. NF200 could

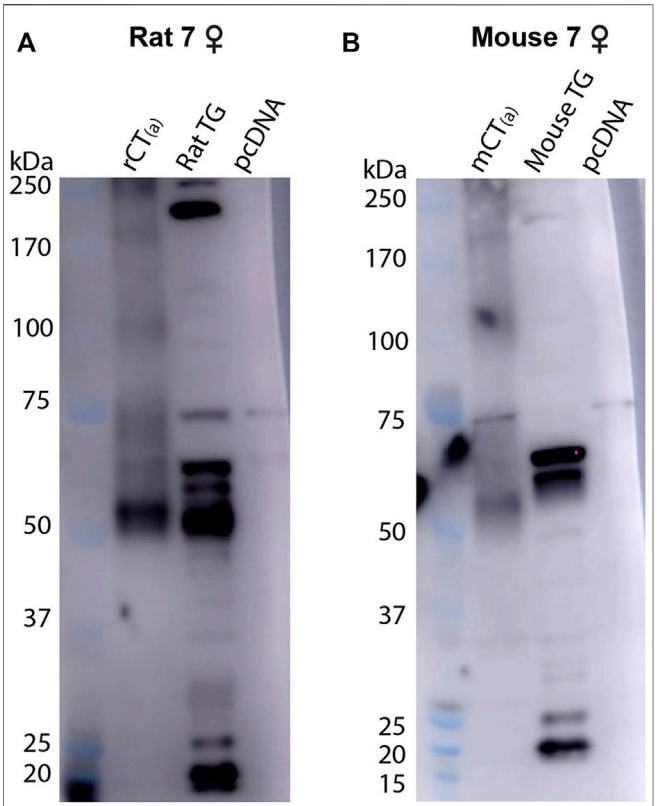

FIGURE 6 | CTR-like immunoreactivity in rat and mouse TG lysate using immunoblotting with pAb188. **(A)** Immunoblots using lysate preparations from adult rat TG (20 µg) and HEK293S cells (0.1 µg) transfected with rat $CT_{(a)}$ or vector alone (pcDNA). **(B)** Immunoblots using lysate preparations from adult mouse TG (20 µg) and HEK293S cells (0.3 µg) transfected with mouse $CT_{(a)}$ or vector alone. Blots were probed with pAb188 (4 µg/ml). MW markers are shown on the left of each blot, with apparent MW in kDa. This image is representative of three western blots using TG lysate from three individual mice or rats (mixed sex) displaying results from a female **(A)** rat and **(B)** mouse. Images were adjusted uniformly for brightness and contrast for presentation purposes.

not be used as a marker for A-fiber neurons as it is not discriminatory for these neuronal subpopulations in humans (Shiers et al., 2020). Pilot results indicated that CTR-LI and CGRP-LI may be present in glia, therefore, co-staining with S100, a glial marker, was also performed. CGRP and CTR results are initially described individually to allow comparisons with the cellular markers, then together to examine their spatial relationship.

3.4.1 CGRP-like Immunoreactivity

CGRP-LI was observed in the cell bodies of small to medium-sized neurons and as varicose fibers, overlapping with β tubulin III (**Figure 8**, **Supplementary Figure S10**). CGRP-LI was diffuse in the soma of smaller to medium-sized neuronal cell bodies or as puncta, indicating presence in vesicles (**Figure 8**; **Supplementary Figure S10**). Qualitative assessment of β tubulin III neuronal size suggests that CGRP-LI neurons are likely C-fiber neurons. Overlapping CGRP-LI and S100-LI was observed for some fibers in all four human cases. This suggests that either a subset of neuronal fibers co-express CGRP and S100 or that a subset of CGRP expressing neuronal fibers is encased by S100 expressing Schwann cells (**Supplementary Figure S10**). CGRP-LI was not observed in the satellite glia surrounding neuronal cell bodies for three of the four human cases, however, overlap of S100, and CGRP was observed in case 15A (**Supplementary Figure S11**). Overall, CGRP staining was relatively consistent across the human cases and agreed with previous studies (Del Fiacco et al., 1991; Eftekhari et al., 2010; Ghanizada et al., 2021).

3.4.2 CTR-like Immunoreactivity

CTR-LI was present but was variable across the human cases (**Figure 8**). In case 3A, a female, intense CTR-LI was observed primarily in smaller neurons with more diffuse and less frequent staining in medium neurons. For cases 4, 8A, males, staining was more commonly observed in medium neurons than smaller neurons. A similar pattern was observed for case 15A, male, however staining of what appeared to be satellite glia was also observed (**Supplementary Figure S11**). No CTR-LI was observed in the neuronal fibers for any of these human cases.

3.4.3 The Relative Distribution of CGRP-like and CTR-like Immunoreactivity

Image analysis and quantification was not possible due to the inherent variation in staining patterns between different human cases. However, apparent colocalization of CTR-LI and CGRP-LI was observed in neuronal cell bodies for all four human cases (**Figure 8**). Neurons that expressed CTR but not CGRP were sometimes located next to neurons that expressed CGRP. CGRP-LI together with CTR-LI followed the pattern of CTR staining, where colocalization was mostly seen in smaller neurons of case 3A and medium neurons for case 4A, 8A, 15A (**Figure 8**). Additionally, colocalization of CTR and CGRP staining was observed in the probable satellite glia of case 15A (**Supplementary Figure S11**).

3.5 Immunoblotting - CTR Expression in Human TG and TN

Western blotting (with mAb31-01) was also used as an orthogonal method to explore CTR expression in human samples. In human TG and TN, bands were observed at ~52, 90 and 130 kDa, with the 52 kDa band having the most intense immunoreactivity of the three (**Figure 9**). Between the 22 and 37 kDa markers, two additional bands were present in the TN but not the TG lysate (**Figure 9**). Interestingly, the immunoreactive bands in the human TG and TN lysates did not directly overlap with the bands in the $hCT_{(a)}$ control lysate. A potential explanation for this is splice variation, which is discussed below.

3.6 CGRP-like and CTR-like Immunoreactivity Distribution Across Species

Images of CGRP-LI and CTR-LI across species are collated in **Figure 10** to enable comparison of staining patterns in the TG of rats, mice and humans. These images underwent additional adjustment of contrast and brightness via the histogram

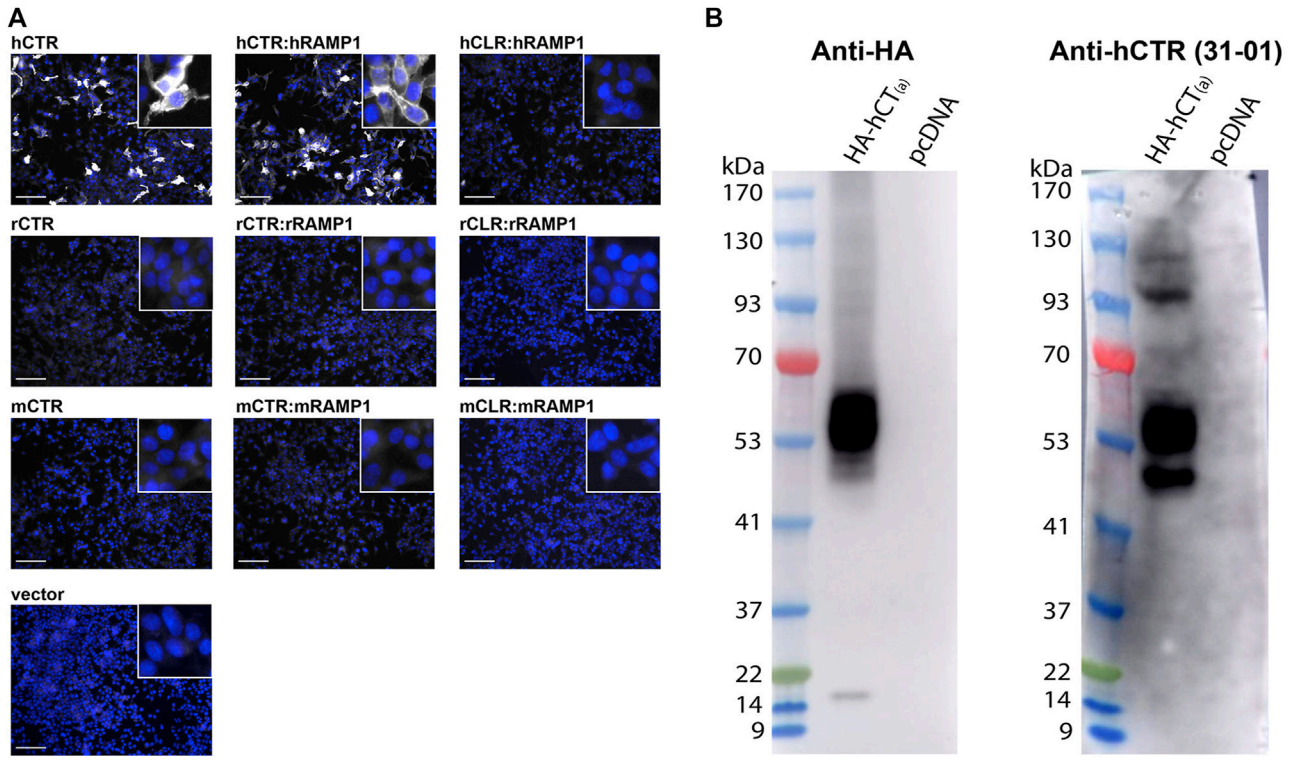

FIGURE 7 | Validation of the anti-human CTR mAb31-01 antibody in transfected HEK293S cells. **(A)** Immunocytochemistry, with immunoreactive staining in greyscale, and nuclear DAPI staining in blue. Scale bar = 100 µm h, human; r, rat; m, mouse. Images are representative of three independent experiments in duplicate wells. **(B)** Immunoblots using cell lysate (10 µg) preparations from HEK293S cells transfected with HA-tagged human CTR or vector alone (pcDNA). Lanes were loaded with 10 µg of protein. Blots were probed with anti-HA (1 µg/ml) or mAb31-01 (2 µg/ml). MW markers are shown on the left of each blot, with apparent MW in kDa. This image is representative of four technical replicates using the same lysate preparations. The brightness and contrast of these images have been enhanced uniformly for presentation purposes.

(contrast stretching) to enable greater visualization of positively stained cells of varying intensities, in line with acceptable image processing guidelines (Johnson, 2012).

Figure 10 shows that staining in all three species is similar, with some cells displaying intense immunoreactivity and others with more moderate immunoreactivity, suggesting varying levels of protein expression. Colocalization of CTR-LI and CGRP-LI in neuronal cell bodies, with occasional immunoreactivity in adjacent neurons, was observed for all three species. Overall, the distribution and colocalization of CGRP-LI and CTR-LI appear to be consistent in the TG of rats, mice and humans.

4 DISCUSSION

4.1 CTR Is Expressed in the Cell Bodies of TG Neurons

In this study, CTR-LI was observed in the TG of three species when examined by immunohistochemistry and western blotting. This extends previous work which detected CTR in the TG of humans and rats and is the first report of CTR expression in mouse TG (Walker et al., 2015; Edvinsson L. et al., 2020). Additionally, this the first report quantifying the proportion of TG neurons that express CTR and provides the novel finding that CGRP may be co-expressed with CTR in individual neurons.

The distribution of CTR-LI in the cell bodies of small to medium-sized TG neurons is consistent with previous reports in rats and humans (Walker et al., 2015; Edvinsson L. et al., 2020). The CTR-LI distribution was similar in mouse TG. The size of neuronal cell bodies in the TG is known to be loosely correlated with the different neuronal subpopulations (Messlinger and Russo, 2019). The size of the CTR-LI neurons, in combination with the less frequent colocalization with NF200, suggests that CTR is expressed predominantly in C-fiber neurons in the TG (Ruscheweyh et al., 2007; Shiers et al., 2020). These data provide further evidence supporting the expression of CTR in the TG, however, the relative distribution across the whole TG remains unknown and should be considered in future studies. CTR-LI was not present in all cells, potentially explaining why studies examining CTR mRNA expression have given variable results (Barrett et al., 2013; Manteniotis et al., 2013; Flegel et al., 2015; Walker et al., 2015; LaPaglia et al., 2018).

The pattern of CTR expression was relatively consistent between individual rats and mice, however, there were differences between human cases, particularly in relation to

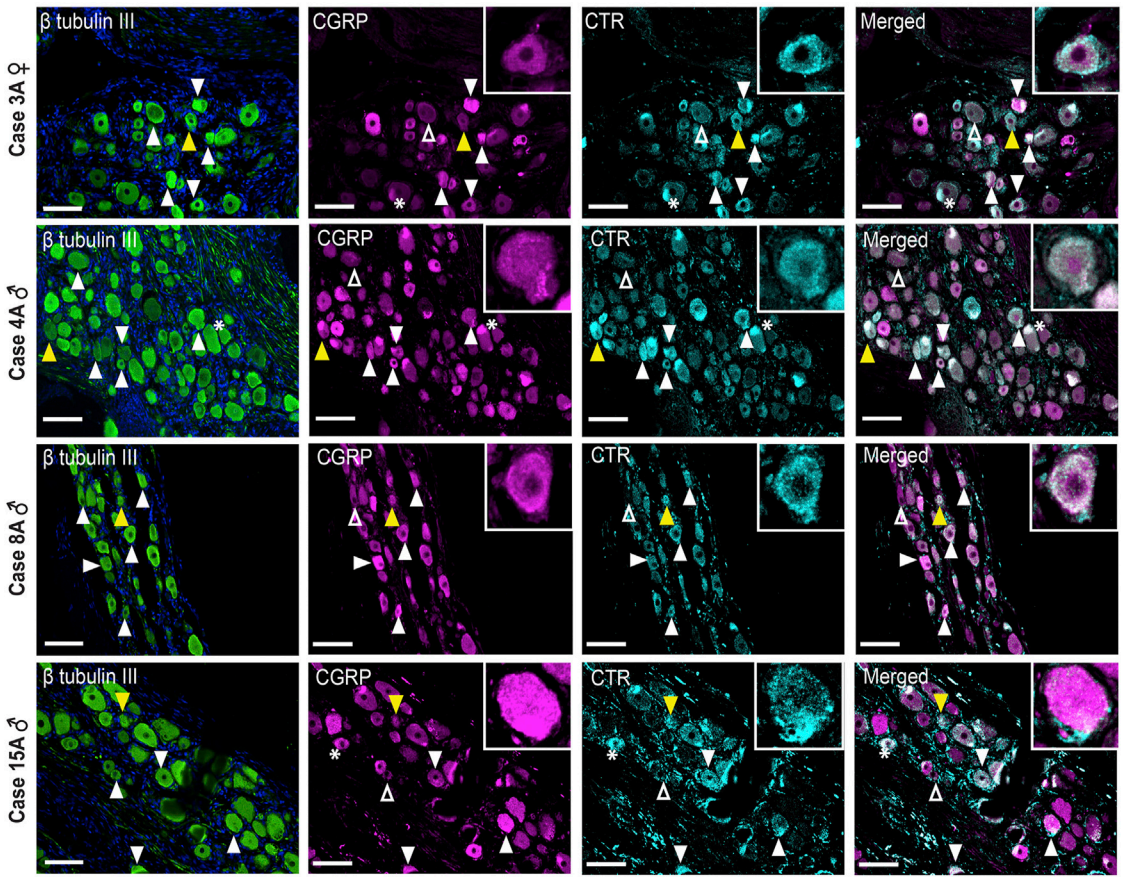

FIGURE 8 | Immunohistochemical localization of CGRP (pAb36001, 10 μg/ml) and CTR (mAb31-01, 4 μg/ml) together with β tubulin III (1.2 μg/ml) in human TG. CGRP and CTR in four human cases (3A, 4A, 8A and 15A). β tubulin III is shown for reference. Filled white arrowheads indicate examples of positive staining; empty arrowheads indicate examples of an absence of staining; yellow arrowheads indicate expression in adjacent neurons. *Indicates examples of autofluorescence due to lipofuscin. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Scale bar = 100 μm. Images are from each of the four human cases.

the size of neurons that contained CTR-LI. A greater variation is not unexpected in the human samples. The case history of migraine in these individuals is unknown. Recently, it was reported that for some migraineurs an amylin agonist, pramlintide, but not CGRP, was able to induce migraine-like attacks (Ghanizada et al., 2021). It is possible that for these patients an amylin receptor, such as the AMY_1 receptor, may make a greater contribution to migraine genesis. Populations of neurons which express amylin-responsive receptors could underlie this sensitivity. However, the sample size in this study was small and further research is required. Future studies should also investigate the presence of RAMP1 and other RAMPs, together with CTR (Walker et al., 2015; Hendrikse et al., 2019). This relies on the availability of antibodies that pass rigorous validation tests. It is also possible that CTR without RAMPs has a role to play in the TG because administration of calcitonin and activation of CTR alone has previously been shown to be anti-nociceptive, including in migraine sufferers (Micieli et al., 1988; Ustdal et al., 1989; Humble, 2011; Ito et al., 2012). It is important to note that in interpreting the presence of CTR in the TG, that CTR pharmacology differs between species with mouse CTR being more responsive to CGRP, especially βCGRP, than human and rat CTR (Garelja et al., 2021a).

In western blotting, the apparent molecular weight of the bands was consistent with the predicted molecular weight of CTR and previous reports (Quiza et al., 1997; Tikellis et al., 2003; Wookey et al., 2012; UniProt, 2021). This also indicated the presence of multiple molecular forms of CTR in the TG, which could be due to splice variants, dimers, post-translational modifications or translation and degradation products (Gorn et al., 1992; Anusaksathien et al., 2001; Seck et al., 2003; Harikumar et al., 2010; Gilabert-Oriol et al., 2017). For example, the size of some of the bands in the TG lysates suggests that the insert positive $CT_{(b)}$ variant, which is approximately 2–5 kDa larger than $CT_{(a)}$ in humans and rodents, may also be present in addition to the $CT_{(a)}$ variant (Albrandt et al., 1993; Moore et al., 1995; Quiza et al., 1997). In addition to several distinct bands which could correspond to specific splice variants, immunoreactivity at approximately 50–52 kDa was often observed as a wide band. This suggests

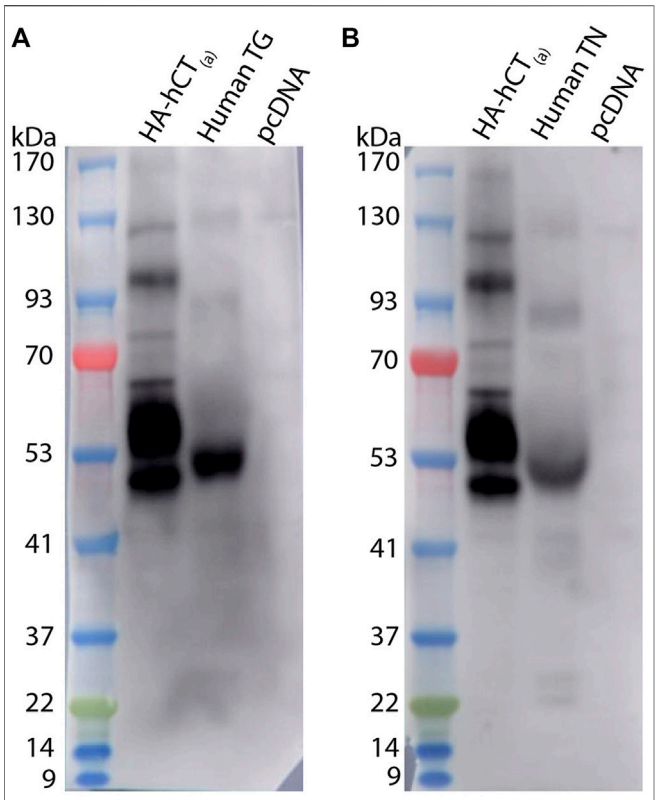

FIGURE 9 | CTR-like immunoreactivity in human TG and TN lysate using immunoblotting. Immunoblots using lysate preparations from human TG and TN (20 µg) or HEK293S cells (10 µg) transfected with HA-tagged human $CT_{(a)}$ or vector alone (pcDNA). Blots were probed with mAb31-01 (2 µg/ml), comparing immunoreactivity in **(A)** TG (case 1322) and **(B)** TN (case 1543). MW markers are shown on the left of each blot, with apparent MW in kDa. This image is from one western blot for two human cases. Images were adjusted uniformly for brightness and contrast for presentation purposes.

that there may be multiple CTR forms of similar molecular weights, such as $CT_{(a)}$ and $CT_{(b)}$, present in the TG. The observation of potential CTR variants in the trigeminovascular system provides an avenue for future investigation because different CTR splice variants have different pharmacological and cell signalling profiles (Houssami et al., 1994; Moore et al., 1995; Qi et al., 2013; Yu et al., 2013; Wnorowski and Jozwiak, 2014; Dal Maso et al., 2018). Interestingly, some differences in immunoreactivity were observed for mAb8B9 and pAb188 in rat and mouse TG lysates which may be due to their monoclonal and polyclonal nature (Lipman et al., 2005).

4.2 CTR and CGRP Colocalize in C-Fiber Neurons

The expression of CGRP was consistent with prior reports. CTR and CGRP colocalized in the cell bodies of several neurons or were observed in adjacent neurons. A similar pattern of CGRP-LI and CTR-LI was evident in mouse, rat and human TG. This cross-species consistency provides support for a conserved mechanism being at play in the function of these proteins. Notably, a similar distribution of CTR-LI was observed in all species, even though a distinct antibody was used in the human samples. Colocalization tended to occur in small to medium sized neurons, suggesting that colocalization occurs in C-fiber neurons (Messlinger and Russo, 2019). This was supported by qualitative assessment indicating that colocalization did not tend to occur in NF200 (an A-fiber marker) expressing neurons (Ruscheweyh et al., 2007). Additionally, single cell mRNA expression data from a TG atlas indicates that CGRP and CTR mRNA are both present in non-A-fiber, peptidergic and non-peptidergic nociceptor neurons in the mouse TG (Yang et al., 2022).

This contrasts with previous studies examining the relative distribution of CLR-LI and CGRP-LI, which consistently report that these are present in distinct neuronal populations (Lennerz et al., 2008; Eftekhari et al., 2010; Eftekhari et al., 2013; Edvinsson et al., 2019). The presence of CTR-LI in or nearby to cells with CGRP-LI suggests that CGRP could act as the local agonist for CTR-based receptors, such as the AMY_1 receptor, in the TG. This implies that CTR-based receptors could mediate some aspects of CGRP function in the TG. It is also possible that other CTR agonists, calcitonin and amylin, could act via this receptor in the TG. However, the source of these agonists is more likely to be systemic than locally produced because the TG is not a notable source of these peptides, compared to that of CGRP (Rosenfeld et al., 1983; Goadsby et al., 1990; Manteniotis et al., 2013; Flegel et al., 2015; Irimia et al., 2020; Rees et al., 2021b; Ghanizada et al., 2021).

The presence of CGRP either in the same neurons as CTR or in neurons close in proximity to CTR-expressing neurons supports the potential involvement of a CTR-based receptor in pain modulation. Their size is consistent with C-fiber neurons, which are reported to have a greater contribution to the long-term potentiation of pain and sensitization in migraine than A-fiber neurons (Henrich et al., 2015; Levy et al., 2019). In particular, the activation of meningeal and dural afferent C-fibers, which project from the TG, have been linked to sensitization (Bartsch and Goadsby, 2003). Additionally, CGRP may possibly promote cortical spreading depression, which has been linked to the development of migraine and its symptoms, via both C- and A-fiber neurons (Charles and Baca, 2013; Close et al., 2019; Filiz et al., 2019).

4.3 A CTR-Based Receptor Could Underlie CGRP's Autoregulatory Action

Autocrine regulation of CGRP in the TG has been speculated for many years (Messlinger et al., 2020). Several studies have reported that genes upregulated by CGRP can, in turn, upregulate CGRP itself (Durham and Russo, 2003; Bellamy et al., 2006; Durham, 2006; Zhang et al., 2007). Furthermore, gold labelled-CGRP appeared to bind to neurons that expressed CGRP and stimulation of TG neuronal cultures with CGRP increases CGRP mRNA (Segond von Banchet et al., 2002; Zhang et al., 2007). Interestingly, in an animal model of chronic migraine, mice chronically treated with nitroglycerine (NTG) have increased CGRP expression in the TG (Iversen and Olesen, 1996; Dieterle et al., 2011; Pradhan et al., 2014; Dallel et al., 2018). TG neuron cultures from this mouse model were also more responsive to CGRP than those treated with vehicle (Guo et al., 2020). However, chronic NTG treatment did not increase the

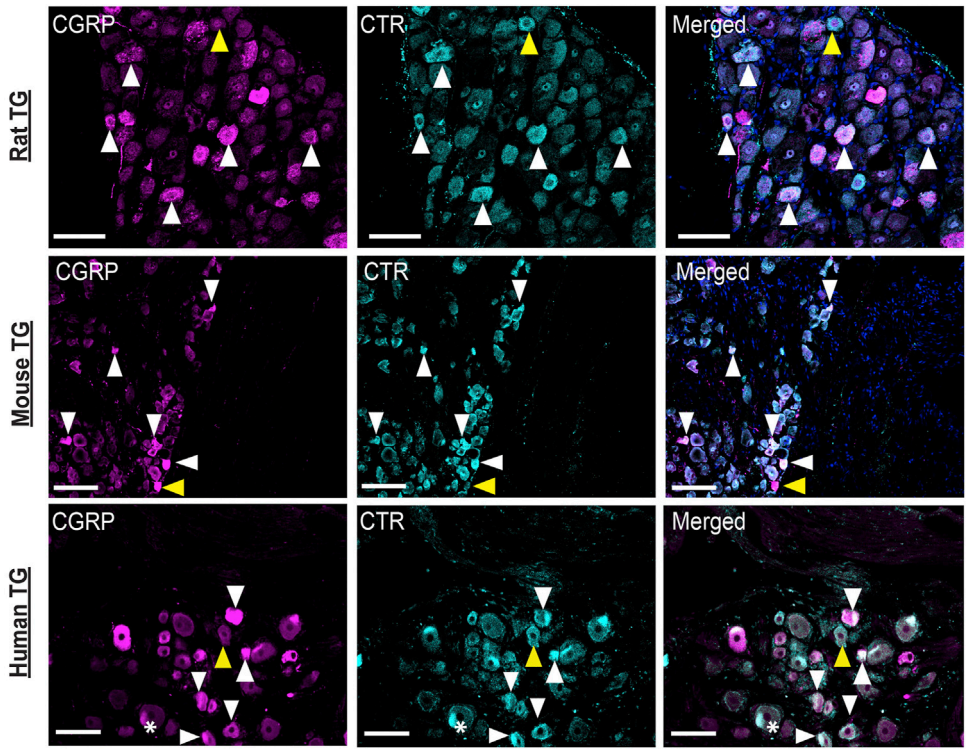

FIGURE 10 | Comparison of CGRP-like and CTR-like immunoreactivity across species. CGRP and CTR in rat, mouse and human TG. White arrowheads indicate colocalization; yellow arrowheads indicate expression in adjacent neurons. *Indicates examples of autofluorescence due to lipofuscin. Image brightness and contrast were adjusted for presentation purposes and merged in FIJI. Additional histogram adjustment (contrast stretching) was performed for these images to convey the frequency and variation in the intensity of the immunoreactivity. Scale bar = 100 µm. Images are representative of six rats and mice (three male and three female). Human case 3A was used for this comparison.

number of neurons expressing CGRP. This suggests that the upregulation of CGRP mRNA previously observed may be primarily within neurons which already express CGRP (Messlinger et al., 2012). Additionally, this increased responsiveness to CGRP appeared to primarily be regulated via C-fiber neurons. How this apparent autoregulation of CGRP is mediated has remained unanswered as CLR and CGRP are not reported to commonly colocalize in TG neurons (Lennerz et al., 2008; Messlinger et al., 2020). It has therefore been previously speculated that co-expression of CTR and CGRP might occur (Guo et al., 2020; Messlinger et al., 2020). Our data indicating co-expression of CTR and CGRP in C-fiber neurons support the hypothesis that a CTR-based receptor, with a strong candidate being the AMY_1 receptor, could mediate CGRP upregulation and response in the TG (Guo et al., 2020).

Differences in the degree of autocrine signaling could occur between individual migraine patients, where enhanced autocrine signaling of CGRP in TG neurons could account for some patients who are unresponsive to anti-CGRP antibody therapy. Although this is speculative, it is based on estimates that significantly more anti-ligand antibody, in the realm of four to eight orders of magnitude greater than the peptide-receptor binding affinity, is required to inhibit autocrine signaling (Forsten and Lauffenburger, 1992a). In these cases, a receptor-targeted approach would be preferable (Forsten and Lauffenburger, 1992b). Currently, erenumab is the only approved anti-receptor antibody for the prevention of migraine, which targets the CGRP receptor, but does some have some ability to block the AMY_1 receptor (Garelja et al., 2021b; Bhakta et al., 2021). Erenumab can potently block CGRP receptor activation of A-fiber neurons to inhibit firing (Melo-Carrillo et al., 2017). However, erenumab does not appear to lower the firing rate of activated C-fiber neurons in response to CGRP (Melo-Carrillo et al., 2017). This raises the question of whether erenumab is sufficient to inhibit the potential autocrine activation of CTR-based receptors, such as the AMY_1 receptor, in C-fiber neurons. The gepants should also be considered for their potential effects, given that they also have affinity for the AMY_1 receptor (Garelja et al., 2021b).

5 CONCLUSION

In this study, we identified that CTR and CGRP can be co-expressed in TG neurons. This may underpin the reported autocrine actions of CGRP, which have been linked to migraine chronification. Additionally, multiple molecular forms of CTR were present in TG, indicating that multiple pharmacologically unique receptors could contribute to craniofacial pain. Although current migraine treatments which target CGRP and the canonical CGRP receptor

provide relief for many migraine patients, there are patients who do not respond, suggesting that there is scope to develop further treatments (Edvinsson et al., 2018; Dubowchik et al., 2020). CTR-based receptors, such as the AMY_1 receptor, have been proposed as potential targets (Walker et al., 2015; Irimia et al., 2020; Ghanizada et al., 2021). Our study supports the hypothesis that such receptors may contribute to migraine and highlights the importance of developing specific antibodies or antagonists targeting CTR-based receptors, such as the AMY_1-receptor, to probe the drug development potential of this receptor in the treatment or prevention of migraine.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Auckland Human Anatomy Laboratory, overseen by the New Zealand Police Inspector of Anatomy under the Human Tissue Act of 2008. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Auckland Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act (1999).

AUTHOR CONTRIBUTIONS

TR, CW, and DH conception and design of research; TR performed experiments; SO sourced and prepared human tissue for histology; TR, CW, AR, and DH analyzed data and interpreted results of experiments; TR prepared figures and drafted manuscript; TR, CW, and DH edited and revised manuscript; TR, CW, SO, AR, and DH approved final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.860037/full#supplementary-material

REFERENCES

Agosti, R. (2018). Migraine Burden of Disease: From the Patient's Experience to a Socio-Economic View. *Headache: J. Head Face Pain* 58 (Suppl. 1), 17–32. doi:10.1111/head.13301

Albrandt, K., Brady, E. M., Moore, C. X., Mull, E., Sierzega, M. E., and Beaumont, K. (1995). Molecular Cloning and Functional Expression of a Third Isoform of the Human Calcitonin Receptor and Partial Characterization of the Calcitonin Receptor Gene. *Endocrinology* 136 (12), 5377–5384. doi:10.1210/endo.136.12.7588285

Albrandt, K., Mull, E., Brady, E. M. G., Herich, J., Moore, C. X., and Beaumont, K. (1993). Molecular Cloning of Two Receptors from Rat Brain with High Affinity for salmon Calcitonin. *FEBS Lett.* 325 (3), 225–232. doi:10.1016/0014-5793(93)81078-e

Ambalavanar, R., and Morris, R. (1992). The Distribution of Binding by Isolectin I-B4 from Griffonia Simplicifolia in the Trigeminal Ganglion and Brainstem Trigeminal Nuclei in the Rat. *Neuroscience* 47 (2), 421–429. doi:10.1016/0306-4522(92)90256-2

Anusaksathien, O., Laplace, C., Li, X., Ren, Y., Peng, L., Goldring, S. R., et al. (2001). Tissue-specific and Ubiquitous Promoters Direct the Expression of Alternatively Spliced Transcripts from the Calcitonin Receptor Gene. *J. Biol. Chem.* 276 (25), 22663–22674. doi:10.1074/jbc.M007104200

Bailey, R., Walker, C., Ferner, A., Loomes, K., Prijic, G., Halim, A., et al. (2012). Pharmacological Characterization of Rat Amylin Receptors: Implications for the Identification of Amylin Receptor Subtypes. *Br. J. Pharmacol.* 166 (1), 151–167. doi:10.1111/j.1476-5381.2011.01717.x

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., et al. (2013). NCBI GEO: Archive for Functional Genomics Data Sets-Update. *Nucleic Acids Res.* 41 (D1), D991–D995. doi:10.1093/nar/gks1193

Bartsch, T., and Goadsby, P. J. (2003). Increased Responses in Trigeminocervical Nociceptive Neurons to Cervical Input after Stimulation of the Dura Mater. *Brain* 126 (Pt 8), 1801–1813. doi:10.1093/brain/awg190

Bellamy, J., Bowen, E. J., Russo, A. F., and Durham, P. L. (2006). Nitric Oxide Regulation of Calcitonin Gene-Related Peptide Gene Expression in Rat Trigeminal Ganglia Neurons. *Eur. J. Neurosci.* 23 (8), 2057–2066. doi:10.1111/j.1460-9568.2006.04742.x

Bhakta, M., Vuong, T., Taura, T., Wilson, D. S., Stratton, J. R., and Mackenzie, K. D. (2021). Migraine Therapeutics Differentially Modulate the CGRP Pathway. *Cephalalgia* 41 (5), 499–514. doi:10.1177/0333102420983282

Bohn, K. J., Li, B., Huang, X., Mason, B. N., Wattiez, A. S., Kuburas, A., et al. (2017). CGRP Receptor Activity in Mice with Global Expression of Human Receptor Activity Modifying Protein 1. *Br. J. Pharmacol.* 174 (12), 1826–1840. doi:10.1111/bph.13783

Bower, R. L., Yule, L., Rees, T. A., Deganutti, G., Hendrikse, E. R., Harris, P. W. R., et al. (2018). Molecular Signature for Receptor Engagement in the Metabolic Peptide Hormone Amylin. *ACS Pharmacol. Transl. Sci.* 1 (1), 32–49. doi:10.1021/acsptsci.8b00002

Buzzi, M., Tassorelli, C., and Nappi, G. (2003). Peripheral and central Activation of Trigeminal Pain Pathways in Migraine: Data from Experimental Animal Models. *Cephalalgia* 23 (Suppl. 1), 1–4. doi:10.1046/j.1468-2982.23.s1.1.x

Charles, A. C., and Baca, S. M. (2013). Cortical Spreading Depression and Migraine. *Nat. Rev. Neurol.* 9 (11), 637–644. doi:10.1038/nrneurol.2013.192

Close, L. N., Eftekhari, S., Wang, M., Charles, A. C., and Russo, A. F. (2019). Cortical Spreading Depression as a Site of Origin for Migraine: Role of CGRP. *Cephalalgia* 39 (3), 428–434. doi:10.1177/0333102418774299

Coester, B., Foll, C. L., and Lutz, T. A. (2020). Viral Depletion of Calcitonin Receptors in the Area Postrema: A Proof-Of-Concept Study. *Physiol. Behav.* 223, 112992. doi:10.1016/j.physbeh.2020.112992

Dal Maso, E., Just, R., Hick, C., Christopoulos, A., Sexton, P. M., Wootten, D., et al. (2018). Characterization of Signalling and Regulation of Common Calcitonin Receptor Splice Variants and Polymorphisms. *Biochem. Pharmacol.* 148, 111–129. doi:10.1016/j.bcp.2017.12.016

Dallel, R., Descheemaeker, A., and Luccarini, P. (2018). Recurrent Administration of the Nitric Oxide Donor, Isosorbide Dinitrate, Induces a Persistent Cephalic Cutaneous Hypersensitivity: A Model for Migraine Progression. *Cephalalgia* 38 (4), 776–785. doi:10.1177/0333102417714032

Dieterle, A., Fischer, M. J., Link, A. S., Neuhuber, W. L., and Messlinger, K. (2011). Increase in CGRP- and nNOS-Immunoreactive Neurons in the Rat Trigeminal Ganglion after Infusion of an NO Donor. *Cephalalgia* 31 (1), 31–42. doi:10.1177/0333102410375725

Dubowchik, G. M., Conway, C. M., and Xin, A. W. (2020). Blocking the CGRP Pathway for Acute and Preventive Treatment of Migraine: The Evolution of Success. *J. Med. Chem.* 63 (13), 6600–6623. doi:10.1021/acs.jmedchem.9b01810

Durham, P. L. (2006). Calcitonin Gene-Related Peptide (CGRP) and Migraine. *Headache* 46 (Suppl. 1), S3–S8. doi:10.1111/j.1526-4610.2006.00483.x

Durham, P. L., and Russo, A. F. (2003). Stimulation of the Calcitonin Gene-Related Peptide Enhancer by Mitogen-Activated Protein Kinases and Repression by an Antimigraine Drug in Trigeminal Ganglia Neurons. *J. Neurosci.* 23 (3), 807–815. doi:10.1523/jneurosci.23-03-00807.2003

Edvinsson, J. C. A., Viganò, A., Alekseeva, A., Alieva, E., Arruda, R., De Luca, C., et al. (2020a). The Fifth Cranial Nerve in Headaches. *J. Headache Pain* 21 (1), 65. doi:10.1186/s10194-020-01134-1

Edvinsson, J. C. A., Warfvinge, K., Krause, D. N., Blixt, F. W., Sheykhzade, M., Edvinsson, L., et al. (2019). C-fibers May Modulate Adjacent Aδ-Fibers through Axon-Axon CGRP Signaling at Nodes of Ranvier in the Trigeminal System. *J. Headache Pain* 20 (1), 105. doi:10.1186/s10194-019-1055-3

Edvinsson, L., Grell, A.-S., and Warfvinge, K. (2020b). Expression of the CGRP Family of Neuropeptides and Their Receptors in the Trigeminal Ganglion. *J. Mol. Neurosci.* 70 (5), 930–944. doi:10.1007/s12031-020-01493-z

Edvinsson, L., Haanes, K. A., Warfvinge, K., and Krause, D. N. (2018). CGRP as the Target of New Migraine Therapies - Successful Translation from Bench to Clinic. *Nat. Rev. Neurol.* 14 (6), 338–350. doi:10.1038/s41582-018-0003-1

Eftekhari, S., Salvatore, C. A., Calamari, A., Kane, S. A., Tajti, J., and Edvinsson, L. (2010). Differential Distribution of Calcitonin Gene-Related Peptide and its Receptor Components in the Human Trigeminal Ganglion. *Neuroscience* 169 (2), 683–696. doi:10.1016/j.neuroscience.2010.05.016

Eftekhari, S., Salvatore, C. A., Johansson, S., Chen, T.-b., Zeng, Z., and Edvinsson, L. (2015). Localization of CGRP, CGRP Receptor, PACAP and Glutamate in Trigeminal Ganglion. Relation to the Blood-Brain Barrier. *Brain Res.* 1600, 93–109. doi:10.1016/j.brainres.2014.11.031

Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of Nerve Fibers Storing CGRP and CGRP Receptors in the Peripheral Trigeminovascular System. *The J. Pain* 14 (11), 1289–1303. doi:10.1016/j.jpain.2013.03.010

Fiacco, M. D., Diaz, G., Floris, A., and Quartu, M. (1991). Substance P?containing and Calcitonin Gene?related Peptide?containing Neurons in the Human Trigeminal Ganglion. *Ann. NY Acad. Sci.* 632, 382–384. doi:10.1111/j.1749-6632.1991.tb33130.x

Filiz, A., Tepe, N., Eftekhari, S., Boran, H. E., Dilekoz, E., Edvinsson, L., et al. (2019). CGRP Receptor Antagonist MK-8825 Attenuates Cortical Spreading Depression Induced Pain Behavior. *Cephalalgia* 39 (3), 354–365. doi:10.1177/0333102417735845

Flegel, C., Schöbel, N., Altmüller, J., Becker, C., Tannapfel, A., Hatt, H., et al. (2015). RNA-seq Analysis of Human Trigeminal and Dorsal Root Ganglia with a Focus on Chemoreceptors. *PLoS One* 10 (6), e0128951. doi:10.1371/journal.pone.0128951

Forsten, K. E., and Lauffenburger, D. A. (1992a). Autocrine Ligand Binding to Cell Receptors. Mathematical Analysis of Competition by Solution "decoys". *Biophysical J.* 61 (2), 518–529. doi:10.1016/S0006-3495(92)81856-8

Forsten, K. E., and Lauffenburger, D. A. (1992b). Interrupting Autocrine Ligand-Receptor Binding: Comparison between Receptor Blockers and Ligand Decoys. *Biophysical J.* 63 (3), 857–861. doi:10.1016/S0006-3495(92)81661-2

Fu, W., Vukojevic, V., Patel, A., Soudy, R., MacTavish, D., Westaway, D., et al. (2017). Role of Microglial Amylin Receptors in Mediating Beta Amyloid (Aβ)-Induced Inflammation. *J. Neuroinflammation* 14 (1), 199. doi:10.1186/s12974-017-0972-9

Furness, S. G. B., Liang, Y.-L., Nowell, C. J., Halls, M. L., Wookey, P. J., Dal Maso, E., et al. (2016b). Ligand-Dependent Modulation of G Protein Conformation Alters Drug Efficacy. *Cell* 167 (3), 739–749. e711. doi:10.1016/j.cell.2016.09.021

Furness, S., Hare, D., Kourakis, A., Turnley, A., and Wookey, P. (2016a). A Novel Ligand of Calcitonin Receptor Reveals a Potential New Sensor that Modulates Programmed Cell Death. *Cel Death Discov.* 2, 16062. doi:10.1038/cddiscovery.2016.62

Garelja, M. L., Bower, R. L., Brimble, M. A., Chand, S., Harris, P. W. R., Jamaluddin, M. A., et al. (2021a). Pharmacological Characterisation of Mouse Calcitonin and Calcitonin Receptor-like Receptors Reveals Differences Compared with Human Receptors. *Br. J Pharmacol.* 179 (3), 416–434. doi:10.1111/bph.15628

Garelja, M. L., Walker, C. S., and Hay, D. L. (2021b). CGRP Receptor Antagonists for Migraine. Are They Also AMY 1 Receptor Antagonists? *Br. J Pharmacol.* 179 (3), 454–459. doi:10.1111/bph.15585

Ghanizada, H., Al-Karagholi, M. A. M., Walker, C. S., Arngrim, N., Rees, T., Petersen, J., et al. (2021). Amylin Analog Pramlintide Induces Migraine-like Attacks in Patients. *Ann. Neurol.* 89 (6), 1157–1171. doi:10.1002/ana.26072

Gilabert-Oriol, R., Furness, S. G. B., Stringer, B. W., Weng, A., Fuchs, H., Day, B. W., et al. (2017). Dianthin-30 or Gelonin versus Monomethyl Auristatin E, Each Configured with an Anti-calcitonin Receptor Antibody, Are Differentially Potent *In Vitro* in High-Grade Glioma Cell Lines Derived from Glioblastoma. *Cancer Immunol. Immunother.* 66 (9), 1217–1228. doi:10.1007/s00262-017-2013-z

Gingell, J. J., Rees, T. A., Hendrikse, E. R., Siow, A., Rennison, D., Scotter, J., et al. (2020). Distinct Patterns of Internalization of Different Calcitonin Gene-Related Peptide Receptors. *ACS Pharmacol. Transl. Sci.* 3 (2), 296–304. doi:10.1021/acsptsci.9b00089

Goadsby, P. J., Edvinsson, L., and Ekman, R. (1990). Vasoactive Peptide Release in the Extracerebral Circulation of Humans during Migraine Headache. *Ann. Neurol.* 28 (2), 183–187. doi:10.1002/ana.410280213

Goda, T., Doi, M., Umezaki, Y., Murai, I., Shimatani, H., Chu, M. L., et al. (2018). Calcitonin Receptors Are Ancient Modulators for Rhythms of Preferential Temperature in Insects and Body Temperature in Mammals. *Genes Dev.* 32 (2), 140–155. doi:10.1101/gad.307884.117

Gorn, A. H., Lin, H. Y., Yamin, M., Auron, P. E., Flannery, M. R., Tapp, D. R., et al. (1992). Cloning, Characterization, and Expression of a Human Calcitonin Receptor from an Ovarian Carcinoma Cell Line. *J. Clin. Invest.* 90 (5), 1726–1735. doi:10.1172/JCI116046

Guo, Z., Czerpaniak, K., Zhang, J., and Cao, Y.-Q. (2020). Increase in Trigeminal Ganglion Neurons that Respond to Both Calcitonin Gene-Related Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide in Mouse Models of Chronic Migraine and Posttraumatic Headache. *Pain* 162 (5), 1483–1499. doi:10.1097/j.pain.0000000000002147

Harikumar, K. G., Ball, A. M., Sexton, P. M., and Miller, L. J. (2010). Importance of Lipid-Exposed Residues in Transmembrane Segment Four for Family B Calcitonin Receptor Homo-Dimerization. *Regul. Peptides* 164 (2-3), 113–119. doi:10.1016/j.regpep.2010.06.001

Hay, D. L., Chen, S., Lutz, T. A., Parkes, D. G., and Roth, J. D. (2015). Amylin: Pharmacology, Physiology, and Clinical Potential. *Pharmacol. Rev.* 67 (3), 564–600. doi:10.1124/pr.115.010629

Hay, D. L., Garelja, M. L., Poyner, D. R., and Walker, C. S. (2018). Update on the Pharmacology of Calcitonin/CGRP Family of Peptides: IUPHAR Review 25. *Br. J. Pharmacol.* 175 (1), 3–17. doi:10.1111/bph.14075

Hay, D. L., Howitt, S. G., Conner, A. C., Schindler, M., Smith, D. M., and Poyner, D. R. (2003). CL/RAMP2 and CL/RAMP3 Produce Pharmacologically Distinct

Adrenomedullin Receptors: a Comparison of Effects of Adrenomedullin22-52 , CGRP8-37 and BIBN4096BS. *Br. J. Pharmacol.* 140 (3), 477–486. doi:10.1038/sj.bjp.0705472

Hendrikse, E. R., Bower, R. L., Hay, D. L., and Walker, C. S. (2019). Molecular Studies of CGRP and the CGRP Family of Peptides in the central Nervous System. *Cephalalgia* 39 (3), 403–419. doi:10.1177/0333102418765787

Hendrikse, E. R., Rees, T. A., Tasma, Z., Le Foll, C., Lutz, T. A., Siow, A., et al. (2022). Calcitonin Receptor Antibody Validation and Expression in the Rodent Brain. *Cephalalgia*. In Press. doi:10.1177/03331024221084029

Henrich, F., Magerl, W., Klein, T., Greffrath, W., and Treede, R.-D. (2015). Capsaicin-sensitive C- and A-Fibre Nociceptors Control Long-Term Potentiation-like Pain Amplification in Humans. *Brain* 138 (Pt 9), 2505–2520. doi:10.1093/brain/awv108

Houssami, S., Findlay, D. M., Brady, C. L., Myers, D. E., Martin, T. J., and Sexton, P. M. (1994). Isoforms of the Rat Calcitonin Receptor: Consequences for Ligand Binding and Signal Transduction. *Endocrinology* 135 (1), 183–190. doi:10.1210/endo.135.1.8013352

Humble, S. R. (2011). Calcitonin for Acute Neuropathic Pain Associated with Spinal Cord Injury. *Anaesth. Intensive Care* 39 (4), 682–686. doi:10.1177/0310057X1103900425

Irimia, P., Martínez-Valbuena, I., Mínguez-Olaondo, A., Domínguez-Vivero, C., Sánchez-Arias, J.-A., Martínez-Vila, E., et al. (2020). Interictal Amylin Levels in Chronic Migraine Patients: A Case-Control Study. *Cephalalgia* 41 (5), 604–612. doi:10.1177/0333102420977106

Ito, A., Takeda, M., Yoshimura, T., Komatsu, T., Ohno, T., Kuriyama, H., et al. (2012). Anti-hyperalgesic Effects of Calcitonin on Neuropathic Pain Interacting with its Peripheral Receptors. *Mol. Pain* 8, 1744–8069. doi:10.1186/1744-8069-8-42

Iversen, H., and Olesen, J. (1996). Headache Induced by a Nitric Oxide Donor (Nitroglycerin) Responds to Sumatriptan. A Human Model for Development of Migraine Drugs. *Cephalalgia* 16 (6), 412–418. doi:10.1046/j.1468-2982.1996.1606412.x

Johnson, J. (2012). Not seeing Is Not Believing: Improving the Visibility of Your Fluorescence Images. *MBoC* 23 (5), 754–757. doi:10.1091/mbc.E11-09-0824

LaPaglia, D. M., Sapio, M. R., Burbelo, P. D., Thierry-Mieg, J., Thierry-Mieg, D., Raithel, S. J., et al. (2018). RNA-seq Investigations of Human post-mortem Trigeminal Ganglia. *Cephalalgia* 38 (5), 912–932. doi:10.1177/0333102417720216

Lennerz, J. K., Rühle, V., Ceppa, E. P., Neuhuber, W. L., Bunnett, N. W., Grady, E. F., et al. (2008). Calcitonin Receptor-like Receptor (CLR), Receptor Activity-Modifying Protein 1 (RAMP1), and Calcitonin Gene-Related Peptide (CGRP) Immunoreactivity in the Rat Trigeminovascular System: Differences between Peripheral and central CGRP Receptor Distribution. *J. Comp. Neurol.* 507 (3), 1277–1299. doi:10.1002/cne.21607

Levy, D., Labastida-Ramirez, A., and MaassenVanDenBrink, A. (2019). Current Understanding of Meningeal and Cerebral Vascular Function Underlying Migraine Headache. *Cephalalgia* 39 (13), 1606–1622. doi:10.1177/0333102418771350

Lipman, N. S., Jackson, L. R., Trudel, L. J., and Weis-Garcia, F. (2005). Monoclonal versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR J.* 46 (3), 258–268. doi:10.1093/ilar.46.3.258

Manteniotis, S., Lehmann, R., Flegel, C., Vogel, F., Hofreuter, A., Schreiner, B. S. P., et al. (2013). Comprehensive RNA-Seq Expression Analysis of Sensory Ganglia with a Focus on Ion Channels and GPCRs in Trigeminal Ganglia. *PLoS One* 8 (11), e79523. doi:10.1371/journal.pone.0079523

May, A., and Goadsby, P. J. (1999). The Trigeminovascular System in Humans: Pathophysiologic Implications for Primary Headache Syndromes of the Neural Influences on the Cerebral Circulation. *J. Cereb. Blood Flow Metab.* 19 (2), 115–127. doi:10.1097/00004647-199902000-00001

Melo-Carrillo, A., Strassman, A. M., Nir, R.-R., Schain, A. J., Noseda, R., Stratton, J., et al. (2017). Fremanezumab-A Humanized Monoclonal Anti-CGRP Antibody-Inhibits Thinly Myelinated (Aδ) but Not Unmyelinated (C) Meningeal Nociceptors. *J. Neurosci.* 37 (44), 10587–10596. doi:10.1523/JNEUROSCI.2211-17.2017

Messlinger, K., Balcziak, L. K., and Russo, A. F. (2020). Cross-talk Signaling in the Trigeminal Ganglion: Role of Neuropeptides and Other Mediators. *J. Neural Transm.* 127 (4), 431–444. doi:10.1007/s00702-020-02161-7

Messlinger, K., Lennerz, J. K., Eberhardt, M., and Fischer, M. J. M. (2012). CGRP and NO in the Trigeminal System: Mechanisms and Role in Headache Generation. *Headache* 52 (9), 1411–1427. doi:10.1111/j.1526-4610.2012.02212.x

Messlinger, K., and Russo, A. F. (2019). Current Understanding of Trigeminal Ganglion Structure and Function in Headache. *Cephalalgia* 39 (13), 1661–1674. doi:10.1177/0333102418786261

Micieli, G., Cavallini, A., Martignoni, E., Covelli, V., Facchinetti, F., and Nappi, G. (1988). Effectiveness of salmon Calcitonin Nasal spray Preparation in Migraine Treatment. *Headache* 28 (3), 196–200. doi:10.1111/j.1526-4610.1988.hed2803196.x

Moore, E. E., Kuestner, R. E., Stroop, S. D., Grant, F. J., Matthewes, S. L., Brady, C. L., et al. (1995). Functionally Different Isoforms of the Human Calcitonin Receptor Result from Alternative Splicing of the Gene Transcript. *Mol. Endocrinol.* 9 (8), 959–968. doi:10.1210/mend.9.8.7476993

Nygaard, S. C., Kuestner, R. E., Moore, E. E., and Stroop, S. D. (1997). Phosphorylation of the Human Calcitonin Receptor by Multiple Kinases Is Localized to the C-Terminus. *J. Bone Miner Res.* 12 (10), 1681–1690. doi:10.1359/jbmr.1997.12.10.1681

Ostrovskaya, A., Hick, C., Hutchinson, D. S., Stringer, B. W., Wookey, P. J., Wootten, D., et al. (2019). Expression and Activity of the Calcitonin Receptor Family in a Sample of Primary Human High-Grade Gliomas. *BMC Cancer* 19 (1), 157. doi:10.1186/s12885-019-5369-y

Pradhan, A. A., Smith, M. L., McGuire, B., Tarash, I., Evans, C. J., and Charles, A. (2014). Characterization of a Novel Model of Chronic Migraine. *Pain* 155 (2), 269–274. doi:10.1016/j.pain.2013.10.004

Qi, T., Dong, M., Watkins, H., Wootten, D., Miller, L., and Hay, D. (2013). Receptor Activity-Modifying Protein-dependent Impairment of Calcitonin Receptor Splice Variant Δ(1-47)hCT(a)function. *Br. J. Pharmacol.* 168 (3), 644–657. doi:10.1111/j.1476-5381.2012.02197.x

Quiza, M., Dowton, M., Perry, K. J., and Sexton, P. M. (1997). Electrophoretic Mobility and Glycosylation Characteristics of Heterogeneously Expressed Calcitonin Receptors1. *Endocrinology* 138 (2), 530–539. doi:10.1210/endo.138.2.4911

Rees, T. A., Hay, D. L., and Walker, C. S. (2021a). Amylin Antibodies Frequently Display Cross-Reactivity with CGRP: Characterization of Eight Amylin Antibodies. *Am. J. Physiology-Regulatory, Integr. Comp. Physiol.* 320 (5), R697–R703. doi:10.1152/ajpregu.00338.2020

Rees, T. A., Hendrikse, E. R., Hay, D. L., and Walker, C. S. (2021b). Beyond CGRP: the Calcitonin Peptide Family as Targets for Migraine and Pain. *Br. J Pharmacol.* 179 (3), 381–399. doi:10.1111/bph.15605

Rosenfeld, M. G., Mermod, J.-J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., et al. (1983). Production of a Novel Neuropeptide Encoded by the Calcitonin Gene via Tissue-specific RNA Processing. *Nature* 304 (5922), 129–135. doi:10.1038/304129a0

Ruscheweyh, R., Forsthuber, L., Schoffnegger, D., and Sandkühler, J. (2007). Modification of Classical Neurochemical Markers in Identified Primary Afferent Neurons with Aβ-, Aδ-, and C-Fibers after Chronic Constriction Injury in Mice. *J. Comp. Neurol.* 502 (2), 325–336. doi:10.1002/cne.21311

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an Open-Source Platform for Biological-Image Analysis. *Nat. Methods* 9 (7), 676–682. doi:10.1038/nmeth.2019

Seck, T., Baron, R., and Horne, W. C. (2003). Binding of Filamin to the C-Terminal Tail of the Calcitonin Receptor Controls Recycling. *J. Biol. Chem.* 278 (12), 10408–10416. doi:10.1074/jbc.M209655200

Segond von Banchet, G., Pastor, A., Biskup, C., Schlegel, C., Benndorf, K., and Schaible, H.-G. (2002). Localization of Functional Calcitonin Gene-Related Peptide Binding Sites in a Subpopulation of Cultured Dorsal Root Ganglion Neurons. *Neuroscience* 110 (1), 131–145. doi:10.1016/s0306-4522(01)00547-4

Shiers, S., Klein, R. M., and Price, T. J. (2020). Quantitative Differences in Neuronal Subpopulations between Mouse and Human Dorsal Root Ganglia Demonstrated with RNAscope *In Situ* Hybridization. *Pain* 161 (10), 2410–2424. doi:10.1097/j.pain.0000000000001973

Tikellis, C., Xuereb, L., Casley, D., Brasier, G., Cooper, M. E., and Wookey, P. J. (2003). Calcitonin Receptor Isoforms Expressed in the Developing Rat Kidney. *Kidney Int.* 63 (2), 416–426. doi:10.1046/j.1523-1755.2003.00754.x

UniProt, C. (2021). UniProt: the Universal Protein Knowledgebase in 2021. *Nucleic Acids Res.* 49 (D1), D480–D489. doi:10.1093/nar/gkaa1100

Üstdal, M., Doǵan, P., Soyuer, A., and Terzi, S. (1989). Treatment of Migraine with salmon Calcitonin : Effects on Plasma β-endorphin, ACTH and Cortisol Levels. *Biomed. Pharmacother.* 43 (9), 687–691. doi:10.1016/0753-3322(89)90088-7

Vogel, C., de Sousa Abreu, R., Ko, D., Le, S. Y., Shapiro, B. A., Burns, S. C., et al. (2010). Sequence Signatures and mRNA Concentration Can Explain Two-thirds of Protein Abundance Variation in a Human Cell Line. *Mol. Syst. Biol.* 6, 400. doi:10.1038/msb.2010.59

Vogel, C., and Marcotte, E. M. (2012). Insights into the Regulation of Protein Abundance from Proteomic and Transcriptomic Analyses. *Nat. Rev. Genet.* 13 (4), 227–232. doi:10.1038/nrg3185

von Buchholtz, L. J., Lam, R. M., Emrick, J. J., Chesler, A. T., and Ryba, N. J. P. (2020). Assigning Transcriptomic Class in the Trigeminal Ganglion Using Multiplex *In Situ* Hybridization and Machine Learning. *Pain* 161 (9), 2212–2224. doi:10.1097/j.pain.0000000000001911

Walker, C. S., Eftekhari, S., Bower, R. L., Wilderman, A., Insel, P. A., Edvinsson, L., et al. (2015). A Second Trigeminal CGRP Receptor: Function and Expression of the AMY 1 Receptor. *Ann. Clin. Transl Neurol.* 2 (6), 595–608. doi:10.1002/acn3.197

Wnorowski, A., and Jozwiak, K. (2014). Homo- and Hetero-Oligomerization of β2-adrenergic Receptor in Receptor Trafficking, Signaling Pathways and Receptor Pharmacology. *Cell Signal.* 26 (10), 2259–2265. doi:10.1016/j.cellsig.2014.06.016

Wookey, P. J., McLean, C. A., Hwang, P., Furness, S. G. B., Nguyen, S., Kourakis, A., et al. (2012). The Expression of Calcitonin Receptor Detected in Malignant Cells of the Brain Tumour Glioblastoma Multiforme and Functional Properties in the Cell Line A172. *Histopathology* 60 (6), 895–910. doi:10.1111/j.1365-2559.2011.04146.x

Wookey, P. J., Zulli, A., Buxton, B. F., and Hare, D. L. (2008). Calcitonin Receptor Immunoreactivity Associated with Specific Cell Types in Diseased Radial and Internal Mammary Arteries. *Histopathology* 52 (5), 605–612. doi:10.1111/j.1365-2559.2008.02979.x

Yang, L., Xu, M., Bhuiyan, S. A., Li, J., Zhao, J., Cohrs, R. J., et al. (2022). Human and Mouse Trigeminal Ganglia Cell Atlas Implicates Multiple Cell Types in Migraine. *Neuron* 2022. doi:10.1016/j.neuron.2022.03.003

Yu, R., Zhong, J., Li, M., Guo, X., Zhang, H., and Chen, J. (2013). PACAP Induces the Dimerization of PAC1 on the Nucleus Associated with the cAMP Increase in the Nucleus. *Neurosci. Lett.* 549, 92–96. doi:10.1016/j.neulet.2013.06.015

Zhang, Z., Winborn, C. S., Marquez de Prado, A. F., and Russo, A. F. (2007). Sensitization of Calcitonin Gene-Related Peptide Receptors by Receptor Activity-Modifying Protein-1 in the Trigeminal Ganglion. *J. Neurosci.* 27 (10), 2693–2703. doi:10.1523/JNEUROSCI.4542-06.2007

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