Vascular Effects of Current and Novel Antimigraine Drugs

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Vascular Effects of Current and Novel Antimigraine Drugs

Vasculaire effecten van huidige en nieuwe antimigraine geneesmiddelen

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En memoria de mis abuelos
Hugo (1927-2019) *y Martha* (1930-2018)

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PART I.

Chapter I.

Introduction

"All our knowledge *begins* with the senses...

Pathophysiology of migraine

Migraine is a highly disabling neurovascular disorder[1] and several theories have arisen regarding its pathophysiology[2]. Currently, migraine is considered a neurovascular disorder that involves activation of the trigeminovascular system[3]. This system comprises both peripheral and central projections, the former via the trigeminal ganglion that sends sensory fibers to the dura mater and the cranial vasculature[4] and the latter via the trigeminocervical complex that consists of the trigeminal nucleus caudalis and the upper two cervical divisions[5]. The activation of this system is considered to result in the release of calcitonin gene-related peptide (CGRP) from the sensory fibers, causing vasodilation of the cranial vasculature and nociceptive transmission[6]. In accordance with this, studies have shown that during a migraine attack, there is an increase in CGRP levels in plasma in the jugular vein[7,8], while treatment with triptans normalizes these levels[9]. Also, intravenous infusions of CGRP are known to provoke migraine-like attacks in migraine patients[10].

Treatment of migraine

Before the discovery of the fundamental role of CGRP in the pathophysiology and treatment of migraine, the main target for selective acutely acting antimigraine drugs was the serotoninergic signaling, with the triptans being the gold standard since the beginning of the 1990's[11]. The prophylactic treatment, however, consisted of medication not developed originally for migraine, but for other diseases, such as hypertension (HT), epilepsy and depression.

A decade later, and due to the important role of CGRP in migraine pathophysiology, CGRP receptor antagonists (gepants) were developed for the acute treatment of migraine and proved to be effective[12,13]. Unfortunately, pharmacokinetic limitations and hepatotoxicity cases did not allow the initial gepants to reach the market[14]. New gepants are currently in Phase II trials for the acute and prophylactic treatment of migraine, with no hepatotoxicity reported[15,16], nevertheless, the concerns about the hepatotoxicity reports led to the development of CGRP (receptor) antibodies for the prophylactic treatment of migraine[17-19], and so far, all clinical trials with these antibodies have shown promising results[20,21].

Migraine, CGRP and cardiovascular risk

CGRP is widely expressed throughout the body, participating not only in migraine pathophysiology, but also in several physiological processes and homeostatic responses during pathophysiological events. Therefore it is important to consider the possible side effects especially after long-term blockade of the CGRP pathway.

To begin with, sensory CGRPergic fibers have been described to innervate the coronary blood vessels as well as the myocardium[22-24]. Several studies have shown that CGRP plays an important role in the regulation of blood pressure and in the homeostatic responses during ischemic events, and it seems to act as a protective/compensatory mechanism during HT[25-31]. Moreover, CGRP is not only involved in peripheral mechanisms, but it also participates in the maintenance of cerebrovascular reactivity during chronic HT by increasing cerebral blood flow[32-35].

CGRP also seems to be involved in the vascular adaptations during pregnancy, as plasma levels increase through the gestation period, reaching their maximum during the last trimester and normalizing after delivery. However, in pre-eclampsia, a pregnancy disorder characterized by high blood pressure and proteinuria, CGRP levels are lower[36].

The role of CGRP as compensatory mechanism in ischemic events and blood pressure regulation poses a concern, especially as numerous studies have shown that migraine patients present an increased risk of hemorrhagic and ischemic stroke, with the risk being higher for women[37-42]. Moreover, a higher risk of myocardial infarction, coronary artery disease and altered arterial function

have also been described[43,44]. Unfortunately, the mechanisms behind these increases are not clear, but they are thought to involve genetic aspects and vascular dysfunction, amongst other factors.

One strategy to understand migraine pathophysiology and its relation with the increase in cardiovascular risk is the use of animal models of migraine, but current animal models, although useful, only represent certain features of this rather complex disorder. Another alternative is the use of models of monogenic disorders that are comorbid with migraine, such as "Autosomal dominant **R**etinal **V**asculopathy with **C**erebral **L**eukodystrophy" (RVCL)[45,46]. Interestingly, this vasculopathy, caused by a mutation in the *TREX1* gene, is associated with endothelial dysfunction[46], and almost two thirds of the patients present migraine without aura[45], providing an unique opportunity to study both the genetic and the vascular interactions in migraine pathophysiology.

References

1. Stovner LJ, Nichols E, Steiner TJ, et al. Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurology 2018;17:954-976.
2. Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiological Reviews 2017;97:553-622.
3. Edvinsson L. The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine. Headache 2017;57 Suppl 2:47-55.
4. Mayberg M, Langer RS, Zervas NT, Moskowitz MA. Perivascular Meningeal Projections from Cat Trigeminal Ganglia: Possible Pathway for Vascular Headaches in Man. Science 1981;213:228-230.
5. Goadsby PJ, Hoskin KL. The distribution of trigeminovascular afferents in the nonhuman primate brain Macaca nemestrina: a c-fos immunocytochemical study. Journal of Anatomy 1997;190:367-375.
6. Goadsby PJ, Lipton RB, Ferrari MD. Migraine - Current Understanding and Treatment. New England Journal of Medicine 2002;346:257-270.
7. Goadsby PJ, Edvinsson L, Ekman R. Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. Annals of Neurology 1988;23:193-196.
8. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Annals of neurology 1990;28:183-187.
9. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: Studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of Neurology 1993;33:48-56.
10. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22:54-61.
11. Rubio-Beltran E, Labastida-Ramirez A, Villalon CM, MaassenVanDenBrink A. Is selective 5-HT_{1F} receptor agonism an entity apart from that of the triptans in antimigraine therapy? Pharmacol Ther 2018.
12. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet 2010;376:645-655.
13. Doods H, Hallermayer G, Wu D, et al. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. British Journal of Pharmacology 2000;129:420-423.
14. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
15. Tepper SJ. Anti-Calcitonin Gene-Related Peptide (CGRP) Therapies: Update on a Previous Review After the American Headache Society 60th Scientific Meeting, San Francisco, June 2018. Headache: The Journal of Head and Face Pain 2018;58:276-290.
16. Holland PR, Goadsby PJ. Targeted CGRP Small Molecule Antagonists for Acute Migraine Therapy. Neurotherapeutics 2018;15:304-312.
17. Deen M, Correnti E, Kamm K, et al. Blocking CGRP in migraine patients - a review of pros and cons. J Headache Pain 2017;18:96.
18. Schuster NM, Vollbracht S, Rapoport AM. Emerging treatments for the primary headache disorders. Neurol Sci 2015;36 Suppl 1:109-113.
19. Wrobel Goldberg S, Silberstein SD. Targeting CGRP: A New Era for Migraine Treatment. CNS Drugs 2015;29:443-452.
20. MaassenVanDenBrink A, Terwindt GM, van den Maagdenberg AMJM. Calcitonin gene-related peptide (receptor) antibodies: an exciting avenue for migraine treatment. Genome medicine 2018;10:10-10.

21. Mitsikostas DD, Reuter U. Calcitonin gene-related peptide monoclonal antibodies for migraine prevention: comparisons across randomized controlled studies. Current Opinion in Neurology 2017;30:272-280.
22. Uddman R, Edvinsson L, Ekblad E, Håkanson R, Sundler F. Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regulatory Peptides 1986;15:1-23.
23. Wimalawansa SJ, MacIntyre I. Calcitonin gene-related peptide and its specific binding sites in the cardiovascular system of rat. Int J Cardiol 1988;20:29-37.
24. Opgaard OS, Gulbenkian S, Bergdahl A, et al. Innervation of human epicardial coronary veins: immunohistochemistry and vasomotility. Cardiovascular Research 1995;29:463-468.
25. Smillie S-J, King R, Kodji X, et al. An Ongoing Role of α-Calcitonin Gene–Related Peptide as Part of a Protective Network Against Hypertension, Vascular Hypertrophy, and Oxidative Stress. Hypertension 2014;63:1056-1062.
26. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. Physiological Reviews 2014;94:1099.
27. Lindstedt IH, Edvinsson ML, Evinsson L. Reduced responsiveness of cutaneous microcirculation in essential hypertension – A pilot study. Blood Pressure 2006;15:275-280.
28. McCulloch J, Uddman R, Kingman TA, Edvinsson L. Calcitonin gene-related peptide: functional role in cerebrovascular regulation. Proceedings of the National Academy of Sciences 1986;83:5731-5735.
29. Edvinsson L, Mulder H, Goadsby PJ, Uddman R. Calcitonin gene-related peptide and nitric oxide in the trigeminal ganglion: Cerebral vasodilatation from trigeminal nerve stimulation involves mainly calcitonin gene-related peptide. Journal of the Autonomic Nervous System 1998;70:15-22.
30. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
31. Keith IM, Tjen-A-Looi S, Kraiczi H, Ekman R. Three-week neonatal hypoxia reduces blood CGRP and causes persistent pulmonary hypertension in rats. American Journal of Physiology - Heart and Circulatory Physiology 2000;279:H1571-H1578.
32. Wang Z, Martorell BC, Wälchli T, et al. Calcitonin Gene-Related Peptide (CGRP) Receptors Are Important to Maintain Cerebrovascular Reactivity in Chronic Hypertension. PLOS ONE 2015;10:e0123697.
33. Sakas DE, Moskowitz MA, Wei EP, Kontos HA, Kano M, Ogilvy CS. Trigeminovascular fibers increase blood flow in cortical gray matter by axon reflex-like mechanisms during acute severe hypertension or seizures. Proceedings of the National Academy of Sciences 1989;86:1401-1405.
34. Moskowitz MA, Sakas DE, Wei EP, et al. Postocclusive cerebral hyperemia is markedly attenuated by chronic trigeminal ganglionectomy. American Journal of Physiology-Heart and Circulatory Physiology 1989;257:H1736-H1739.
35. Zhang J-y, Yan G-t, Liao J, et al. Leptin attenuates cerebral ischemia/reperfusion injury partially by CGRP expression. European Journal of Pharmacology 2011;671:61-69.
36. Yadav S, Yadav YS, Goel MM, Singh U, Natu SM, Negi MPS. Calcitonin gene- and parathyroid hormone-related peptides in normotensive and preeclamptic pregnancies: a nested case–control study. Archives of Gynecology and Obstetrics 2014;290:897-903.
37. Sacco S, Ornello R, Ripa P, Pistoia F, Carolei A. Migraine and hemorrhagic stroke: a meta-analysis. Stroke 2013;44:3032-3038.
38. Chang CL, Donaghy M, Poulter N. Migraine and stroke in young women: case-control study. BMJ : British Medical Journal 1999;318:13-18.
39. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.
40. Schurks M, Rist PM, Bigal ME, Buring JE, Lipton RB, Kurth T. Migraine and cardiovascular disease: systematic review and meta-analysis. BMJ: British Medical Journal 2009;339:b3914.
41. Spector JT, Kahn SR, Jones MR, Jayakumar M, Dalal D, Nazarian S. Migraine headache and ischemic stroke risk: an updated meta-analysis. The American journal of medicine 2010;123:612-624.
42. Tzourio C, Tehindrazanarivelo A, Iglesias S, et al. Case-control study of migraine and risk of ischaemic stroke in young women. BMJ: British Medical Journal 1995;310:830-833.
43. Vanmolkot FH, Van Bortel LM, de Hoon JN. Altered arterial function in migraine of recent onset. Neurology 2007;68:1563-1570.
44. Scher AI, Terwindt GM, Picavet HSJ, Verschuren WMM, Ferrari MD, Launer LJ. Cardiovascular risk factors and migraine: The GEM population-based study. Neurology 2005;64:614-620.
45. Stam AH, Haan J, van den Maagdenberg A, Ferrari MD, Terwindt GM. Migraine and Genetic and Acquired Vasculopathies. Cephalalgia 2009;29:1006-1017.
46. de Boer I, Stam AH, Buntinx L, et al. RVCL-S and CADASIL display distinct impaired vascular function. Neurology 2018;91:e956-e963.

Chapter II.

Aims

Based on the questions posed in **Chapters I**, **III** and **VI**, the objective of this thesis was to investigate the vascular effects of current and novel antimigraine drugs. For this purpose, the following objectives were defined:

1. The gold standard for the acute treatment of migraine are the triptans. It has been shown that triptans cause vasoconstriction of the middle meningeal artery and, unfortunately, of the coronary arteries, due to their affinity for the 5-HT_{1B} receptors present in vascular smooth muscle cells. Therefore, novel serotonergic antimigraine drugs without vasoconstrictive properties and devoid of affinity for the 5-HT_{1B} receptor are needed. In **Chapter IV** we investigated the binding, functional activity and contractile responses of the selective 5-HT_{1F} receptor agonist lasmiditan, a novel antimigraine drug that is effective for the acute treatment of migraine.

2. Studies have shown that CGRP plays an important role in migraine pathophysiology. This has led to the development of antagonists (ubrogepant and atogepant) and an antibody (erenumab) against the CGRP receptor. We characterized the effect of ubrogepant, atogepant **(Chapter V)** and erenumab **(Chapter VIII)** in human cranial and coronary arteries to investigate the possible mechanism of their therapeutic effect, as well as their potential coronary side effects.

3. Before the arrival of the antibodies against CGRP or its receptor, prophylactic migraine treatment was not developed specifically for this disorder, but for hypertension, epilepsy or depression. Although these treatments are effective, their mechanism of action in migraine treatment unfortunately has not been studied in detail. Propranolol is one of the most widely prescribed drugs for prophylactic treatment of migraine, therefore we set out to investigate the effect of propranolol in the modulation of the trigeminovascular system in our human model of trigeminal nerve-mediated vasodilation **(Chapter IX)**. Since most of migraine patients are females and we have previously shown that trigeminovascular responses can be modulated by sex hormones, we stratified our data to see whether the responses between males and females were comparable.

4. Not all patients respond to treatments based on current targets. Based on the current knowledge of migraine pathophysiology, new drugs could act at receptors that are also activated by CGRP or that inhibit CGRP release. In **Chapter XI**, we investigated the role of imidazoline receptors in the inhibition produced by moxonidine and agmatine on the vasodepressor sensory CGRPergic outflow in pithed rats.

5. Migraine pathophysiology remains largely unknown. Current animal models, although useful, only represent certain features of this rather complex disorder. "Autosomal dominant **R**etinal **V**asculopathy with **C**erebral **L**eukodystrophy" (RVCL), caused by a mutation in the *TREX1* gene, is a vasculopathy that presents migraine as its earliest manifestation. Monogenic diseases such as RVCL provide an opportunity to study the genetic and vascular mechanisms involved in migraine pathophysiology. In **Chapter XII** we assessed whether RVCL-KI mice have features in line with the pathology seen in patients, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and the induction of experimental stroke).

...*proceeds* then to the understanding...

PART II:

Acute treatment of migraine

Chapter III.

Is selective 5-HT$_{1F}$ receptor agonism an entity apart from that of the triptans in antimigraine therapy?

Based on: ***E Rubio-Beltrán****[*], A Labastida-Ramírez[*], CM Villalón, A MaassenVanDenBrink (2018) Pharmacology & Therapeutics; 186:88-97.*

[*]Both authors contributed equally

Abstract

Migraine is a neurovascular disorder that involves activation of the trigeminovascular system and cranial vasodilation mediated by release of calcitonin gene-related peptide (CGRP).

The gold standard for acute migraine treatment are the triptans, 5-$HT_{1B/1D/(1F)}$ receptor agonists. Their actions are thought to be mediated through activation of: (i) 5-HT_{1B} receptors in cranial blood vessels with subsequent cranial vasoconstriction; (ii) prejunctional 5-HT_{1D} receptors on trigeminal fibres that inhibit trigeminal CGRP release; and (iii) 5-$HT_{1B/1D/1F}$ receptors in central nervous system involved in (anti)nociceptive modulation. Unfortunately, coronary arteries also express 5-HT_{1B} receptors whose activation would produce coronary vasoconstriction; hence, triptans are contraindicated in patients with cardiovascular disease. In addition, since migraineurs have an increased cardiovascular risk, it is important to develop antimigraine drugs devoid of vascular (side) effects.

Ditans, here defined as selective 5-HT_{1F} receptor agonists, were developed on the basis that most of the triptans activate trigeminal 5-HT_{1F} receptors, which may explain part of the triptans' antimigraine action. Amongst the ditans, lasmiditan: (i) fails to constrict human coronary arteries; and (ii) is effective for the acute treatment of migraine in preliminary Phase III clinical trials. Admittedly, the exact site of action is still unknown, but lasmiditan possess a high lipophilicity, which suggests a direct action on the central descending antinociceptive pathways. Furthermore, since 5-HT_{1F} receptors are located on trigeminal fibres, they could modulate CGRP release.

This review will be focussed on the similarities and differences between the triptans and the ditans, their proposed sites of action, side effects and their cardiovascular risk profile.

Introduction

Migraine is a debilitating neurovascular disorder characterized by recurring unilateral pulsating headaches of moderate to severe intensity, associated with nausea, photophobia and/or phonophobia, lasting from 4 to 72 hours[1]. In the Global Burden of Disease Study, migraine was ranked as the third disabler in women, sixth disabler when taking both genders into account, and the most disabling of all neurological disorders, affecting approximately 15% of the world population[2], with a profound negative effect on the patient's quality of life[3]. Furthermore, this neurovascular disorder represents an economic loss of €20 billion in Europe every year[4]. Thus, migraine is a public health problem that affects both the individual and society.

Pathophysiology of migraine

Throughout the years, several theories regarding the pathophysiology of migraine have emerged[5]. In the late 1930's and early 1940's, Wolff's group described migraine as a disorder of vascular origin, with an intense extracranial vasodilation as the cause of migraine pain[6,7]. Decades later, Moskowitz introduced the neurogenic theory, where trigeminovascular axons from blood vessels of the dura mater released vasoactive peptides producing an sterile inflammatory response followed by pain[8]. Nowadays, migraine is considered as a neurovascular disorder that involves activation of the trigeminovascular system[9,10], presumably followed by vasodilation mainly mediated by the release of calcitonin gene-related peptide (CGRP), a neuropeptide present in perivascular sensory fibres[11,12].

Treatment of migraine

Despite the long history of migraine treatment, effective antimigraine drugs have been, until very recently, limited in number (for references see[13]). Basically, pharmacological treatment of migraine can be divided into prophylactic drugs, designed to reduce the frequency and severity of migraine attacks, and acutely acting drugs, aimed to reverse the attack once it has begun, including the

associated symptoms. The majority of migraine patients only need acute treatment, nevertheless, migraineurs that suffer from frequent attacks or have contraindications for the use of acutely acting drugs, are also prescribed prophylactic drugs[14]. The prophylactic treatment will not be discussed here as it is falls beyond the scope of the present review.

The acute treatment can be further subdivided in specific (ergot derivatives, triptans, gepants, "ditans"), and non-specific (nonsteroidal anti-inflammatory drugs, analgesics) antimigraine drugs[14,15]. While non-specific drugs aim to treat migraine as a general headache or other pain, specific treatment is developed based on the neurovascular basis of migraine. Thus, these drugs target the modulation of the trigeminovascular system, the CGRP-mediated vasodilation (i.e. extracranial vasoconstriction, inhibition of CGRP release, CGRP receptor antagonism) and/or the pain perception pathway, amongst others. On this basis, CGRP receptor antagonists (gepants) are a likely candidate for the acute treatment of migraine, and indeed, they were effective in clinical trials[12,16,17]; however, due to pharmacokinetic and/or hepatotoxic limitations none of the gepants have yet reached the market[18]. Currently, a potential concern with gepants includes the cardiovascular side effects when used chronically, considering the physiological protective role of CGRP in maintaining cardiovascular homeostasis in ischemic events (for further references see[19]). Several drugs targeting the CGRP receptor are presently under development for the acute and prophylactic treatment of migraine[19,20].

During the last 40 years, the target for selective antimigraine drugs has been the serotoninergic signalling. Long before the discovery of CGRP and its fundamental role in migraine, increased urinary and plasma levels of serotonin (5-hydroxytryptamine, 5-HT) and its metabolites were described[21,22]. Further studies showed that intravenous infusion of 5-HT was capable of aborting migraine attacks[23] and that antimigraine drugs like methysergide and ergotamine were acting on, amongst other receptors, 5-HT_1 receptors[24,25]. In this review, the main focus will be on the triptans, 5-$HT_{1B/1D/(1F)}$ receptor agonists, that are currently considered the gold standard for acute migraine treatment, and on the novel "ditans" (in this review defined as selective 5-HT_{1F} receptor agonists), not only developed based on the neurovascular origin of migraine, but also in view of the cardiovascular risk profile of migraine patients[26-34] .

Triptans

As mentioned in the above section, the role of serotoninergic neurotransmission in migraine led to the design of antimigraine drugs that targeted the 5-HT receptors[21-23]; however, the exact 5-HT receptors involved in the relief of migraine attacks were unknown. Indeed, intravenous infusion of 5-HT was able to abort migraine attacks, but considering that there are fourteen 5-HT receptors[35], and they were all activated, numerous side effects were observed[23]. After several studies using selective agonists and antagonists it was demonstrated that the therapeutic action of 5-HT was mediated by "5-HT_1-like receptors" that constricted cranial blood vessels[24,25,36], and the first triptan was developed: sumatriptan[37]. In the early 1990s, sumatriptan was officially introduced to the market[38]. In view of the low oral bioavailability and lipophilicity of sumatriptan[39], as well as the vast market potential, "second generation" triptans (zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan and avitriptan) were developed, with a chemical structure similar to sumatriptan (see Fig. 1), but with higher oral availability and lipophilicity (see Table 1), as well as a longer plasma half-life[35,40,41].

Mechanism of action

Initially, it was described that the therapeutic action of 5-HT on migraine was mediated by activation of "5-HT_1-like" receptors[36]. Years later, based on structural, transductional and operational criteria, these receptors were classified into 5-HT_{1B} and 5-HT_{1D} receptors (for further references see[42,43]).

Triptans are 5-$HT_{1B/1D}$ receptor agonists, and a grand majority of them are also $5HT_{1F}$ receptor agonists (see Table 2, Fig. 2). Sumatriptan, as previously mentioned, has low lipophilicity (see Table 1) and cannot cross the blood brain barrier (BBB). For a drug to be considered able to cross the BBB, it should have a distribution coefficient at physiological pH ($logD_{pH7.4}$) higher than -1[44,45]. Notably, second generation triptans were developed with higher lipophilicity[39] but their ability to cross the BBB is in controversy, since their reported $logD_{pH7.4}$ values are not consistent amongst studies (see Table 1). Furthermore, it is important to consider the possible interactions between triptans and BBB efflux transporters (*e.g.* P-glycoprotein) that limit the central actions of triptans, as it has been reported for eletriptan[46].

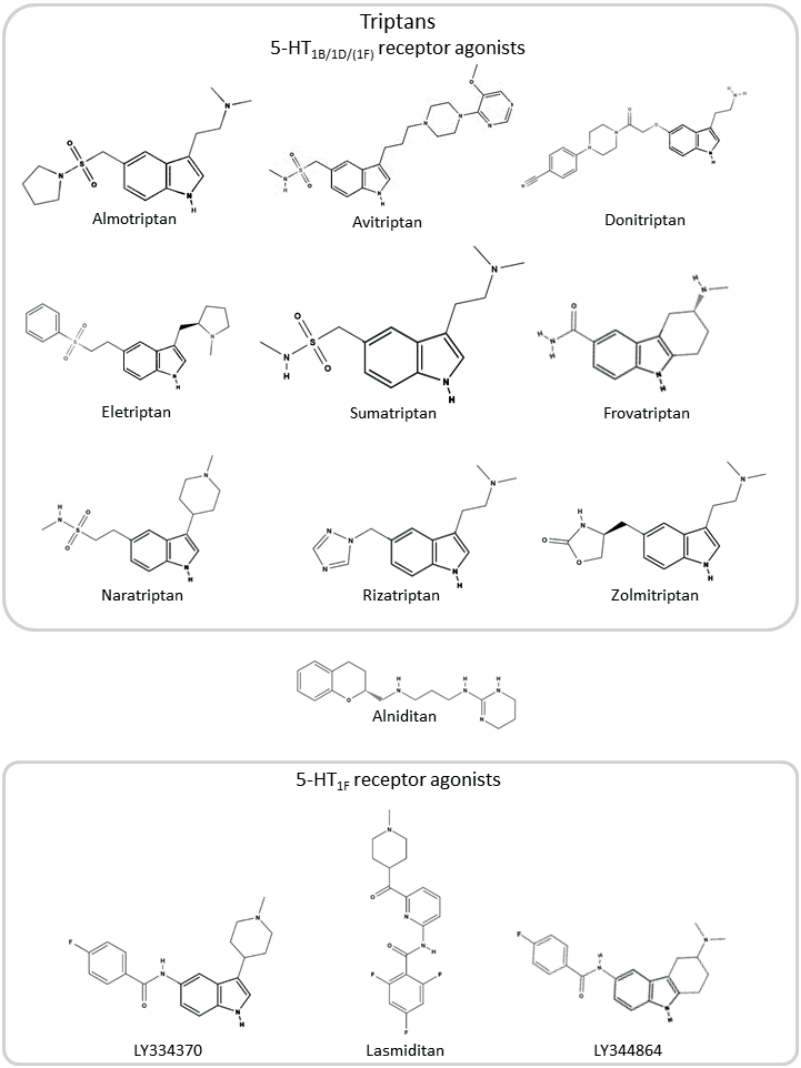


Fig. 1. Chemical structures of the triptans, alniditan and the selective 5-HT_{1F} receptor agonists. It is worth remarking the presence of the indole group in all the structures, with exception of lasmiditan and alniditan[35].

Table 1. Reported LogD$_{pH7.4}$ values for triptans. Compounds with values higher than -1 are considered to be able to cross the BBB.

	Sumatriptan	Zolmitriptan	Naratriptan	Rizatriptan	Almotriptan	Eletriptan	Frovatriptan
Fox[58]	-1.5	-1	-0.2	-0.7	ND	ND	-1
Ferrari, *et al*[59]	-1.3	-0.7	-0.2	-0.7	+0.35	+0.5	ND
Glennon and Dukat[60]	-1.7	-0.8	-1.2	-1.4	-0.5	+0.2	-2.1
Milton, *et al*[61]	ND	ND	ND	ND	ND	+1.1	ND
Pascual and Muñoz[62]	-1.3	-0.7	-0.2	-0.7	-2.1	+0.5	ND
Cheng, *et al*[63]	-1.4	-1.5	ND	-0.8	+0.4	+0.2	-1.9

ND. Not defined

Table 2. Summary of pEC$_{50}$ values of cAMP (5-HT$_1$ and 5-HT$_7$) and IP (5-HT$_2$) assays of individual antimigraine drugs at 5-HT receptors[64]. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response is exerted. *pEC$_{50}$ values correspond to [^{35}S]GTPγS assay.

	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$*	5-ht$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_7$
Ergotamine tartrate	9.78	9.94	9.43	5.95	5.97	9.25	8.72	7.09
Sumatriptan succinate	<5	7.32	8.30	5.99	8.03	<5	<5	5.22
Zolmitriptan	<5	7.87	9.51	8.18	8.00	<5	<5	6.28
Naratriptan hydrochloride	<5	8.05	8.80	7.75	8.38	<5	<5	<5
Rizatriptan benzoate	<5	7.08	8.11	7.34	6.54	<5	5.49	<5
Almotriptan malate	<5	7.08	7.75	<5	7.79	<5	5.20	<5
Eletriptan hydrobromide	<5	8.00	9.04	7.53	8.13	6.07	6.81	6.45
Frovatriptan racemate	<5	7.98	8.36	5.04	7.10	<5	<5	7.42
Donitriptan hydrochloride	5.94	9.96	9.51	<5	<5	8.10	7.61	5.23
Avitriptan fumarate	<5	8.57	9.27	5.52	7.09	6.91	6.41	5.38
Alniditan dihydrochloride	7.00	8.87	8.20	5.68	5.92	<5	7.15	6.32
Lasmiditan hemisuccinate	<5	<5	6.64	6.17	8.43	<5	<5	<5
LY334370 hydrochloride	5.84	6.52	6.92	7.53	9.08	<5	<5	<5
LY344864 hydrochloride	<5	<5	6.93	6.22	8.72	<5	<5	<5

Cardiovascular (side) effects

The initially proposed therapeutic action of triptans is through the selective vasoconstriction of cranial blood vessels due to the high expression of 5-HT$_{1B}$ receptors in this vasculature[36,47] in comparison with peripheral blood vessels[48]. In agreement with this, *in vitro* studies have shown that at therapeutic concentrations triptans contract the middle meningeal artery (MMA)[48]. Furthermore, magnetic resonance angiography studies demonstrated that migraine attacks are associated with dilation of the extra- and intracerebral arteries, ipsilateral to the headache side; and that contraction of dural (but not intracranial) arteries by triptans, is associated with headache relief[49], although it is worth mentioning that further results of the same group have been inconsistent[50-52] and it is still a matter of debate to what extent the vascular action of the triptans contributes to their therapeutic efficacy. Unfortunately, studies have consistently shown that triptans induce an increase in blood pressure[53] and contraction of coronary arteries[48,54-56], which is more pronounced in the distal than in the proximal portion of the human coronary artery[57].

Currently, although there is still a debate on whether the therapeutic action of triptans relays on their vasoconstrictive properties, it is clear that coronary vasoconstriction is a drug class effect of the triptans as 5-HT_{1B} receptor agonists[65]. Therefore, triptans are contraindicated in migraine patients with cardiovascular disease[66]. Additionally, it is important to consider that migraineurs are known to have an increased risk of haemorrhagic[29] and ischemic[26-28,30,31] stroke, with women presenting a higher risk[34]. Also, an altered arterial function[32], and a higher risk of myocardial infarction and coronary heart disease[33] have been described. Nonetheless, the use of triptans does not seem to increase the risk of stroke, myocardial infarction, cardiovascular death, ischemic heart disease or mortality[67-69]. However, taken together, their coronary vasoconstrictor potential justifies the contraindication of the triptans in patients with cardiovascular risk factors.

Neuronal effects

Although sumatriptan does not cross the BBB[59], it has long been speculated that during a migraine attack there would be a disruption of the BBB, which would enable the triptans, even those with a low lipophilicity, to exert a central effect. However, it has recently been demonstrated that there is no disruption of the BBB during a migraine attack[70,71], thus excluding a central action for the triptans, except for those with a high lipophilicity. Besides the potential role for blood vessels in the pathophysiology of migraines, this suggests that actions of the triptans can well be mediated through neuronal structures that are not protected by the BBB, as it is the case for the pituitary gland, choroid plexus and, most importantly, the trigeminal ganglion (TG)[72], a key structure in migraine pathophysiology. Accordingly, treatment with sumatriptan reduces CGRP plasma levels as migraine lessens[73]. More recently, sumatriptan was also shown to inhibit capsaicin-induced trigeminal CGRP release in healthy volunteers[74]. Furthermore, expression of prejunctional 5-HT_{1D} receptors has been described in trigeminovascular nociceptive neurons[47,75,76] which could suggest a role in the modulation of CGRP release, as well as plasma protein extravasation[77]. Remarkably, based on the prejunctional location of 5-HT_{1D} receptors in the TG, PNU-142633, a selective 5-HT_{1D} receptor agonist, was developed for the acute treatment of migraine, and showed a superior potency over sumatriptan for blocking plasma protein extravasation. Unfortunately, it was not effective in the acute treatment of migraine[78]. It is worth mentioning that PNU-142633 was developed based on the gorilla $5HT_{1D}$ receptor, which could explain its lack of efficacy, and thus a role for the 5-HT_{1D} receptor in the treatment of migraine cannot categorically be ruled out. Besides, it has been shown that activation of 5-HT_{1B}, but not 5-HT_{1D} receptors inhibits CGRP release in the pithed rat model[79], indicating that, while 5-HT_{1D} receptor activation may contribute to the therapeutic actions of triptans, it may not be their main site of action. Further studies are needed to completely elucidate the role of 5-HT_{1D} receptor activation in migraine treatment.

As discussed earlier, second generation triptans were developed with higher lipophilicity than sumatriptan[40,41]. Therefore, some of them may be able to cross the BBB[59,80]. Although the lipophilicity of triptans correlates with central side effects, it does not seem to be related to their efficacy[62], and there is no consistency in the lipophilicity reported in literature (see Table 2). Nevertheless, we cannot categorically exclude additional therapeutic actions mediated via activation of 5-$HT_{1B/1D/(1F)}$ receptors in the central nervous system by highly lipophilic triptans[818181]. In accordance with this, it has been shown that vasodilation of the canine external carotid artery induced by intracarotid administration of capsaicin, is inhibited by spinal (but not intravenous) administration of sumatriptan via activation of 5-HT_{1B} receptors; in contrast, intravenous administration of the highly lipophilic donitriptan inhibits the capsaicin-induced vasodilation, also mediated by activation of 5-HT_{1B} receptors[80,82].

Several studies have described 5-HT$_{1B}$ and (presynaptic) 5-HT$_{1D}$ receptors in the dorsal horn of the spinal cord (DHSC)[83], substantia nigra, globus pallidus[83,84], nucleus tractus solitarius (NTS), trigeminal nucleus caudalis (TNC)[47,83], periaqueductal grey area (PAG)[83,85], the ventroposteromedial nucleus of the thalamus[86], hypothalamic paraventricular nucleus (PVN)[87] and the rostral ventromedial medulla[88], structures previously associated with nociceptive and anti-nociceptive pathways[89-97]. Furthermore, it has been shown that spinal 5-HT$_{1B/1D/1F}$ receptors are involved in the serotonergic descending inhibitory pain system[98-102]. Of special interest, intravenous administration of naratriptan[103,104] results in inhibition of the spinal TNC. Also, the PAG, more specifically the ventrolateral division, is activated by afferents from the TG[105-107], and microinjection of naratriptan in this structure inhibits nociceptive dural responses[85]. Moreover, the PVN, that has been previously shown to participate in the endogenous modulation of pain[97,108] sends projections to the PAG[109] and the spinal trigeminal nucleus[87], and microinjection of naratriptan in this structure, attenuates dural-evoked trigeminovascular responses[87]. Thus, indeed highly lipophilic triptans could not only act through the vasoconstriction of extracranial vasculature and inhibition of the release of CGRP, but also through the activation of the descending inhibitory pain system and/or the inhibition of nociceptive transmission (see Fig. 3). Interestingly, most of these studies were performed using naratriptan, a highly lipophilic triptan that has a high affinity for the 5-HT$_{1F}$ receptor (see Table 2). Furthermore, although antagonists were used to confirm the role of 5-HT$_{1B/1D}$ receptors, there is no selective antagonist commercially available for the 5-HT$_{1F}$ receptor yet. Therefore, the involvement of this receptor in the neuronal actions of triptans can neither be confirmed nor excluded and requires further studies.

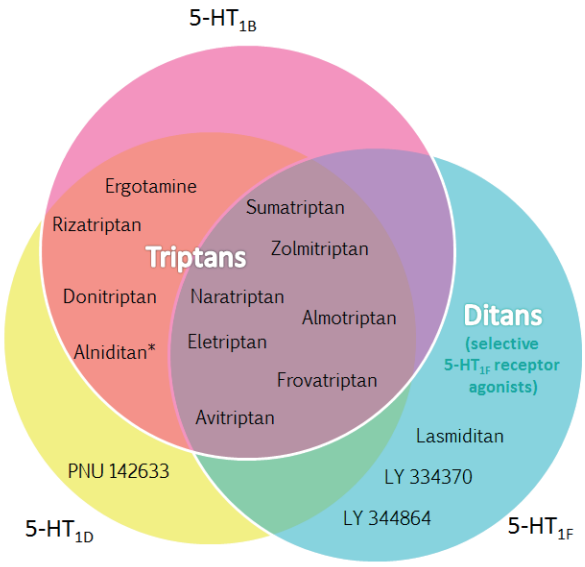


Fig. 2. Summary of agonist profiles of triptans, ditans (here considered as selective 5-HT$_{1F}$ receptor agonists) and other 5-HT receptor ligands, for 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ receptors (pEC$_{50}$>7). *In view of the high affinity of alniditan for the 5-HT$_{1B/1D}$ receptors (pEC$_{50}$ cAMP 8.87 and 8.20, respectively), we classified it as a triptan, despite its generic name[64].

Ditans and the 5-HT$_{1F}$ receptor

Triptan monotherapy is ineffective for approximately 25% of migraineurs and in 40% of acute migraine attacks[110]. Several studies have tried to elucidate the lack of efficacy in some migraineurs, but were unable to explain why some patients are responders to the triptans while others are nonresponders. Whereas a difference in efficacy could be due to pharmacokinetic factors for some of the oral triptans, pharmacokinetics did not seem to be responsible for differences in efficacy in response to subcutaneous sumatriptan[111], nor polymorphisms in the 5-HT$_{1B}$ and 5-HT$_{1F}$ receptor genes were able to explain differences in clinical responses to sumatriptan[112-114]. Moreover, as previously discussed, migraine patients are known to have an increased cardiovascular risk; therefore, it is important to develop novel effective antimigraine drugs that are devoid of cardiovascular side effects.

Several triptans bind to the 5-HT$_{1F}$ receptor (see Table 2). Also, as will be discussed later, 5HT$_{1F}$ receptors are expressed in several structures associated with migraine pathophysiology and with the (neuronal) therapeutic actions of triptans. This led to the development of selective 5-HT$_{1F}$ receptor agonists as possible option for migraine treatment. Several selective 5-HT$_{1F}$ receptor agonists have been developed (see Fig. 1), including LY344864, an aminocarbazole, LY334370, a 4-(3-indolyl)piperidine[60] and lasmiditan (COL-144, LY573144), a piridinoyl-piperidine[115]. While the selective 5-HT$_{1F}$ receptor agonists were referred to as SSOFRAs (Selective Serotonin One F Receptor Agonists) for some time, in the last years the term "ditan" has been accepted as a synonymous of selective 5-HT$_{1F}$ receptor agonist[116]. In this context, it is important to consider alniditan, a 5-HT$_{1A/1B/1D}$ receptor agonist with only low 5-HT$_{1F}$ receptor affinity (see Table 2), suggesting that the suffix "ditan" is merely to distinguish novel acutely acting (5-HT$_1$ receptor agonists) antimigraine drugs from triptans, without any structural (see Fig. 2) and/or pharmacological criteria (see Table 2). As the following paragraph is only focused on 5-HT$_{1F}$ receptor agonists, alniditan will not be further discussed when ditans are mentioned.

Location

The human 5-HT$_{1F}$ receptor was first identified and cloned in 1993[117]. It has been described in the TG[75,118,119], globus pallidus, NTS, substantia nigra, PAG, DHSC[83], caudate nucleus[120], caudate putamen, nucleus accumbens[121], and the TNC[83,120,122,123]. Interestingly, it has also been shown to be expressed on cerebrovascular tissues[118] as well as peripheral arteries[54].

Preclinical studies

Selective 5-HT$_{1F}$ receptor agonists were developed as a novel therapeutic option for migraineurs, including those with increased cardiovascular risk. Therefore, the main concern was to study the (lack of) vascular effects, as well as their efficacy on predictive models of migraine treatment.

Cardiovascular (side) effects

An established *in vitro* model used to analyze potential contraction of human arteries is the contraction of the rabbit saphenous vein[124]. LY334370[125,126], LY344864[126,127] and lasmiditan[115] lacked vasoconstrictor effects in this model. Furthermore, in two *in vivo* studies in dogs, intracarotid administration of LY344864 did not affect carotid blood flow[128], and responses to continuous intravenous infusions of lasmiditan, in escalating cumulative doses, failed to constrict the coronary and carotid arteries; sumatriptan, on the other hand, constricted both arteries at clinically relevant doses[129]. Furthermore, in *in vitro* studies in human mammary and coronary arteries, lasmiditan was also devoid of vasoconstrictor properties, while sumatriptan was shown to contract already at subtherapeutic concentrations[64,129]. It is worth mentioning that in this study, also a threshold stimulation with the thromboxane A$_2$ analogue U46619 was performed to potentially "unmask"

vasoconstrictor responses in the internal mammary artery; notably, sumatriptan contracted at even lower concentrations, while lasmiditan failed to produce vasoconstriction. Similarly, pharmacological activation of 5-HT$_{1F}$ receptors failed to produce vasoconstriction in human cerebral and MMA[130,131].

Neuronal effects

A number of studies have shown that intravenous administration of the selective 5-HT$_{1F}$ receptor agonists LY334370[125], LY344864[132] and lasmiditan[115] inhibits protein plasma extravasation in the dura mater. However, it is important to consider that the selective 5HT$_{1D}$ receptor agonist PNU-142633 also showed a high potency for inhibiting protein plasma extravasation, but was not effective in the acute treatment of migraine[78]. Similar failures in the acute treatment of migraine were observed with endothelin receptor antagonists[133] and the highly potent inhibitor of protein plasma extravasation CP 122,288[134]. Therefore, it seems likely that 5-HT$_{1F}$ receptor agonists act through additional pathways, for instance, modulation of the trigeminovascular system, inhibition of the CGRP-mediated vasodilation and/or modulation of the pain perception pathway (see Fig. 3). In accordance with these potential mechanisms, activation of 5-HT$_{1F}$ receptors has been shown to inhibit the activation of second order neurons in the TNC in mice[135], rats[115,136-138] and cats[139], suggesting a modulation of the trigeminovascular system. Moreover, an *in vitro* study with LY344864 showed an inhibition of CGRP release in rat dura mater, but not in TNC or TG[122]. In contrast, a recent study showed that lasmiditan inhibits CGRP release in mouse dura mater, TG and TNC[140], although only supratherapeutic concentrations were studied. Therefore, more *in vitro* and *in vivo* studies (e.g. closed cranial window model) are required to confirm 5-HT$_{1F}$ receptor involvement in the inhibition of CGRP release. Nonetheless, in the pithed rat model it has been shown that activation of 5-HT$_{1F}$ receptors inhibits the release of CGRP from perivascular nerve fibres[141].

Furthermore, the expression of 5-HT$_{1F}$ receptors in the globus pallidus, NTS, the DHSC[83], caudate nucleus[120], putamen and the nucleus accumbens[121], structures associated with (anti)nociceptive pathways[89,91,93,94,142,143], suggests a possible role for 5-HT$_{1F}$ receptor activation in the modulation of nociceptive impulses.

Clinical studies

Twenty years have passed since the development of LY334370[136]; nowadays, two more 5-HT$_{1F}$ receptor agonists have been synthesized, namely, LY344864[132] and lasmiditan[115]. Only LY334370 and lasmiditan have reached clinical trials.

LY334370

The prototype for the 5-HT$_{1F}$ receptor agonists reached phase II of clinical trials[144], and the results were favourable for the acute treatment of migraine, as sustained headache response rates were higher on 60 mg (37%) and 200 mg (52%) compared to placebo (8%; $p<0.001$). However, further trials were halted due to observed liver damage in beagle dogs after treatment for longer than one month[145]. It is worth mentioning that liver damage was not reported in other species, discarding hepatotoxicity as a drug class effect.

Lasmiditan

Considered as the most promising of the 5-HT$_{1F}$ receptor agonists, lasmiditan differs from LY334370 and LY344864, as it does not possess the indole group (see Fig. 1).

Five phase I trials have been completed[146-148] for intravenous and oral formulations for safety, bioavailability, tolerability and pharmacokinetic studies. In 2007, a phase II study for the intravenous formulation was conducted[149], and later on, in 2009 for the oral formulation[150]. In both cases, lasmiditan was shown to be safe and effective in the acute treatment of migraine. Phase III trials

are ongoing, with preliminary statements showing positive results as the percentage of patients pain free at two hours was higher for 100 mg (28%) and 200 mg (32%), compared to placebo (15%; p<0.001[151]). It is worth mentioning, that the first phase III trial ("SAMURAI", NCT02439320) included a majority of migraineurs (80%) that had cardiovascular conditions or cardiovascular risk factors (*i.e.* obesity, hypertension, hyperlipidemia), the main group of patients that would benefit from the lack of vasoconstrictive properties. Two more phase III trials ("SPARTAN", NCT02605174: "GLADIATOR", NCT02565186) are under way and are aimed to compare different doses of lasmiditan, and to evaluate the safety and efficacy of long term use, respectively. Recently, preliminary results from SPARTAN have been released and showed that at two hours following the first dose of lasmiditan, the percentage of pain-free patients was statistically significantly higher for 50 mg (28%, p=0.003); 100 mg (31%, p<0.001) and 200 mg (38%, p<0.001) compared to placebo (21%)[152]. Only patients that received lasmiditan in previous trials are allowed to be included in the GLADIATOR trial. It is worth mentioning that no direct comparison has been performed between triptans and lasmiditan in clinical studies. Future phase III trials with a triptan as an active comparator would allow a better evaluation of their efficacy.

Conclusions

Triptans are 5-$HT_{1B/1D/(1F)}$ receptors agonists and are considered as the gold standard for acute migraine treatment that have been proven effective. Unfortunately, they are contraindicated in patients with cardiovascular diseases due to their vasoconstrictor (side) effects[55,65]. Furthermore, triptans are not effective in 25% of migraine patients[110]; thus, it is important to develop new antimigraine drugs that are cardiovascularly completely safe and at least equally effective. The vasoconstrictor properties of triptans are thought to be mediated via activation of 5-HT_{1B} receptors in blood vessels; this has led, in view of the contraindications in patients with cardiovascular pathologies, to the development of antimigraine drugs targeting the 5-HT_{1D} and 5-HT_{1F} receptors. While 5-HT_{1D} receptor activation was not effective in the acute treatment of migraine[78], the 5HT_{1F} receptor agonists have shown to be effective[149,150]. Furthermore, predictive preclinical models of migraine have shown that lasmiditan does not cause vasoconstriction and that its antimigraine effects are likely mediated via neural modulation[115,129,138,140]. Thus, 5-HT_{1F} receptor agonists may provide migraine patients with another type of specific acutely acting antimigraine drug, with a cardiovascular safety advantage over the triptans, and with a mechanism of action that is likely to be, at least partly, different from that of the triptans.

Based on the lack of vasoconstrictive properties and its presumably neuronal mode of action, 5-HT_{1F} receptor agonists can be considered as an entity apart from that of the triptans in antimigraine therapy.

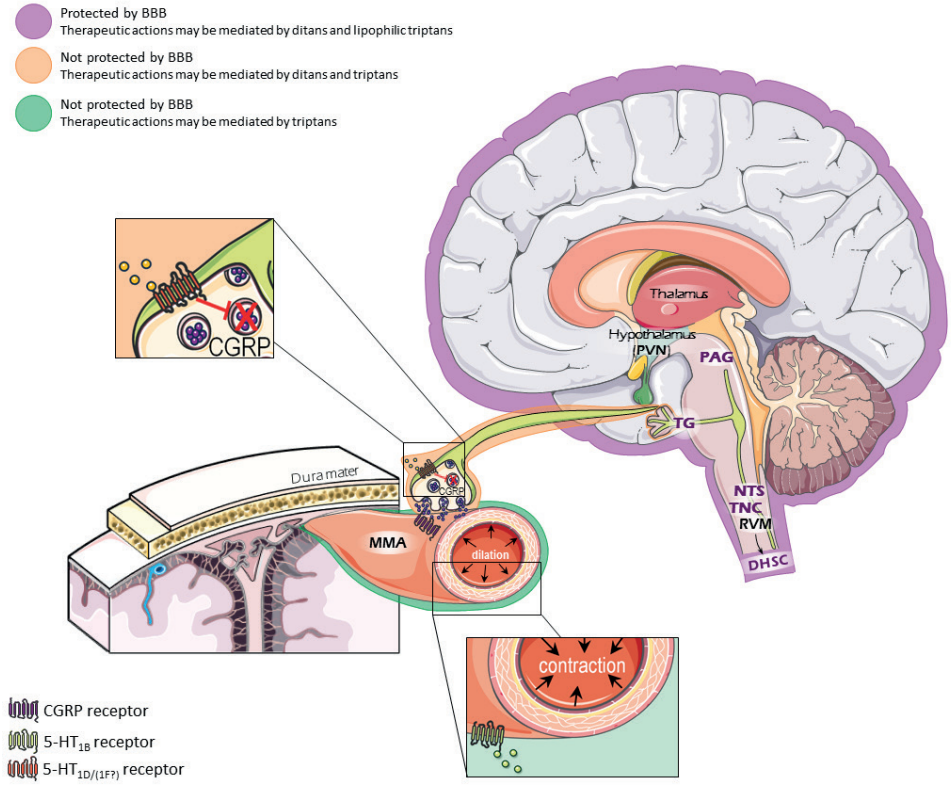


Fig. 3. Structures associated with migraine pathophysiology and/or treatment. The proposed therapeutic action of triptans is through the selective vasoconstriction of the MMA (green), as well as the inhibition of CGRP release from the sensory fibres and the modulation of the TG, since they are not protected by the BBB, where also 5-HT$_{1F}$ receptors have been described (orange). Highly lipophilic triptans could also act on the ventroposteromedial nucleus of the thalamus, PVN, PAG, LC, NTS, TNC, RVM and the (DH) SC, where 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors have been described and whose activation could also modulate the activity of the TG and/or the (anti)nociception pathways (purple). Furthermore, 5-HT$_{1F}$ receptors have also been reported in PAG, TG, TNC and the (DH)SC, which suggests a role in the modulation of the trigeminal responses as well as possible action on the (anti)nociceptive pathways (**BOLD** letters). BBB: blood brain barrier; CGRP: calcitonin-like related peptide; DHSC: dorsal horn of the spinal cord; MMA: middle meningeal artery; NTS: nucleus tractus solitarius; PAG: periaqueductal grey area; PVN: paraventricular nucleus of the hypothalamus; RVM: rostral ventromedial medulla; TG: trigeminal ganglion; TNC: trigeminal nucleus caudalis.

References

1. Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia 2013;33:629-808.
2. Steiner TJ, Stovner LJ, Birbeck GL. Migraine: the seventh disabler. The journal of headache and pain 2013;14:1.
3. Ruiz de Velasco I, González N, Etxeberria Y, Garcia-Monco JC. Quality of Life in Migraine Patients: A Qualitative Study. Cephalalgia 2003;23:892-900.
4. Gustavsson A, Svensson M, Jacobi F, et al. Cost of disorders of the brain in Europe 2010. European Neuropsychopharmacology 2011;21:718-779.
5. Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiological Reviews 2017;97:553-622.
6. Ray BS, Wolff HG. Experimental studies on headache: Pain-sensitive structures of the head and their significance in headache. Archives of Surgery 1940;41:813-856.
7. Graham JR, Wolff HG. Mechanism of migraine headache and action of ergotamine tartrate. Archives of Neurology & Psychiatry 1938;39:737-763.
8. Moskowitz MA, Romero J, Reinhard JF, Melamed E, Pettibone DJ. Neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine? The Lancet 1979;314:883-885.
9. Edvinsson L. The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine. Headache 2017;57 Suppl 2:47-55.
10. Edvinsson L, Uddman R. Neurobiology in primary headaches. Brain Research Brain research reviews 2005;48:438-456.
11. Goadsby PJ, Lipton RB, Ferrari MD. Migraine - Current Understanding and Treatment. New England Journal of Medicine 2002;346:257-270.
12. Villalón CM, Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacology & Therapeutics 2009;124:309-323.
13. Villalón CM, Centurión D, Valdivia LF, de Vries P, Saxena PR. Migraine: Pathophysiology, Pharmacology, Treatment and Future Trends. Current Vascular Pharmacology 2003;1:71-84.
14. Dib M. Optimizing prophylactic treatment of migraine: Subtypes and patient matching. Therapeutics and Clinical Risk Management 2008;4:1061-1078.
15. Marmura MJ, Silberstein SD, Schwedt TJ. The acute treatment of migraine in adults: the american headache society evidence assessment of migraine pharmacotherapies. Headache 2015;55:3-20.
16. Doods H, Hallermayer G, Wu D, et al. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. British Journal of Pharmacology 2000;129:420-423.
17. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet 2010;376:645-655.
18. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
19. Tso AR, Goadsby PJ. Anti-CGRP Monoclonal Antibodies: the Next Era of Migraine Prevention? Current Treatment Options in Neurology 2017;19:27.
20. Schuster NM, Rapoport AM. Calcitonin Gene-Related Peptide-Targeted Therapies for Migraine and Cluster Headache: A Review. Clinical Neuropharmacology 2017;40:169-174.
21. Somerville BW. Platelet-bound and free serotonin levels in jugular and forearm venous blood during migraine. Neurology 1976;26:41.
22. Sicuteri F, Testi A, Anselmi B. Biochemical Investigations in Headache: Increase in the Hydroxyindoleacetic Acid Excretion During Migraine Attacks. International Archives of Allergy and Immunology 1961;19:55-58.
23. Kimball RW, Friedman AP, Vallejo E. Effect of serotonin in migraine patients. Neurology 1960;10:107-111.
24. Apperley E, Feniuk W, Humphrey PPA, Levy GP. Evidence for two types of excitatory receptors for 5-hydroxytryptamine in dog isolated vasculature. British Journal of Pharmacology 1980;68:215-224.
25. Humphrey PPA, Feniuk W, Perren MJ, et al. GR43175, a selective agonist for the 5-HT_1-like receptor in dog isolated saphenous vein. British Journal of Pharmacology 1988;94:1123-1132.
26. Chang CL, Donaghy M, Poulter N. Migraine and stroke in young women: case-control study. BMJ : British Medical Journal 1999;318:13-18.
27. Schurks M, Rist PM, Bigal ME, Buring JE, Lipton RB, Kurth T. Migraine and cardiovascular disease: systematic review and meta-analysis. BMJ: British Medical Journal 2009;339:b3914.
28. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.

29. Sacco S, Ornello R, Ripa P, Pistoia F, Carolei A. Migraine and hemorrhagic stroke: a meta-analysis. Stroke 2013;44:3032-3038.
30. Tzourio C, Tehindrazanarivelo A, Iglesias S, et al. Case-control study of migraine and risk of ischaemic stroke in young women. BMJ: British Medical Journal 1995;310:830-833.
31. Spector JT, Kahn SR, Jones MR, Jayakumar M, Dalal D, Nazarian S. Migraine headache and ischemic stroke risk: an updated meta-analysis. The American journal of medicine 2010;123:612-624.
32. Vanmolkot FH, Van Bortel LM, de Hoon JN. Altered arterial function in migraine of recent onset. Neurology 2007;68:1563-1570.
33. Scher AI, Terwindt GM, Picavet HSJ, Verschuren WMM, Ferrari MD, Launer LJ. Cardiovascular risk factors and migraine: The GEM population-based study. Neurology 2005;64:614-620.
34. Linstra KM, Ibrahimi K, Terwindt GM, Wermer MJH, MaassenVanDenBrink A. Migraine and cardiovascular disease in women. Maturitas 2017;97:28-31.
35. Villalón CM, MaassenVanDenBrink A. The Role of 5-Hydroxytryptamine in the Pathophysiology of Migraine and its Relevance to the Design of Novel Treatments. Mini-Reviews in Medicinal Chemistry 2017;17:928-938.
36. Feniuk W, Humphrey PPA. The development of a highly selective 5-HT_1 receptor agonist, sumatriptan, for the treatment of migraine. Drug Development Research 1992;26:235-240.
37. Humphrey PPA. The Discovery of a New Drug Class for the Acute Treatment of Migraine. Headache: The Journal of Head and Face Pain 2007;47:S10-S19.
38. The Subcutaneous Sumatriptan International Study Group Treatment of migraine attacks with sumatriptan. New England Journal of Medicine 1991;325:316-321.
39. Fowler PA, Lacey LF, Thomas M, Keene ON, Tanner RJN, Baber NS. The Clinical Pharmacology, Pharmacokinetics and Metabolism of Sumatriptan. European Neurology 1991;31:291-294.
40. de Vries P, Villalón CM, Saxena PR. Pharmacology of triptans. Emerging Drugs 1999;4:107-125.
41. Tfelt-Hansen P, De Vries P, Saxena PR. Triptans in Migraine. Drugs 2000;60:1259-1287.
42. Hoyer D, Clarke DE, Fozard JR, et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev 1994;46:157-203.
43. Saxena PR, de Vries P, Villalón CM. 5-HT_1-like receptors: a time to bid goodbye. Trends in Pharmacological Sciences 1998;19:311-316.
44. Levin VA. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. Journal of Medicinal Chemistry 1980;23:682-684.
45. Brodie BB, Kurz H, Schanker LS. The importance of dissociation constant and lipid-solubility in influencing the passage of drugs into the cerebrospinal fluid. Journal of Pharmacology and Experimental Therapeutics 1960;130:20-25.
46. Evans DC, O'Connor D, Lake BG, Evers R, Allen C, Hargreaves R. Eletriptan metabolism by human hepatic CYP450 enzymes and transport by human P-glycoprotein. Drug Metabolism and Disposition 2003;31:861-869.
47. Longmore J, Shaw D, Smith D, et al. Differential distribution of $5HT_{1D}$- and $5HT_{1B}$-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. Cephalalgia 1997;17:833-842.
48. MaassenVanDenBrink A, van den Broek RW, de Vries R, Bogers AJ, Avezaat CJ, Saxena PR. Craniovascular selectivity of eletriptan and sumatriptan in human isolated blood vessels. Neurology 2000;55:1524-1530.
49. Asghar MS, Hansen AE, Amin FM, et al. Evidence for a vascular factor in migraine. Annals of Neurology 2011;69:635-645.
50. MaassenVanDenBrink A, Ibrahimi K, Edvinsson L. Intracranial and extracranial arteries in migraine. The Lancet Neurology 2013;12:847-848.
51. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. The Lancet Neurology 2013;12:454-461.
52. Benemei S, Cortese F, Labastida-Ramírez A, et al. Triptans and CGRP blockade – impact on the cranial vasculature. The Journal of Headache and Pain 2017;18:103.
53. Hoon JNJM, Willigers JM, Troost J, Struijker-Boudier HAJ, Bortel LMAB. Vascular effects of 5-$HT_{1B/1D}$-receptor agonists in patients with migraine headaches. Clinical Pharmacology & Therapeutics 2000;68:418-426.
54. Nilsson T, Longmore J, Shaw D, et al. Characterisation of 5-HT receptors in human coronary arteries by molecular and pharmacological techniques. European Journal of Pharmacology 1999;372:49-56.
55. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.

56. MacIntyre PD, Bhargava B, Hogg KJ, Gemmill JD, Hillis WS. Effect of subcutaneous sumatriptan, a selective $5HT_1$ agonist, on the systemic, pulmonary, and coronary circulation. Circulation 1993;87:401-405.
57. Chan KY, de Vries R, Leijten FPJ, et al. Functional characterization of contractions to tegaserod in human isolated proximal and distal coronary arteries. European Journal of Pharmacology 2009;619:61-67.
58. Fox AW. Comparative Tolerability of Oral 5-$HT_{1B/1D}$ Agonists. Headache: The Journal of Head and Face Pain 2000;40:521-527.
59. Ferrari MD, Goadsby PJ, Roon KI, Lipton RB. Triptans (serotonin, 5-$HT_{1B/1D}$ agonists) in migraine: detailed results and methods of a meta-analysis of 53 trials. Cephalalgia 2002;22:633-658.
60. Glennon RA, Dukat M. Serotonin receptors and drugs affecting serotonergic neurotransmission. In: Lemke TL, Williams DA, eds. Foye's principles of medicinal chemistry. USA: Lippincott Williams & Wilkins, 2002: 365-396.
61. Milton KA, Scott NR, Allen MJ, et al. Pharmacokinetics, Pharmacodynamics, and Safety of the 5-$HT_{1B/1D}$ Agonist Eletriptan following Intravenous and Oral Administration. The Journal of Clinical Pharmacology 2002;42:528-539.
62. Pascual J, Muñoz P. Correlation Between Lipophilicity and Triptan Outcomes. Headache: The Journal of Head and Face Pain 2005;45:3-6.
63. Cheng Z, Liu H, Yu N, et al. Hydrophilic anti-migraine triptans are substrates for OATP1A2, a transporter expressed at human blood-brain barrier. Xenobiotica 2012;42:880-890.
64. Rubio-Beltrán E, Labastida-Ramírez A, van den Bogaerdt A, et al. In vitro characterization of agonist binding and functional activity at a panel of serotonin receptor subtypes for lasmiditan, triptans and other 5-HT receptor ligands and activity relationships for contraction of human isolated coronary artery. Cephalalgia 2017;37:363.
65. MaassenVanDenBrink A, Saxena PR. Coronary Vasoconstrictor Potential of Triptans: A Review of In Vitro Pharmacologic Data. Headache: The Journal of Head and Face Pain 2004;44:S13-S19.
66. Dodick D, Lipton RB, Martin V, et al. Consensus statement: cardiovascular safety profile of triptans (5-HT agonists) in the acute treatment of migraine. Headache 2004;44:414-425.
67. Hall GC, Brown MM, Mo J, MacRae KD. Triptans in migraine: The risks of stroke, cardiovascular disease, and death in practice. Neurology 2004;62:563-568.
68. Wammes-van der Heijden EA, Rahimtoola H, Leufkens HGM, Tijssen CC, Egberts ACG. Risk of ischemic complications related to the intensity of triptan and ergotamine use. Neurology 2006;67:1128-1134.
69. Chan KY, Vermeersch S, de Hoon J, Villalón CM, MaassenVanDenBrink A. Potential mechanisms of prospective antimigraine drugs: A focus on vascular (side) effects. Pharmacology & Therapeutics 2011;129:332-351.
70. Amin FM, Hougaard A, Cramer SP, et al. Intact blood–brain barrier during spontaneous attacks of migraine without aura: a 3T DCE-MRI study. European Journal of Neurology 2017;24:1116-1124.
71. Hougaard A, Amin FM, Christensen CE, et al. Increased brainstem perfusion, but no blood-brain barrier disruption, during attacks of migraine with aura. Brain 2017;140:1633-1642.
72. Eftekhari S, Salvatore CA, Johansson S, Chen TB, Zeng Z, Edvinsson L. Localization of CGRP, CGRP receptor, PACAP and glutamate in trigeminal ganglion. Relation to the blood-brain barrier. Brain Research 2015;1600:93-109.
73. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: Studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of Neurology 1993;33:48-56.
74. Ibrahimi K, Danser AHJ, Terwindt GM, van den Meiracker AH, MaassenVanDenBrink A. A human trigeminovascular biomarker for antimigraine drugs: A randomised, double-blind, placebo-controlled, crossover trial with sumatriptan. Cephalalgia 2016;37:94-98.
75. Classey JD, Bartsch T, Goadsby PJ. Distribution of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptor expression in rat trigeminal and dorsal root ganglia neurons: relevance to the selective anti-migraine effect of triptans. Brain Research 2010;1361:76-85.
76. Hou M, Kanje M, Longmore J, Tajti J, Uddman R, Edvinsson L. 5-HT_{1B} and 5-HT_{1D} receptors in the human trigeminal ganglion: co-localization with calcitonin gene-related peptide, substance P and nitric oxide synthase. Brain Research 2001;909:112-120.
77. Buzzi MG, Moskowitz MA. The antimigraine drug, sumatriptan (GR43175), selectively blocks neurogenic plasma extravasation from blood vessels in dura mater. British Journal of Pharmacology 1990;99:202-206.
78. Gómez-Mancilla B, Cutler NR, Leibowitz MT, et al. Safety and Efficacy of PNU-142633, a Selective 5-HT_{1D} Agonist, in Patients with Acute Migraine. Cephalalgia 2001;21:727-732.
79. González-Hernández A, Muñoz-Islas E, Lozano-Cuenca J, et al. Activation of 5-HT_{1B} receptors inhibits the vasodepressor sensory CGRPergic outflow in pithed rats. European Journal of Pharmacology 2010;637:131-137.

80. Muñoz-Islas E, Gupta S, Jiménez-Mena LR, et al. Donitriptan, but not sumatriptan, inhibits capsaicin-induced canine external carotid vasodilatation via 5-HT1B rather than 5-HT_{1D} receptors. British Journal of Pharmacology 2006;149:82-91.
81. Deleu D, Hanssens Y. Current and Emerging Second-Generation Triptans in Acute Migraine Therapy: A Comparative Review. The Journal of Clinical Pharmacology 2000;40:687-700.
82. Muñoz-Islas E, Lozano-Cuenca J, González-Hernández A, et al. Spinal sumatriptan inhibits capsaicin-induced canine external carotid vasodilatation via 5-HT1B rather than 5-HT_{1D} receptors. European Journal of Pharmacology 2009;615:133-138.
83. Castro ME, Pascual J, Romón T, Del Arco C, Del Olmo E, Pazos A. Differential Distribution of [^{3}H]Sumatriptan Binding Sites (5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} Receptors) in Human Brain: Focus on Brainstem and Spinal Cord. Neuropharmacology 1997;36:535-542.
84. Lindhe Ö, Almqvist P, Kågedal M, et al. Autoradiographic Mapping of 5-H $T_{1B/1D}$ Binding Sites in the Rhesus Monkey Brain Using [carbonyl-$^{(11)}$C]zolmitriptan. International Journal of Molecular Imaging 2011;2011:694179.
85. Bartsch T, Knight YE, Goadsby PJ. Activation of 5-$HT_{1B/1D}$ receptor in the periaqueductal gray inhibits nociception. Annals of Neurology 2004;56:371-381.
86. Shields KG, Goadsby PJ. Serotonin receptors modulate trigeminovascular responses in ventroposteromedial nucleus of thalamus: A migraine target? Neurobiology of Disease 2006;23:491-501.
87. Robert C, Bourgeais L, Arreto CD, et al. Paraventricular hypothalamic regulation of trigeminovascular mechanisms involved in headaches. Journal of Neuroscience 2013;33:8827-8840.
88. Vera-Portocarrero LP, Ossipov MH, King T, Porreca F. Reversal of Inflammatory and Non-Inflammatory Visceral Pain by Central or Peripheral Actions of Sumatriptan. Gastroenterology 2008;135:1369-1378.
89. Loher T, Burgunder J, Weber S, Sommerhalder R, Krauss J. Effect of chronic pallidal deep brain stimulation on off period dystonia and sensory symptoms in advanced Parkinson's disease. Journal of Neurology, Neurosurgery, and Psychiatry 2002;73:395-399.
90. Baumeister AA, Anticich TG, Hawkins MF, Liter JC, Thibodeaux HF, Guillory EC. Evidence that the substantia nigra is a component of the endogenous pain suppression system in the rat. Brain Research 1988;447:116-121.
91. Lewis JW, Baldrighi G, Akil H. A possible interface between autonomic function and pain control: opioid analgesia and the nucleus tractus solitarius. Brain Research 1987;424:65-70.
92. Olszewski J. On the anatomical and functional organization of the spinal trigeminal nucleus. Journal of Comparative Neurology 1950;92:401-413.
93. Fields HL, Basbaum AI, Clanton CH, Anderson SD. Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. Brain Research 1977;126:441-453.
94. Foreman RD, Beall JE, Coulter JD, Willis WD. Effects of dorsal column stimulation on primate spinothalamic tract neurons. Journal of Neurophysiology 1976;39:534-546.
95. Reynolds DV. Surgery in the Rat during Electrical Analgesia Induced by Focal Brain Stimulation. Science 1969;164:444-445.
96. Gerhart KD, Yezierski RP, Fang ZR, Willis WD. Inhibition of primate spinothalamic tract neurons by stimulation in ventral posterior lateral (VPLc) thalamic nucleus: possible mechanisms. Journal of Neurophysiology 1983;49:406-423.
97. Condés-Lara M, Rojas-Piloni G, Martínez-Lorenzana G, Rodríguez-Jiménez J, López Hidalgo M, Freund-Mercier MJ. Paraventricular hypothalamic influences on spinal nociceptive processing. Brain Research 2006;1081:126-137.
98. Kayser V, Aubel B, Hamon M, Bourgoin S. The antimigraine 5-$HT_{(1B/1D)}$ receptor agonists, sumatriptan, zolmitriptan and dihydroergotamine, attenuate pain-related behaviour in a rat model of trigeminal neuropathic pain. British Journal of Pharmacology 2002;137:1287-1297.
99. Kayser V, Latremoliere A, Hamon M, Bourgoin S. N-methyl-D-aspartate receptor-mediated modulations of the anti-allodynic effects of 5-$HT_{1B/1D}$ receptor stimulation in a rat model of trigeminal neuropathic pain. European Journal of Pain 2011;15:451-458.
100. Ávila-Rojas SH, Velázquez-Lagunas I, Salinas-Abarca AB, Barragán-Iglesias P, Pineda-Farías JB, Granados-Soto V. Role of spinal 5-HT_{5A}, and 5-$HT_{1A/1B/1D}$, receptors in neuropathic pain induced by spinal nerve ligation in rats. Brain Research 2015;1622:377-385.
101. Vidal-Cantú GC, Jiménez-Hernández M, Rocha-González HI, Villalón CM, Granados-Soto V, Muñoz-Islas E. Role of 5-HT_{5A} and 5-$HT_{1B/1D}$ receptors in the antinociception produced by ergotamine and valerenic acid in the rat formalin test. European Journal of Pharmacology 2016;781:109-116.

102. Granados-Soto V, Argüelles CF, Rocha-González HI, Godínez-Chaparro B, Flores-Murrieta FJ, Villalón CM. The role of peripheral 5-$HT_{1A,}$ 5-$HT_{1B,}$ 5-$HT_{1D,}$ 5-HT_{1E} and 5-HT_{1F} serotonergic receptors in the reduction of nociception in rats. Neuroscience 2010;165:561-568.
103. Cumberbatch MJ, Hill RG, Hargreaves RJ. Differential effects of the $5HT_{1B/1D}$ receptor agonist naratriptan on trigeminal versus spinal nociceptive responses. Cephalalgia 1998;18:659-663.
104. Goadsby PJ, Knight Y. Inhibition of trigeminal neurones after intravenous administration of naratriptan through an action at 5-hydroxy-tryptamine (5-$HT_{1B/1D}$) receptors. British Journal of Pharmacology 1997;122:918-922.
105. Hoskin KL, Bulmer DCE, Lasalandra M, Jonkman A, Goadsby PJ. Fos expression in the midbrain periaqueductal grey after trigeminovascular stimulation. Journal of Anatomy 2001;198:29-35.
106. Keay KA, Bandler R. Vascular head pain selectively activates ventrolateral periaqueductal gray in the cat. Neuroscience Letters 1998;245:58-60.
107. Knight YE, Goadsby PJ. The periaqueductal grey matter modulates trigeminovascular input: a role in migraine? Neuroscience 2001;106:793-800.
108. DeLaTorre S, Rojas-Piloni G, Martínez-Lorenzana G, Rodríguez-Jiménez J, Villanueva L, Condés-Lara M. Paraventricular oxytocinergic hypothalamic prevention or interruption of long-term potentiation in dorsal horn nociceptive neurons: Electrophysiological and behavioral evidence. PAIN 2009;144:320-328.
109. Condés-Lara M, Martínez-Lorenzana G, Rubio-Beltrán E, Rodríguez-Jiménez J, Rojas-Piloni G, González-Hernández A. Hypothalamic paraventricular nucleus stimulation enhances c-Fos expression in spinal and supraspinal structures related to pain modulation. Neuroscience research 2015;98:59-63.
110. Diener H-C, Limmroth V. Advances in pharmacological treatment of migraine. Expert Opinion on Investigational Drugs 2001;10:1831-1845.
111. Visser WH, Burggraaf J, Muller LM, et al. Pharmacokinetic and pharmacodynamic profiles of sumatriptan in migraine patients with headache recurrence or no response. Clin Pharmacol Ther 1996;60:452-460.
112. MaassenVanDenBrink A, Vergouwe MN, Ophoff RA, Saxena PR, Ferrari MD, Frants RR. 5-HT_{1B} Receptor Polymorphism and Clinical Response to Sumatriptan. Headache: The Journal of Head and Face Pain 1998;38:288-291.
113. MaassenVanDenBrink A, Vergouwe MN, Ophoff RA, et al. Chromosomal localization of the 5-HT_{1F} receptor gene: no evidence for involvement in response to sumatriptan in migraine patients. American journal of medical genetics 1998;77:415-420.
114. Mehrotra S, Vanmolkot KR, Frants RR, van den Maagdenberg AM, Ferrari MD, MaassenVanDenBrink A. The phe-124-Cys and A-161T variants of the human 5-HT1B receptor gene are not major determinants of the clinical response to sumatriptan. Headache 2007;47:711-716.
115. Nelson DL, Phebus LA, Johnson KW, et al. Preclinical pharmacological profile of the selective 5-HT_{1F} receptor agonist lasmiditan. Cephalalgia 2010;30:1159-1169.
116. Hoffmann J, Goadsby PJ. Emerging Targets in Migraine. CNS Drugs 2014;28:11-17.
117. Adham N, Kao HT, Schecter LE, et al. Cloning of another human serotonin receptor (5-HT_{1F}): a fifth 5-HT_{1} receptor subtype coupled to the inhibition of adenylate cyclase. Proceedings of the National Academy of Sciences of the United States of America 1993;90:408-412.
118. Bouchelet I, Cohen Z, Case B, Séguéla P, Hamel E. Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. Molecular Pharmacology 1996;50:219-223.
119. Kovalchin J, Ghiglieri A, Zanelli E, Ings R, Mathers T. Lasmiditan Acts Specifically on the 5-HT_{1F} Receptor in the Central Nervous System. Cephalalgia 2016;36:103.
120. Waeber C, Moskowitz MA. [^{3}H]sumatriptan labels both 5-HT_{1D} and 5-HT_{1F} receptor binding sites in the guinea pig brain: an autoradiographic study. Naunyn-Schmiedeberg's Archives of Pharmacology 1995;352:263-275.
121. Lucaites VL, Krushinski JH, Schaus JM, Audia JE, Nelson DL. [^{3}H]LY334370, a novel radioligand for the 5-HT_{1F} receptor. II. Autoradiographic localization in rat, guinea pig, monkey and human brain. Naunyn-Schmiedeberg's Archives of Pharmacology 2005;371:178-184.
122. Amrutkar DV, Ploug KB, Hay-Schmidt A, Porreca F, Olesen J, Jansen-Olesen I. mRNA expression of 5-hydroxytryptamine 1B, 1D, and 1F receptors and their role in controlling the release of calcitonin gene–related peptide in the rat trigeminovascular system. PAIN® 2012;153:830-838.
123. Bruinvels AT, Landwehrmeyer B, Gustafson EL, et al. Localization of 5-HT_{1B}, 5-$HT_{1Dα}$, 5-HT_{1E} and 5-HT_{1F} receptor messenger RNA in rodent and primate brain. Neuropharmacology 1994;33:367-386.
124. Cohen ML, Johnson KW, Schenck KW, Phebus LA. Migraine Therapy. Cephalalgia 1997;17:631-638.
125. Johnson KW, Schaus JM, Durkin MM, et al. 5-HT_{1F} receptor agonists inhibit neurogenic dural inflammation in guinea pigs. NeuroReport 1997;8:2237-2239.

126. Cohen ML, Schenck K. Contractile responses to sumatriptan and ergotamine in the rabbit saphenous vein: effect of selective 5-HT$_{(1F)}$ receptor agonists and PGF$_{(2α)}$. British Journal of Pharmacology 2000;131:562-568.
127. Cohen ML, Schenck K. 5-Hydroxytryptamine$_{(1F)}$ receptors do not participate in vasoconstriction: Lack of vasoconstriction to LY344864, a selective serotonin$_{(1F)}$ receptor agonist in rabbit saphenous vein. Journal of Pharmacology and Experimental Therapeutics 1999;290:935-939.
128. Centurión D, Sánchez-López A, De Vries P, Saxena PR, Villalón CM. The GR127935-sensitive 5-HT$_1$ receptors mediating canine internal carotid vasoconstriction: resemblance to the 5-HT$_{1B}$, but not to the 5-HT$_{1D}$ or 5-ht$_{1F}$, receptor subtype. British Journal of Pharmacology 2001;132:991-998.
129. Rubio-Beltrán E, Haanes K, Labastida A, et al. Lasmiditan and sumatriptan: Comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries. Cephalalgia 2016;36:104-105.
130. Bouchelet I, Case B, Olivier A, Hamel E. No contractile effect for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. British Journal of Pharmacology 2000;129:501-508.
131. Razzaque Z, Heald MA, Pickard JD, et al. Vasoconstriction in human isolated middle meningeal arteries: determining the contribution of 5-HT$_{(1B)}$- and 5-HT$_{(1F)}$-receptor activation. British Journal of Clinical Pharmacology 1999;47:75-82.
132. Phebus LA, Johnson KW, Zgombick JM, et al. Characterization of LY344864 as a pharmacological tool to study 5-HT$_{1F}$ receptors: Binding affinities, brain penetration and activity in the neurogenic dural inflammation model of migraine. Life Sciences 1997;61:2117-2126.
133. May A, Gijsman HJ, Wallno¨fer A, Jones R, Diener HC, Ferrari MD. Endothelin antagonist bosentan blocks neurogenic inflammation, but is not effective in aborting migraine attacks. Pain 1996;67:375-378.
134. Roon KI, Olesen J, Diener HC, et al. No acute antimigraine efficacy of CP-122,288, a highly potent inhibitor of neurogenic inflammation: Results of two randomized, double-blind, placebo-controlled clinical trials. Annals of Neurology 2000;47:238-241.
135. Mitsikostas DD, Sánchez Del Río M, Waeber C. 5-Hydroxytryptamine$_{1B/1D}$ and 5-hydroxytryptamine$_{1F}$ receptors inhibit capsaicin-induced c-fos immunoreactivity within mouse trigeminal nucleus caudalis. Cephalalgia 2002;22:384-394.
136. Shepheard S, Edvinsson L, Cumberbatch M, et al. Possible Antimigraine Mechanisms of Action of the 5HT$_{1F}$ Receptor Agonist LY334370. Cephalalgia 1999;19:851-858.
137. Mitsikostas DD, Sánchez del Río M, Moskowitz MA, Waeber C. Both 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors modulate c-fos expression within rat trigeminal nucleus caudalis. European Journal of Pharmacology 1999;369:271-277.
138. Vila-Pueyo M, Strother L, Page K, et al. Lasmiditan inhibits trigeminovascular nociceptive transmission. Cephalalgia 2016;36:152-153.
139. Goadsby PJ, Classey JD. Evidence for serotonin (5-HT)$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor inhibitory effects on trigeminal neurons with craniovascular input. Neuroscience 2003;122:491-498.
140. Labastida-Ramírez A, Rubio-Beltrán E, Garrelds IM, et al. Lasmiditan inhibits CGRP release in the mouse trigeminovascular system. Cephalalgia 2017; In Press.
141. González-Hernández A, Manrique-Maldonado G, Lozano-Cuenca J, et al. The 5-HT$_{(1)}$ receptors inhibiting the rat vasodepressor sensory CGRPergic outflow: further involvement of 5-HT$_{(1F)}$, but not 5-HT$_{(1A)}$ or 5-HT$_{(1D)}$, subtypes. European Journal of Pharmacology 2011;659:233-243.
142. Lineberry CG, Vierck CJ. Attenuation of pain reactivity by caudate nucleus stimulation in monkeys. Brain Research 1975;98:119-134.
143. Ikeda H, Takasu S, Murase K. Contribution of anterior cingulate cortex and descending pain inhibitory system to analgesic effect of lemon odor in mice. Molecular Pain 2014;10:14-14.
144. Goldstein DJ, Roon KI, Offen WW, et al. Selective serotonin 1F (5-HT$_{1F}$) receptor agonist LY334370 for acute migraine: a randomised controlled trial. The Lancet 2001;358:1230-1234.
145. Ramadan NM, Skljarevski V, Phebus LA, Johnson KW. 5-HT$_{1F}$ Receptor Agonists in Acute Migraine Treatment: A Hypothesis. Cephalalgia 2003;23:776-785.
146. Raffaelli B, Israel H, Neeb L, Reuter U. The safety and efficacy of the 5-HT $_{1F}$ receptor agonist lasmiditan in the acute treatment of migraine. Expert Opinion on Pharmacotherapy 2017:1-7.
147. Pilgrim AJ, Dussault B, Rupniak NM, White J, Mazur D, DiSanto AR. COL-144, an orally bioavailable selective 5-HT$_{1F}$ receptor agonist for acute migraine therapy. Cephalalgia 2009;29:24-25.
148. Liefaard L, Drenth HJ, Pilgrim AJ, et al. Prediction of therapeutically effective dose of COL-144 based on relationship between plasma concentrations and headache response. Cephalalgia 2009;29:24.
149. Ferrari MD, Farkkila M, Reuter U, et al. Acute treatment of migraine with the selective 5-HT$_{1F}$ receptor agonist lasmiditan-a randomised proof-of-concept trial. Cephalalgia 2010;30:1170-1178.

150. Farkkila M, Diener HC, Geraud G, et al. Efficacy and tolerability of lasmiditan, an oral 5-HT_{1F} receptor agonist, for the acute treatment of migraine: a phase 2 randomised, placebo-controlled, parallel-group, dose-ranging study. Lancet Neurology 2012;11:405-413.
151. CoLucid Pharmaceuticals Inc. CoLucid Pharmaceuticals Announces Achievement of Both Primary and Key Secondary Endpoints in the SAMURAI Phase 3 Pivotal Trial of Lasmiditan in Migraine [online]. Available at: https://globenewswire.com/news-release/2016/09/06/869611/0/en/CoLucid-Pharmaceuticals-Announces-Achievement-of-Both-Primary-and-Key-Secondary-Endpoints-in-the-SAMURAI-Phase-3-Pivotal-Trial-of-Lasmiditan-in-Migraine.html. Accessed 14-08-2017.
152. Wietecha LA, Kuca B, Case MG, Selzler KJ, Aurora SK. Phase 3 Study (SPARTAN) of Lasmiditan compared to placebo for acute treatment of migraine. Cephalalgia 2017; In press.

Chapter IV.

Characterization of binding, functional activity and contractile responses of the selective 5 HT_{1F} receptor agonist lasmiditan

Based on: ***E Rubio-Beltrán,*** *A Labastida-Ramírez, KA Haanes, A vd Bogaerdt, AJJC Bogers, E Zanelli, L Meeus, AHJ Danser, MR Gralinski, PB Senese, KW Johnson, J Kovalchin, CM Villalón and A MaassenVanDenBrink (2019) British Journal of Pharmacology; In Press*

Abstract

Background and purpose. Triptans are 5-$HT_{1B/1D}$ receptor agonists (that also display 5-HT_{1F} receptor affinity) with antimigraine action, contraindicated in patients with coronary artery disease due to their vasoconstrictor properties. Conversely, lasmiditan was developed as an antimigraine 5-HT_{1F} receptor agonist. To assess the selectivity and cardiovascular effects of lasmiditan, we investigated the binding, functional activity and in vitro/in vivo vascular effects of lasmiditan, and compared it to sumatriptan.

Experimental approach. Binding and second messenger activity assays of lasmiditan and other serotoninergic agonists were performed for human 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, 5-HT_{1P}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{7} receptors, and the results were correlated with their potency to constrict human isolated coronary arteries (HCA). Furthermore, concentration-response curves to lasmiditan and sumatriptan were performed in proximal and distal HCA, internal mammary and middle meningeal arteries. Finally, anesthetized female Beagle dogs received intravenous infusions of lasmiditan or sumatriptan in escalating cumulative doses, and carotid and coronary artery diameters were measured.

Key results. Lasmiditan showed high selectivity for 5-HT_{1F} receptors. Moreover, the functional potency of the analyzed compounds to inhibit cAMP increase through 5-HT_{1B} receptor activation positively correlated with their potency to contract HCA. In human isolated arteries, sumatriptan, but not lasmiditan, induced contractions. Likewise, in vivo, sumatriptan decreased coronary and carotid artery diameters at clinically relevant doses, while lasmiditan was devoid of vasoconstrictor activity at all doses tested.

Conclusions and implications. Lasmiditan is a selective 5-HT_{1F} receptor agonist devoid of vasoconstrictor activity. This may represent a cardiovascular safety advantage when compared to the triptans.

Introduction

Migraine is a neurologic disease characterized by throbbing unilateral headaches of moderate to severe intensity, accompanied by nausea, vomiting, photophopbia and/or phonophobia (Headache Classification Committee of the International Headache Society, 2018). It has an estimated prevalence of 15% in the global population, with women being three times more affected than men[1,2].

Currently, the specific therapies for acute antimigraine treatment are the triptans, selective 5-$HT_{1B/1D}$ receptor agonists that also display varying levels of 5-HT_{1F} receptor affinity. Unfortunately, not all patients respond to triptans [3] and they are contraindicated in patients with cardiovascular disease, due to their contractile properties via activation of 5-HT_{1B} receptors in coronary arteries[4-7]. Therefore, there is a need for novel antimigraine drugs for the patients that are not respondent to the current available treatments, but also, for those patients with cardiovascular disease.

Based on results from preclinical studies, the 5-HT_{1F} receptor agonist, lasmiditan, was developed for acute antimigraine treatment[8,9] and Phase III trials showed positive results[10]. Considering the increased cardiovascular risk of migraine patients[1,11-13], and the presence of 5-HT_{1F} receptors in the vasculature[14-16], it is important to determine whether lasmiditan lacks affinity for *human* 5-HT_{1B} receptors and whether activation of 5-HT_{1F} receptors will result in vasoconstrictive responses. On this basis, the aim of this study was to investigate the pharmacological properties of lasmiditan, and in particular: (i) to assess the selectivity and functional activity of lasmiditan, triptans and other 5-HT receptor ligands on various *human* 5-HT receptors; (ii) to analyze its potential to induce vasoconstriction in *in vitro* (human isolated proximal and distal coronary, internal mammary and middle meningeal arteries) and *in vivo* (carotid and coronary artery diameters in anesthetized dogs) preclinical models; and (iii) to compare our findings with lasmiditan to those obtained with sumatriptan, one of the most prescribed triptans to treat acute migraine.

We hypothesise that, unlike sumatriptan, lasmiditan selectively activates the *human* 5-HT_{1F} receptor and does not induce vasoconstriction in the above *in vitro* (including human coronary arteries) and *in vivo* vascular models.

Materials and methods

Cell membrane preparation

CHO-K1 cells expressing the human recombinant 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B} or 5-HT_7 receptors, were grown prior to the test in media without antibiotic at Ogeda S.A (Gosselies, Belgium). Cells were prepared using a protocol from Ogeda. In brief, cells were harvested by scraping from the culture vessels in ice-cold Ca^{2+}- and Mg^{2+}-free Phosphate-buffered saline (PBS). The cells were then centrifuged for 10 min at 5,000 x g and 4°C and the pellets were suspended in buffer A (15 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA) and homogenized in a glass-glass homogenizer. The crude membrane fraction was collected by 2 consecutive centrifugation steps at 35,000 x g and 4°C for 30 min separated by a washing step in buffer A. The final membrane pellet was suspended in buffer B (75 mM Tris-HCl pH 7.5; 12.5 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA; 250 mM sucrose) and flash-frozen in liquid nitrogen. Protein content was determined by the BCA method (Interchim, UP40840A).

Radioligand binding competition assay

Competition binding was performed in duplicate in the wells of a 96-well plate containing binding buffer (optimized for each receptor), cells membrane extracts (approximately 20,000 cells distributed in the 96-well plate), radiotracer and test agonist. Nonspecific binding was determined by co-incubation with 200-fold excess of competitor. Cells were incubated and exposed to varying concentrations (1 pM to 10 µM) of a range of displacer agonists (see below). The samples were incubated in a final volume of 0.1 mL and then filtered over Unifilter plates (Perkin Elmer, Massachusetts, United States) pre-treated for 2 hours to limit tracer nonspecific binding. Filters were washed five times with 0.5 mL of ice-cold washing buffer (tris 50 mM pH 7.4) and 50µL of Microscint 20 (Perkin Elmer, Massachusetts, United States) were added to each filter. The plates were incubated 15 min at room temperature on an orbital shaker and the counted with a TopCount™ (Perkin Elmer, Massachusetts, United States) for 1 min per well.

cAMP HTRF assay for G_i coupled receptors

Concentration-response curves were performed in parallel with the agonists. For agonist tests, 12 µl of cells were mixed with 6 µl of the test compound (at increasing concentrations) and 6µl of forskolin, then incubated 30 min at room temperature. After addition of the lysis buffer and 1 h incubation, cAMP concentrations were estimated according to the manufacturer specification with the HTRF kit (Cisbio International, Codelet, France). In brief, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (cAMP-Cryptate and anti-cAMP-d_2 reagents) and diluted in lysis buffer, followed by incubation at room temperature. As 5-HT_{1B} receptors have been reported in naïve CHO cells[18], we also tested 5-carboxamidotryptamine (5-CT, the reference agonist for the 5-HT_{1B} receptor), in CHO cells transfected with a non-5-HT, G protein-coupled receptor.

cAMP HTRF assay for G_s coupled receptors

Concentration-response curves were performed in parallel. For agonist tests, 12 µl of cells were mixed with 12 µl of the test compound at increasing concentrations and then incubated 30 min at room temperature. After addition of the lysis buffer and 60 min incubation, cAMP concentrations

were estimated, according to the manufacturer specification, with the HTRF kit (Cisbio International, Codelet, France). Briefly, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (cAMP-Cryptate and anti-cAMP-d_2 reagents) and diluted in lysis buffer, followed by incubation at room temperature.

IPOne HTRF assay

The assay was performed on adherent cells. For agonist testing, the medium was removed and 20 µl of assay buffer plus 20 µl of the studied agonist or the reference agonist were added in each well. The plate was incubated for 60 min at 37°C with 5% CO_2. IP_1-D2 reagent and anti-IP_1 cryptate reagents were dispensed in the wells and IP_1 concentrations were then measured following the manufacturer instructions (IPOne HTRF assay kit; Cisbio International, Codolet, France). In brief, increasing concentrations of agonists were added to stably transfected cells in buffer in an Optiplate (PerkinElmer Life Sciences, Massachusetts, United States). The plates were incubated, and cells were then lysed by the addition of HTRF reagents (IP_1-D2 reagent and anti-IP_1 cryptate reagents) and diluted in lysis buffer, followed by incubation at room temperature.

GTPγ^{35}[S] assay

For agonist testing, membrane extracts expressing the receptor of interest was mixed with GDP. In parallel, $GTP_\gamma[^{35}S]$ was mixed with the beads just before starting the reaction. The following reagents were successively added in the wells of an Optiplate (Perkin Elmer, Massachusetts, United States): 50 µl of reference ligand, 10 µl of assay buffer, 20 µl of the cells:GDP mix, and 20 µl of the $GTP_\gamma[^{35}S]$:beads mix. The plate was incubated for 60 min, then centrifuged and counted with a PerkinElmer TopCount™ reader.

Agonists tested

Serotonin (5-hydroxytryptamine; 5-HT), 5-CT, ergotamine, sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan, alniditan, lasmiditan, LY334370 and LY344864 were tested. The radioligands and reference compounds used for the radioligand and second messenger studies are specified in Suppl. Tables 1 and 2.

Human isolated arteries collection

Coronary arteries

Coronary arteries were obtained from six "heart beating" organ donors (three males and three females; 48-62 years), who died of non-cardiac disorders less than 24 h before the tissue was taken to the laboratory. The hearts were provided by the Heart Valve Bank Beverwijk Bank (nowadays ETB-BISLIFE Tissue Bank) at that time still located in Rotterdam, from Dutch post-mortem donors, after donor mediation via Bio Implant Services/Eurotransplant Foundation (Leiden, The Netherlands), following removal of the aortic and pulmonary valves for homograft valve transplantation. Immediately after circulatory arrest, the hearts were stored at 4°C in a sterile organ protecting solution and were brought to the laboratory within the first 24 hours of death. After arrival at the laboratory, the right proximal (internal diameter 3–5 mm) and distal (internal diameter 0.5–1 mm) portions of the coronary artery were dissected and placed in a cold, oxygenated (95% O2/5% CO2) Krebs buffer solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 8.3 mM glucose; pH 7.4.

Internal mammary arteries

Internal mammary arteries (internal diameter 2–3 mm) were obtained peri-operatively from eighteen patients (sixteen males and two females; 51–80 years) undergoing coronary bypass

surgery. The tissue was immediately placed in a sterile organ-protecting solution and was brought to the laboratory within 15 min. Subsequently, the artery was cleaned of connective tissue and placed in a cold, oxygenated Krebs buffer solution (for composition, see above).

Middle meningeal arteries

Middle meningeal arteries (internal diameter 0.5–1.5 mm) were obtained from the dura mater of six patients (two males and four females; 12-68 years) who underwent neurosurgery. The dura mater, together with a small piece of the meningeal artery, was collected in a sterile organ-protecting solution and immediately transported to the laboratory. The dura mater and connective tissue were dissected and the artery was placed in a cold, oxygenated Krebs solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_2$ and 11.1 mM glucose; pH 7.4.

All arteries were used on the same day or stored overnight and used the following day for functional experiments. The studies on coronary arteries were approved by the Scientific Advisory Board of the Rotterdam Heart Valve Bank. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study protocols with regard to mammary arteries and middle meningeal arteries.

Isometric tension measurements

Proximal coronary arteries were cut into segments of 2–4 mm length, excluding distinct, macroscopically visible atherosclerotic lesions. The segments were mounted on stainless steel hooks in 15 mL organ baths filled with oxygenated Krebs buffer solution at 37°C. After equilibration for at least 30 min and a wash every 15 min, the vessel segments were stretched to a stable tension of about 15 mN, with the optimal pretension as determined earlier[5]. Changes in tissue tension were measured with an isometric force transducer (Harvard, South Natick, MA, U.S.A.) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The distal coronary, internal mammary and middle meningeal arteries were cut into circular 1-2 mm long segments and mounted in Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) between two parallel small stainless-steel wires (40 µm). All the baths were filled with warm Krebs buffer and aerated with carbogen. The tension was normalized to 90% of I_{100} for all segments, the diameter when transmural pressure equals 100 mm Hg[19]. Data were recorded using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK).

Experimental protocols

A paired parallel set up (*i.e.* all compounds were tested in different segments obtained from the same artery) was used. Initially, all segments were exposed to 30 mM KCl to 'prime' the tissue for stable contractions. After washout, the tissue was exposed to 100 mM KCl to determine the maximal contractile response. After further washout, a concentration-response curve to vehicle, sumatriptan or lasmiditan was constructed, using whole logarithmic steps from 1 nM up to 10 µM. After finishing the curve and washing several times until reaching equilibrium, the functional integrity of the endothelium was verified by observing relaxation to substance P (10 nM; coronary and meningeal arteries) or bradykinin (1 µM; mammary arteries), after precontraction with thromboxane A_2 analogue U46619 (10-100 nM)[4,5].

Furthermore, in the internal mammary arteries, a concentration-response curve to lasmiditan and sumatriptan was also constructed after adding threshold concentrations of U46619 (i.e. concentrations eliciting a contraction of ~10% of 100 mM KCl response, determined in quarter logarithmic steps), used to unmask contractile properties of some agonists in the presence of an increased tension, as previously described[20]. These contractile responses were evaluated post-hoc

in the absence (relaxation to bradykinin <18%) or presence (relaxation to bradykinin >18%) of functional endothelium; for this, endothelial function data was divided in percentiles, where values below the 50th percentile were considered without endothelium, and above the 50th percentile were considered with endothelium. Also, segments were preincubated with clinically relevant concentrations of sumatriptan (0.3 μM) or lasmiditan (1 μM), and followed by a concentration-response curve to lasmiditan or sumatriptan, respectively, to evaluate the possible interactions (i.e. augmented vasoconstriction) between agonists. The clinically relevant concentration of sumatriptan was calculated as previously described[5]; in the case of lasmiditan, it was estimated based on the C_{max} observed in humans following a 100 mg dose (0.25 μM)[21].

Correlation between binding (pK$_i$) and the contractile potency of lasmiditan and other triptans

We related previous[6,22-24] and current data obtained (see Results) to the potency of these compounds to contract the human isolated coronary artery. In case a compound failed to contract human coronary artery, a fixed pEC_{50} value of 5 was set. Our pK_i values used for this correlation are in agreement with those previously published in the literature (Suppl. Table 4, Suppl. Fig. 1).

Animal preparations

Although *in vitro* experiments with human isolated arteries provide invaluable information on vasoconstrictive responses in specific vascular beds, to discard hemodynamic changes after systemic administration of novel experimental therapeutic compounds an in vivo model is necessary. The beagle dog is a well-accepted species that has been in use for several years to predict human cardiovascular responses to novel experimental therapeutic compounds[25]. Therefore, a total of eighteen adult female Beagle dogs (*Canis familiaris*) were selected from the CorDynamics, Inc. animal colony. These animals were obtained from Marshall BioResources (North Rose, N.Y., U.S.A.). Upon receipt at the Biologic Resources Laboratory (BRL) of the University of Illinois-Chicago, dogs were examined by a BRL veterinary personnel to ensure acceptable health status. Veterinary care was provided by the veterinarians and staff employed by the BRL. Dogs were acclimatized for at least 7 days prior to use, and were pair-housed in runs (meeting the size requirement set forth by the USDA Animal Welfare Act) with various cage-enrichment devices. Room temperature set at 18-27°C, humidity at 30–70%, and fluorescent lights timed to give a 12 hour-light and 12 hour-dark cycle. Harlan Certified Canine food (25% Protein Diet #2025C, Harlan Teklad, Madison, WI) was fed daily (500 grams per day) and water was freely available in their runs. At the day of their terminal experiment, the animals were 10.0 to 11.5 months old, and their body weights ranged from 5.4 to 7.9 kg. Body weights were measured twice (approximately one week between measurements) prior to each animal's terminal procedure. Dogs were fasted for 16-18 hours prior to dosing.

All experimental protocols were approved and conducted by CorDynamics in compliance with the US FDA Good Laboratory Practice guidelines (21 CFR Part 58), the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, the Office of Laboratory Animal Welfare, ARRIVE guidelines for reporting experiments involving animals[26]; and the current guidance on experimental design and analysis for the British Journal of Pharmacology[27]. For more specific details on design and statistical analysis, see below the sections entitled: sample size calculation, randomization and blinding; and data presentation and statistical evaluation.

General methods

Dogs were dosed with morphine subcutaneously (s.c., 1 mg·kg^{-1}) approximately 10-20 min prior to administration of propofol anaesthesia i.v. (5-6 mg·kg^{-1}) to allow tracheal intubation. They were placed on a ventilator with isoflurane delivered at 1-2% in oxygen to maintain anaesthesia

throughout the experiment and s.c. morphine (0.5 mg·kg^{-1}) was administered approximately every two hours while under anaesthesia. The local anaesthetic bupivacaine was infiltrated into the incision sites. A continuous 0.9% NaCl solution for injection drip (approximately 10 mL·kg^{-1}·hour^{-1}) was maintained until the start of dosing at which time it was discontinued. Dogs were placed on a heating pad set to maintain the animal's body temperature at approximately 37ºC and their body temperature was monitored throughout the experiment by placing a rectal temperature probe. Additionally, surface ECG leads were placed for anaesthesia monitoring throughout the experimental protocol.

A mid-lateral neck incision was made and the left common carotid artery was exposed. A Transonic Systems Inc. (Ithaca, N.Y., U.S.A.) blood flow probe and two Sonometric Corporation (London, Ontario, Canada) crystals for arterial dimensional analysis were affixed to the artery. A left lateral thoracotomy (6th intercostal space) was performed and the left circumflex coronary (LCX) artery was exposed. A Transonic Systems Inc. blood flow probe and two Sonometric Corporation crystals for arterial dimensional analysis were affixed to the artery. A solid-state high-fidelity pressure catheter (Millar Inc., Houston, TX, U.S.A.) for measurement of arterial pressure (mean, MAP; systolic, SAP; and diastolic, DAP) and heart rate was inserted into a femoral artery and secured in place with silk suture. An indwelling catheter was placed into the femoral vein for collecting blood (2 mL) prior to the start of dosing and at the end of each 20 min infusion period (see experimental protocol) for bioanalytical analysis.

Experimental protocol

Upon completion of the general instrumentation, a 15 min equilibrium period was allowed for a stable hemodynamic condition. Baseline values (defined as the average of the three 5 min values at the aforementioned 15 min) of MAP, heart rate, and carotid and left circumflex coronary artery diameter and flow were determined. Subsequently, the 18 dogs were randomly assigned into three groups (n=6 each), which received vehicle (saline), lasmiditan (0.03, 0.13, 1.13, 4.13 and 11.13 mg·kg^{-1}) or sumatriptan (0.03-11.3 mg·kg^{-1}), respectively. All treatments were filtered through a 0.2 µM membrane and administered i.v. in the escalating cumulative doses mentioned above. Dose-intervals amongst different treatments were administered each 20 min. At the end of the experiment, dogs were euthanized while under anaesthesia via a barbiturate overdose.

Sample size calculation, randomization and blinding

Sample size calculation. The animal sample size (n=6 each group) was calculated by CorDynamics based on their previous studies[28]. For the *in vitro* studies, we based the experimental number (n=5-7 each group) on previous studies from our group[5,29].

Randomization. For the *in vitro* experiments, all artery segments were cut into rings and randomly assigned to a bath, then the treatment group was designed by using a table of random numbers. For the *in vivo* experiments, the animals initially divided in sets (n=6 each group as described above), were randomly assigned to study groups by CorDynamics staff.

Blinding. For the radioligand and second messenger assays, the analyst was not blinded to the compounds but to the research hypothesis. For the vascular *in vitro* experiments, values were calculated using the dose-response auto-analyze selection feature of LabChart. During the analysis, the investigator was unaware of which concentration response curve was being analyzed. The *in vivo* experimental values (*i.e.* the changes in MAP or artery diameter) in each group of animals were simultaneously obtained by at least two different CorDynamics investigators, with at least one of the investigators blinded.

Data presentation and statistical analysis

All data in the text and Fig.s are presented as the mean±SEM from (n) experiments, as shown in the Fig. legends. Data and statistical analysis comply with the recommendations on analysis and experimental design in pharmacology[27].

Radioligand binding and second messenger activity

Reference compounds were tested at several concentrations in duplicate to obtain a concentration-response curve, and an estimated pEC_{50} (negative logarithm of the concentration eliciting 50% of the maximal contractile response, *i.e.* E_{max}) or pIC_{50} value (negative logarithm of the concentration that displaced 50% of the radioligand) was calculated using XLFit (IDBS, Guildford, United Kingdom). Additionally, the reference values obtained were compared to historical values obtained from the same receptor and used to validate the experimental session. A session was considered as valid only if the reference value was found to be within a 0.5 log interval from the historical value, for assays where historical values (determined in at least 5 experiments) were available[30-32]. For the new assays developed in this study (*i.e.* 5-ht_{1E} receptor), the two independent pIC_{50} determined must be concordant with a 1 log unit interval for the assays to be validated. When less than 50% inhibition of binding or second messenger activation was obtained at 10 µM a pIC_{50}/pEC_{50} of "<5" was set.

Human isolated arteries experiments

For the human vessels *in vitro* studies, concentration-response curves were analyzed using GraphPad software (GraphPad Software Inc., San Diego, CA, U.S.A.) to determine pEC_{50} values as previously reported[5]. When a plateau in the concentration-response curve was not reached, the response observed with the highest concentration used (*i.e.* 100 µM) was considered as E_{max}. Differences between pEC_{50} and E_{max} values of the compounds were evaluated with Tukey's test, once an analysis of variance (ANOVA) for paired data had revealed that the samples represented different populations. Values of $P<0.05$ were considered to indicate significant differences.

In vivo *studies*

In the *in vivo* studies, each hemodynamic parameter was analyzed with a repeated measure analysis of covariance (RANCOVA) for changes from baseline at time intervals of 5, 10, 15, and 20 min for each of the 5 dose levels. The model factored the treatment (TRT), the time after dose (TIME), and the interaction of time after dose with treatment (TRT*TIME). The SAS® procedure PROC MIXED was used for analysis with TIME as the repeated effect and ANIMAL as the subject. The covariance between errors from the same animal at different time points was selected based on the corrected Akaike's Information Criterion from selected covariance structures of VC, AR(1), UN, and CS. Non-monotonic dose-responses were evaluated. Within the framework of the RANCOVA, comparisons were made for vehicle *vs*. lasmiditan-treated animals and for vehicle *vs*. sumatriptan-treated animals. If TRT*TIME was significant, the comparisons were conducted for each time interval using an analysis of covariance (ANCOVA) model with an effect for treatment and baseline as a covariate. If only the TRT effect was significant, the comparison was conducted across the pooled time intervals for the overall phase only. These non-monotonic treatment group comparisons were conducted at the $p=0.01$ significance level. Baseline data was analyzed with an ANOVA for each time interval. Factors in the model included treatment (TRT). All statistical analyses were conducted with SAS® version 9.2. After the database lock, post-hoc analyses for coronary artery diameter and carotid artery diameter (primary endpoints) at the clinically relevant time interval 20 min (completion of dose administration) for each cumulated dose (0.03-11.13 $mg{\cdot}kg^{-1}$) were performed for comparisons between sumatriptan and vehicle. A significance level of $p\leq0.025$ was used for the RANCOVA using Bonferroni correction of two tests.

Compounds

The compounds used in the present study (obtained from the sources indicated) were: 5-HT hydrochloride, naratriptan hydrochloride, almotriptan malate, avitriptan fumarate and sumatriptan succinate (Sigma Chemical Co., St. Louis, MO, U.S.A.); lasmiditan hemisuccinate (Eli Lilly & Co., Indianapolis, IN, U.S.A.); 5-CT maleate, sumatriptan succinate, zolmitriptan, rizatriptan benzoate, eletriptan hydrobromide, donitriptan hydrochloride, LY334370 hydrochloride and LY344864 hydrochloride (Tocris Bioscience Co., Park Ellisville, MO, U.S.A.); ergotamine tartrate (TEVA Pharmaceutical Industries Ltd., Petach Tivka, Israel) and alniditan salt (kind gift of Janssen Pharmaceutica, Beerse, Belgium).

All compounds were dissolved in distilled water or physiological saline for the *in vitro* and *in vivo* studies, respectively. These vehicles had no effect on the baseline MAP values or artery diameter (not shown). Fresh solutions were prepared for each experiment. The doses mentioned in this text refer to the free base of substances.

Results

Pharmacological characterization of lasmiditan

As shown in Table 1, radioligand studies revealed that lasmiditan selectively binds to the human 5-HT_{1F} receptor. On the other hand, the triptans almotriptan, avitriptan, eletriptan, frovatriptan, naratriptan, sumatriptan and zolmitriptan showed affinity for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors; while alniditan, donitriptan, ergotamine and rizatriptan had affinity for 5-HT_{1B} and 5-HT_{1D} receptors. Most importantly, when analyzing their second messenger activity, we observed that ergotamine is an agonist of the 5-$HT_{1A/B/D}$, 5-$HT_{2A/B}$ and 5-HT_{7} receptors (but not 5-HT_{1F} receptor). Similar as above, sumatriptan, zolmitriptan, naratriptan, almotriptan, eletriptan, frovatriptan and avitriptan are agonists of the 5-$HT_{1B/1D/1F}$ receptors. Lasmiditan, as well as LY344864, displayed a high potency only for the 5-HT_{1F} receptor (Table 2). In Fig.1, the agonistic profile of the different antimigraine drugs tested on the 5-$HT_{1B/D/F}$ receptors (*i.e.* relevant for migraine therapy), are represented. When comparing our results with those previously published, our values are in agreement with those in the literature (see Suppl. Tables 4-5, Suppl. Fig. 1). Moreover, no functional responses to 5-CT were observed in the CHO cells transfected with an unrelated G protein-coupled receptor (Suppl. Fig. 2).

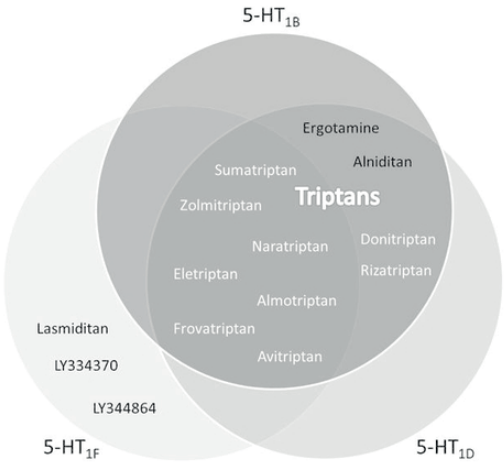


Fig. 1. Summary of the agonist profiles (pEC_{50} >7) of the antimigraine drugs tested on the 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors. Redrawn from[37].

Table 1. Summary of pIC_{50} (negative logarithm of the molar concentration of these compounds at which 50% of the radioligand is displaced) and pK_i (negative logarithm of the molar concentration of the dissociation constant) values of individual antimigraine drugs at 5-HT receptors. The lesser than symbol (<) indicates that less than 50% inhibition of binding was obtained at 10 μM. The radioligands used and their concentrations are described in Suppl. Table 1.

Agonist	5-HT_{1A}		5-HT_{1B}		5-HT_{1D}		5-ht_{1E}		5-HT_{1F}		5-HT_{2A}		5-HT_{2B}		5-HT_{7}	
	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i	pIC_{50}	pK_i
Ergotamine tartrate	9.19	9.70	8.87	9.34	8.63	9.31	6.08	6.39	6.71	7.13	7.62	8.14	7.73	7.94	7.13	7.23
Sumatriptan succinate	6.63	7.14	7.81	8.29	8.31	9.00	5.42	5.72	7.13	7.55	<5	<5	<5	<5	6.10	6.19
Zolmitriptan	7.28	7.79	8.85	9.33	9.28	9.97	7.51	7.81	7.13	7.55	<5	<5	<5	<5	6.97	7.06
Naratriptan hydrochloride	7.31	7.82	8.75	9.22	8.62	9.30	7.83	8.13	8.33	8.75	<5	<5	<5	5.08	5.84	5.93
Rizatriptan benzoate	6.81	7.32	7.51	7.99	8.15	8.83	6.48	6.78	6.4	6.82	<5	<5	5.30	5.51	<5	<5
Almotriptan malate	6.23	6.73	7.97	8.45	7.57	8.26	<5	<5	7.15	7.57	<5	<5	<5	<5	6.36	6.46
Eletriptan hydrobromide	8.20	8.71	8.80	9.28	9.31	9.99	6.91	7.21	7.35	7.77	5.42	5.94	6.14	6.35	6.61	6.70
Frovatriptan racemate	6.83	7.34	8.09	8.57	8.10	8.78	<5	5.18	6.50	6.92	<5	<5	<5	<5	6.88	6.97
Donitriptan hydrochloride	7.42	7.93	9.29	9.77	9.18	9.86	5.47	5.77	<5	5.18	5.83	6.35	5.88	6.09	6.12	6.21
Avitriptan fumarate	7.20	7.71	8.32	8.80	8.42	9.11	5.15	5.45	6.69	7.11	5.11	5.63	5.73	5.94	6.03	6.12
Alniditan dihydrochloride	8.81	9.32	8.93	9.41	8.66	9.35	5.98	6.28	6.02	6.44	<5	5.43	6.67	6.88	7.16	7.26
Lasmiditan hemisuccinate	5.88	6.39	5.54	6.02	5.62	6.31	5.54	5.84	8.09	8.51	<5	<5	5.01	5.22	<5	<5
LY334370 hydrochloride	7.98	8.49	6.74	7.21	6.24	6.92	6.83	7.13	9.03	9.45	5.11	5.63	5.98	6.19	5.66	5.75
LY344864 hydrochloride	6.12	6.63	6.13	6.61	5.83	6.52	6.05	6.35	8.38	8.80	5.11	5.63	5.31	5.52	5.69	5.78

Table 2. Summary of pEC_{50} values of cAMP (5-$HT_{1A/1B/1E/1F}$ and 5-HT_7), GTPγS (5-$HT_{1A/1B/1D/1E/1F}$) and IP (5-HT_2) assays of individual antimigraine drugs at 5-HT receptors. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response is exerted. The lesser than symbol (<) indicates that less than 50% response was obtained at 10 μM. The reference compounds used and their concentrations are described in Suppl. Table 2.

Agonist	5-HT_{1A}		5-HT_{1B}		5-HT_{1D}	5-ht_{1E}		5-HT_{1F}		5-HT_{2A}	5-HT_{2B}	5-HT_{7}
	cAMP	GTPγS	cAMP	GTPγS	GTPγS	cAMP	GTPγS	cAMP	GTPγS	IP	IP	cAMP
Ergotamine tartrate	9.78	9.63	9.94	9.52	9.43	5.95	5.74	5.97	6.30	9.25	8.72	7.09
Sumatriptan succinate	<5	<5	7.32	7.91	8.30	5.99	5.79	8.03	6.80	<5	<5	5.22
Zolmitriptan	<5	5.52	7.87	8.42	9.51	8.18	7.81	8.00	6.67	<5	<5	6.28
Naratriptan hydrochloride	<5	6.52	8.05	8.86	8.80	7.75	8.17	8.38	8.05	<5	<5	<5
Rizatriptan benzoate	<5	<5	7.08	7.56	8.11	7.34	6.90	6.54	5.91	<5	5.49	<5
Almotriptan malate	<5	5.48	7.08	7.85	7.75	<5	<5	7.79	6.90	<5	5.20	<5
Eletriptan hydrobromide	5.74	6.38	8.00	8.09	9.04	7.53	6.90	8.13	6.88	6.07	6.81	6.45
Frovatriptan racemate	<5	6.12	7.98	8.14	8.36	5.04	<5	7.10	6.35	<5	<5	7.42
Donitriptan hydrochloride	5.94	6.74	9.96	9.52	9.51	<5	<5	<5	<5	8.10	7.61	5.23
Avitriptan fumarate	<5	6.19	8.57	8.68	9.27	5.52	<5	7.09	6.05	6.91	6.41	5.38
Alniditan dihydrochloride	7.00	7.29	8.87	8.90	8.20	5.68	5.21	5.92	5.17	<5	7.15	6.32
Lasmiditan hemisuccinate	<5	<5	<5	<5	6.64	6.17	5.34	8.43	7.80	<5	<5	<5
LY334370 hydrochloride	5.84	6.96	6.52	5.80	6.92	7.53	6.95	9.08	9.38	<5	<5	<5
LY344864 hydrochloride	<5	<5	<5	5.82	6.93	6.22	6.12	8.72	7.85	<5	<5	<5

Human isolated arteries

In the human isolated coronary arteries, sumatriptan induced significant contractions in a concentration dependent manner in the proximal (E_{max} 39±12%, pEC_{50} 6.4±0.2; n=6) and distal (E_{max} 59±41%, pEC_{50} 6.02±0.2; n=6) coronary portions, even at clinically relevant concentrations (Fig. 2). In contrast, lasmiditan was devoid of any significant contractile effects in both coronary portions, even at its highest concentration. Moreover, Fig. 3 shows the effects of lasmiditan and sumatriptan on internal mammary arteries in the absence and presence of endothelium. Sumatriptan induced concentration-dependent contractions in segments with (E_{max} 46±18%, pEC_{50} 6.07±0.07; n=5) and without functional endothelium (E_{max} 31±12%, pEC_{50} 5.6±0.94; n=7). After precontraction with threshold concentrations of U46619, sumatriptan also produced concentration-dependent contractions in the presence (E_{max} 63±19%, pEC_{50} 6.83±0.05, n=5) and absence (E_{max} 59±16%, pEC_{50} 6.02±0.59; n=7) of functional endothelium. In contrast, lasmiditan was devoid of any significant contractile effects in internal mammary arteries, even after a modest precontraction with U46619.

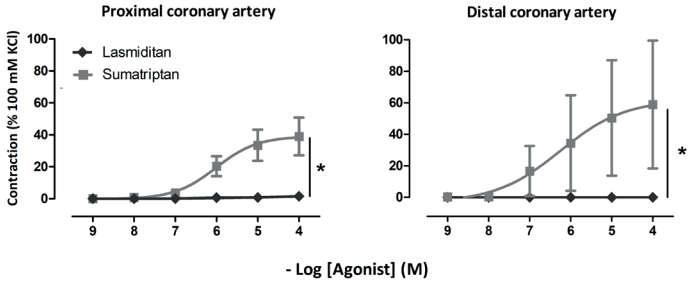


Fig. 2. Contractile responses to lasmiditan and sumatriptan (1 nM – 100 μM) in the human isolated proximal (left) and distal (right) coronary arteries; *P<0.05; n=6 each.

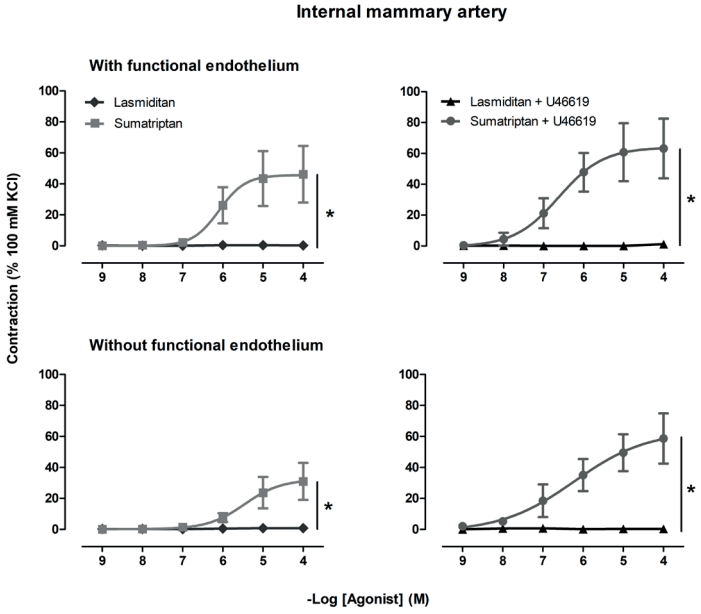


Fig. 3. Contractile responses to sumatriptan and lasmiditan (1 nM–100 μM) in the absence (left) and presence (right) of a threshold precontraction with U46619 (1–10 nM) in human isolated internal mammary arteries with (upper panel, n=5) and without (lower panel, n=7) functional endothelium; *P<0.05.

In middle meningeal arteries, sumatriptan induced significant concentration-dependent contractions (E_{max} 73±13%, pEC_{50} 6.32±0.15; n=6), whereas lasmiditan did not induce any significant contraction at all concentrations tested (E_{max} 0±0%, Fig. 4).

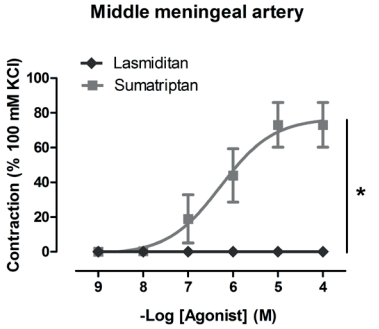


Fig. 4. Contractile responses to sumatriptan and lasmiditan (1 nM – 100 μM) in the human isolated middle meningeal arteries; *P<0.05; n=6 each.

Interaction experiments

As shown in Fig. 5, in human isolated internal mammary arteries, after preincubation with lasmiditan (1 μM), no changes in the contractile responses to sumatriptan were observed when compared to the concentration-response curve to sumatriptan alone (E_{max} 59±16%, pEC_{50} 5.34±0.1 *vs*. E_{max} 51±19%, pEC_{50} 5.71±0.7; n=5 each). In addition, the highest concentration of lasmiditan produced a non-significant vasodilation when preincubated with sumatriptan's clinically relevant concentration (0.3 μM) when compared to the concentration-response curve to lasmiditan without sumatriptan (E_{max} -4.8±5.95% *vs*. E_{max} 0±0% respectively; n=5 each).

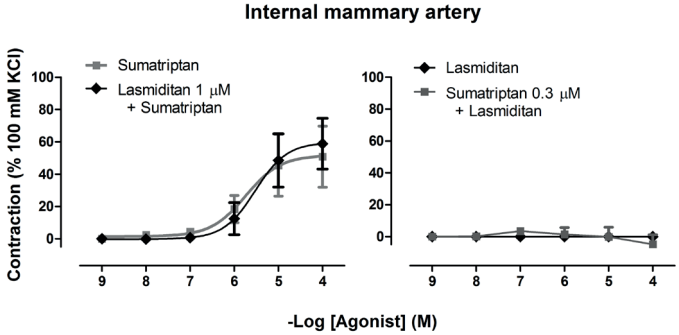


Fig. 5. Contractile responses to sumatriptan and lasmiditan (1 nM – 100 μM) in the internal mammary artery, after preincubation with the clinically relevant concentration of sumatriptan (0.3 μM) or lasmiditan (1 μM), and followed by a concentration-response curve to lasmiditan or sumatriptan, respectively (n=6 each).

Correlation between binding (pK_i) and the contractile potency of lasmiditan and other triptans

As shown in Fig. 6, the potency of the compounds tested to contract the human isolated coronary artery was positively correlated with their potency to bind the 5-HT_{1B} receptor, whereas it was negatively correlated for the 5-HT_{1F} receptor. This was also observed when correlating the pEC_{50} values obtained in our second messenger assays and the contractile potency of the compounds tested in the human coronary arteries (Suppl. Fig. 3).

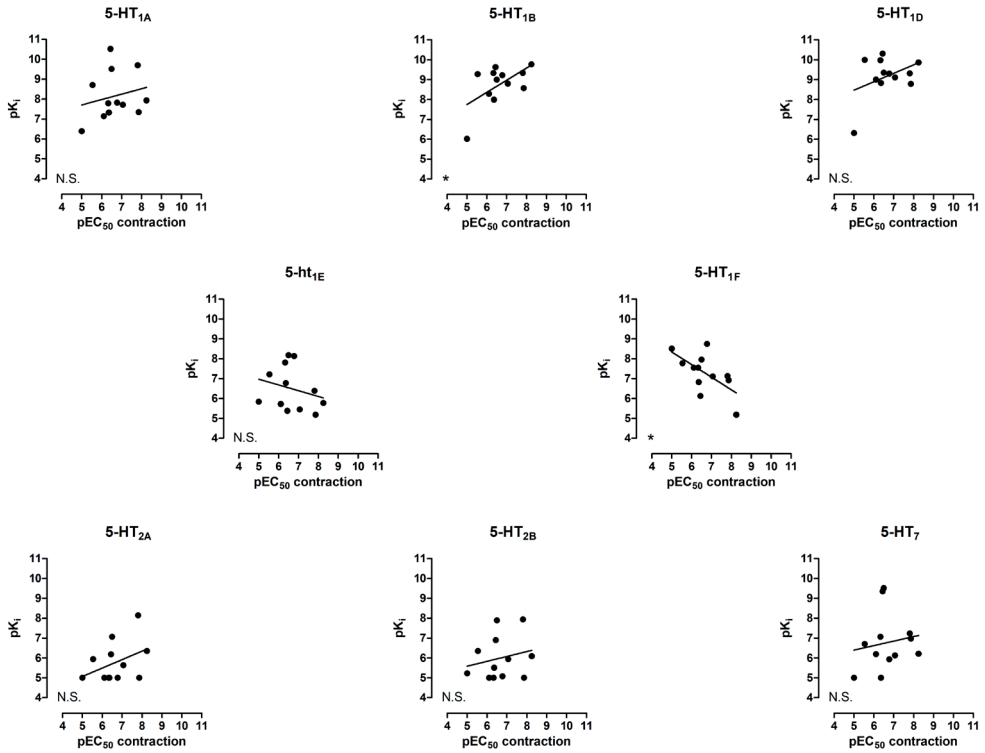


Fig. 6. Correlation between the pK_i values obtained in our study and the contractile potency of lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, alniditan, 5HT, 5-carboxamidotryptamine) in human isolated coronary arteries; N.S., non-significant; $^{*}P<0.05$.

In vivo *studies*

In anaesthetized dogs, a directly proportional relationship was observed between lasmiditan and sumatriptan cumulative i.v. doses and their plasma concentrations; these latter values were used for validating the concentrations used in the *in vitro* studies (data not shown). Moreover, as shown in Fig. 7, changes in carotid artery diameter were not statistically significant in the lasmiditan-treated group as compared to the time-matched vehicle control group. In contrast, as expected, sumatriptan induced dose-dependent decreases in carotid artery diameter, although these effects were statistically significant only at the doses of 0.13 mg·kg^{-1} (clinically relevant) and 11.13 mg·kg^{-1} (Fig. 7). In the LCX coronary artery, lasmiditan failed to induce any statistically significant change in diameter at any dose. Conversely, statistically significant decreases in the LCX coronary artery diameter were observed at all doses in sumatriptan-treated animals as compared to the time-matched vehicle control animals, even at the lowest dose of 0.03 mg·kg^{-1}, which already corresponds to a clinically relevant dose.

Carotid blood flow was not significantly different after vehicle or clinically relevant doses of lasmiditan (0.03-1.13 mg·kg^{-1}). Lasmiditan decreased carotid blood flow significantly, but only after the supratherapeutic cumulative doses of 4.13 mg·kg^{-1} and 11.13 mg·kg^{-1}. In contrast, sumatriptan elicited a statistically significant rapid, dose-dependent, decrease in carotid blood flow at all doses tested. Regarding coronary blood flow, the administration of vehicle, lasmiditan or sumatriptan did

not elicit any statistically significant changes (data not shown). Heart rate was stable over the course of the study and no significant changes were observed in the lasmiditan or vehicle groups. In the sumatriptan–treated group, cumulative doses of 4.13 and 11.13 mg·kg^{-1} elicited dose-dependent decreases in heart rate which were statistically significant, with a peak decrease at 90 min of 16.5±6 bpm (data not shown). MAP, SAP and DAP showed no significant changes in either sumatriptan or lasmiditan-treated groups as compared to the time-matched vehicle group at cumulative doses of up to 4.13 mg·kg^{-1}. At higher doses, both lasmiditan and sumatriptan-treated groups showed a dose-dependent trend to decrease MAP, SAP and DAP; however, these changes were not statistically significant (Fig. 7).

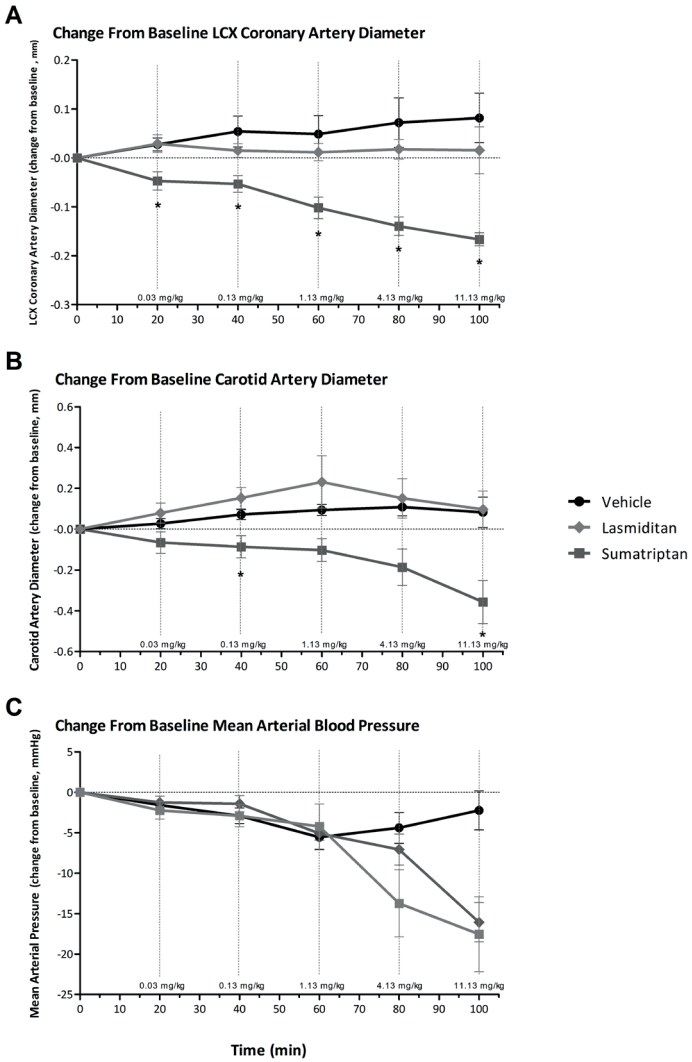


Fig. 7. Changes in the left circumflex (LCX) coronary artery diameter (A), carotid artery diameter (B) and mean arterial blood pressure (C) after the continuous infusion of lasmiditan and sumatriptan (0.03-11.13 mg·kg^{-1} each) or the corresponding infusion volumes of vehicle in female beagle dogs (n=6 each). *P<0.025 vehicle *vs.* sumatriptan post hoc analysis.

Discussion and conclusions

The current study was designed to investigate the selectivity and vasoconstrictor profile of lasmiditan, which belongs to a novel class of acute antimigraine drugs, the ditans. According to its binding and functional activity, it was confirmed that lasmiditan is a highly selective agonist of the 5-HT_{1F} receptor. Moreover, since lasmiditan was developed based on the premise that coronary vasoconstriction is a side effect of the triptans attributed to 5-HT_{1B} receptors, we studied the vasoconstrictor potential of 5-HT_{1F} agonism in two different vascular models and compared our *in vitro* and *in vivo* results to those obtained with sumatriptan, since it is the 'gold standard' triptan for acute antimigraine treatment. This allowed us to compare the results from the current study with results obtained earlier. In accordance with our previous work[4], sumatriptan induced a concentration-dependent contraction in human isolated coronary arteries, which tended to be larger in distal than in proximal coronary artery segments. This contraction was apparent at clinically relevant concentrations, and is most likely due to activation of 5-HT_{1B} receptors in vascular smooth muscle[4]. In contrast, lasmiditan did not induce a contraction at concentrations up to 100 μM (≥ 100x the clinically relevant concentrations) in either proximal or distal coronary arteries. Although moderate to intense expression of mRNA encoding the 5-HT_{1F} receptor in human coronary arteries has been described[16], presence of mRNA does not necessarily mean protein expression, which may well be the case. Thus, the physiological role of this receptor in blood vessels remains to be determined.

Subsequently, we performed more in-depth experiments in the internal mammary artery, where we studied the influence of endothelial functional quality, and the effects of a precontraction induced by the thromboxane A_2 analogue U46619, since such a precontraction is known to 'unmask' or augment contractions to other ligands, such as sumatriptan[20]. As in the coronary artery, sumatriptan contracted the internal mammary artery, similarly in both segments with and without functionally active endothelium. In accordance with earlier observations[20], the contractions to sumatriptan were augmented in the presence of U46619. In contrast, lasmiditan did not induce any contraction in the absence or presence of U46619 in either vessel segments with or without endothelium. Interestingly, in the rabbit saphenous vein, precontraction with $PGF_{2\alpha}$ unmasked a contractile response to the 5-HT_{1F} receptor agonists, LY334370 and LY344864, but only after high concentrations (>10 μM), and therefore likely due to activation of vascular 5-HT_{1B} receptors[33]. Hence, the absence of contractile responses with high concentrations of lasmiditan, even in precontracted vessels, is surprising, given the difference in affinity between sumatriptan and lasmiditan to the 5-HT_{1B} receptor. However, binding affinity does not always correlate with second messenger activation and biological response[34]. Therefore, while our radioligand studies (Table 1) showed a ~100-fold binding difference to the 5-HT_{1B} receptor between sumatriptan (pIC_{50}=7.81) and lasmiditan (pIC_{50}=5.54), our cAMP assays (Table 2) showed that the functional potency (pEC_{50}) of sumatriptan was 7.32 and lasmiditan was <5. As we could not determine the precise pEC_{50} value of lasmiditan, the potency difference between both compounds could be larger than 1000 fold and thus, explain the complete absence of vasoconstrictive responses even at supra-therapeutic concentrations such as 100 μM. This could represent a cardiovascular safety advantage over its triptan predecessors.

Additionally, as contraction of the meningeal artery is thought to contribute to the antimigraine effects of the triptans[35-37], but is not a class effect of all anti-migraine drugs (e.g. gepants), we investigated the contractions to sumatriptan and lasmiditan in human meningeal arteries. In accordance to the previously described craniovascular selectivity of the triptans[22, 38], contractions to sumatriptan were larger in this dural artery than those in the proximal coronary artery. However, as

we have also previously shown, contractions to sumatriptan in distal coronary artery (and internal mammary artery) were not significantly different from those in meningeal artery[4]. In contrast, lasmiditan was devoid of vascular effects in this cranial vessel. Therefore, the efficacy of lasmiditan as acute migraine treatment may be due to inhibition of CGRP release from perivascular fibres or direct central (antinociceptive) modulation[37].

Our binding studies showed that, as mentioned previously, most of the triptans available in the market, namely, almotriptan, frovatriptan, naratriptan, sumatriptan and zolmitriptan are also agonists of the 5-HT_{1F} receptor (Fig. 1). Furthermore, the correlation between binding and the contractile potency of the compounds tested revealed that the potency of the agonists to contract the HCA positively correlated to their potency to bind the 5-HT_{1B} receptor, whereas it negatively correlated for the 5-HT_{1F} receptor (Fig. 6) and this was also observed when correlating the contractile potency and second messenger activation (Suppl. Fig. 3). Moreover, when analyzing the correlation between second messenger activation by 5-HT_{1B} *vs*. 5-HT_{1F} receptors, also a negative correlation was observed (Supplementary Fig. 4). These results, together with our *in vitro* data suggest that, although the mRNA of both receptor subtypes has been described in human vasculature[4,14-16,22,23,39], only activation of the 5-HT_{1B} receptor will result in vasoconstriction of, at least, coronary, mammary and meningeal arteries, whereas activation of the 5- HT_{1F} receptor will not. This could suggest that 5-HT_{1F} receptors in human vasculature are not functional or, that 5-HT_{1F} receptor mRNA is not translated to protein.

When considering the acute hemodynamic effect of sumatriptan in humans, it is well-known that after subcutaneous administration, there are vasopressor responses in the systemic arterial circulation and coronary artery vasoconstriction[40]. Although we observed carotid and coronary vasoconstriction in anesthetized dogs, there were no significant increases in blood pressure, as previously reported in this animal model[41]. In fact, after high doses of sumatriptan a non-significant tendency to decrease blood pressure and significant decreases in heart rate were observed, most probably due to inhibition of vascular and cardiac sympathetic outflows via the stimulation of prejunctional 5-$HT_{1B/1D}$ receptors on perivascular[42] and cardiac[43,44] sympathetic nerves. Lasmiditan only showed a trend to decrease blood pressure at the highest (supratherapeutic dose) which, based on the affinity of lasmiditan (see Table 1), could be due to a non-selective activation of prejunctional 5-HT_{1D} receptors and subsequent inhibition of sympathetic perivascular nerves[42]. Admittedly, this has not been shown directly in dogs but in pithed Wistar rats and, in patients, no changes in blood pressure have been observed[45,46]. Further experiments, falling beyond the scope of the present study, would be required to shed more light on the mechanisms behind these responses, which are only observed at non clinically relevant doses.

In summary, our *in vitro* and *in vivo* results indicate that lasmiditan is devoid of contractile properties in isolated human and anesthetized dog arteries, respectively. This might be of particular relevance in migraine patients who have a high risk of developing cardiovascular disease, such as subjects with hemiplegic migraine, prolonged migraine with aura, or with established cardiovascular disease. Clearly, further studies are needed to evaluate the safety of lasmiditan in these specific patient populations and its effectiveness compared with triptans. Finally, clinical trials have shown that lasmiditan is effective for migraine treatment[10], suggesting a mechanism of action (partially) different to that of the triptans[27].

In conclusion, our data support our initial hypothesis that lasmiditan is a high-affinity and highly selective agonist for the *human* 5-HT_{1F} receptor that is devoid of contractile properties in human isolated blood vessels and in anesthetized canines.

References

1. Kurth T, Winter AC, Eliassen AH, et al. Migraine and risk of cardiovascular disease in women: prospective cohort study. BMJ 2016;353:i2610.
2. Vos T, Flaxman AD, Naghavi M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2163-2196.
3. Diener H-C, Limmroth V. Advances in pharmacological treatment of migraine. Expert Opinion on Investigational Drugs 2001;10:1831-1845.
4. Chan KY, Labruijere S, Ramirez Rosas MB, et al. Cranioselectivity of sumatriptan revisited: pronounced contractions to sumatriptan in small human isolated coronary artery. CNS Drugs 2014;28:273-278.
5. Labruijere S, Chan KY, de Vries R, et al. Dihydroergotamine and sumatriptan in isolated human coronary artery, middle meningeal artery and saphenous vein. Cephalalgia 2015;35:182-189.
6. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.
7. Dodick D, Lipton RB, Martin V, et al. Consensus statement: cardiovascular safety profile of triptans (5-HT agonists) in the acute treatment of migraine. Headache 2004;44:414-425.
8. Nelson DL, Phebus LA, Johnson KW, et al. Preclinical pharmacological profile of the selective 5-HT_{1F} receptor agonist lasmiditan. Cephalalgia 2010;30:1159-1169.
9. Johnson KW, Schaus JM, Durkin MM, et al. 5-HT_{1F} receptor agonists inhibit neurogenic dural inflammation in guinea pigs. NeuroReport 1997;8:2237-2239.
10. Kuca B, Silberstein SD, Wietecha L, Berg PH, Dozier G, Lipton RB. Lasmiditan is an effective acute treatment for migraine. A phase 3 randomized study 2018.
11. Sacco S, Kurth T. Migraine and the risk for stroke and cardiovascular disease. Curr Cardiol Rep 2014;16:524.
12. Buse DC, Reed ML, Fanning KM, Kurth T, Lipton RB. Cardiovascular Events, Conditions, and Procedures Among People With Episodic Migraine in the US Population: Results from the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2017;57:31-44.
13. Schurks M, Rist PM, Bigal ME, Buring JE, Lipton RB, Kurth T. Migraine and cardiovascular disease: systematic review and meta-analysis. BMJ 2009;339:b3914.
14. Bouchelet I, Case B, Olivier A, Hamel E. No contractile effect for 5-HT_{1D} and 5-HT_{1F} receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. British Journal of Pharmacology 2000;129:501-508.
15. Bouchelet I, Cohen Z, Case B, Séguéla P, Hamel E. Differential expression of sumatriptan-sensitive 5-hydroxytryptamine receptors in human trigeminal ganglia and cerebral blood vessels. Molecular Pharmacology 1996;50:219-223.
16. Nilsson T, Longmore J, Shaw D, et al. Characterisation of 5-HT receptors in human coronary arteries by molecular and pharmacological techniques. European Journal of Pharmacology 1999;372:49-56.
17. Alexander SPH, Christopoulos A, Davenport AP, et al. THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: G protein-coupled receptors. British Journal of Pharmacology 2017;174:S17-S129.
18. George SE, Bungay PJ, Naylor LH. Functional Coupling of Endogenous Serotonin (5-HT_{1B}) and Calcitonin (C1a) Receptors in CHO Cells to a Cyclic AMP-Responsive Luciferase Reporter Gene. Journal of Neurochemistry 1997;69:1278-1285.
19. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 1977;41:19-26.
20. MaassenVanDenBrink A, van den Broek RWM, de Vries R, Upton N, Parsons AA, Saxena PR. The Potential Anti-Migraine Compound SB-220453 does not Contract Human Isolated Blood Vessels or Myocardium; A Comparison with Sumatriptan. Cephalalgia 2000;20:538-545.
21. Kovalchin J, Ghiglieri A, Zanelli E, Ings R, Mathers T. Lasmiditan Acts Specifically on the 5-HT_{1F} Receptor in the Central Nervous System. Cephalalgia 2016;36:103.
22. van den Broek RW, MaassenVanDenBrink A, Mulder PG, et al. Comparison of contractile responses to donitriptan and sumatriptan in the human middle meningeal and coronary arteries. Eur J Pharmacol 2002;443:125-132.
23. Parsons AA, Raval P, Smith S, et al. Effects of the Novel High-Affinity 5-$HT_{1B/1D}$-Receptor Ligand Frovatriptan in Human Isolated Basilar and Coronary Arteries. Journal of Cardiovascular Pharmacology 1998;32:220-224.
24. MaassenVanDenBrink A, Reekers M, Bax WA, Saxena PR. Human Isolated Coronary Artery Contraction to Sumatriptan Characterised by the Selective 5-$HT_{1B/1D}$ Receptor Antagonist GR55562. Pharmacology & Toxicology 2000;86:287-290.

25. Cason MDBrian A, Verrier MDEdward D, London MDMartin J, Mangano PDMDDennis T, Hickey MDRobert F. Effects of Isoflurane and Halothane on Coronary Vascular Resistance and Collateral Myocardial Blood FlowTheir Capacity to Induce Coronary Steal. Anesthesiology 1987;67:665-675.
26. McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 2010;160:1573-1576.
27. Curtis Michael J, Bond Richard A, Spina D, et al. Experimental design and analysis and their reporting: new guidance for publication in BJP. British Journal of Pharmacology 2015;172:3461-3471.
28. Cushing DJ, Cooper WD, Gralinski MR, Lipicky RJ, Kudenchuk PJ, Kowey PR. Comparison of the Cardiac Electrophysiology and General Toxicology of Two Formulations of Intravenous Amiodarone in Dogs. Cardiovascular Toxicology 2009;9:126-133.
29. Chan KY, Baun M, de Vries R, et al. Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery. Cephalalgia 2011;31:181-189.
30. Abourashed EA, Koetter U, Brattström A. In vitro binding experiments with a Valerian, Hops and their fixed combination extract (Ze91019) to selected central nervous system receptors. Phytomedicine 2004;11:633-638.
31. Barac YD, Bar-Am O, Liani E, et al. I_1 Imidazoline Receptor: Novel Potential Cytoprotective Target of TVP1022, the S-Enantiomer of Rasagiline. PLOS ONE 2012;7:e47890.
32. Sun W, Blanton MP, Gabriel JL, Canney DJ. Biososteric Replacement in the Design and Synthesis of Ligands for Nicotinic Acetylcholine Receptors. Medicinal Chemistry Research 2005;14:241-259.
33. Cohen ML, Schenck K. Contractile responses to sumatriptan and ergotamine in the rabbit saphenous vein: effect of selective 5-$HT_{(1F)}$ receptor agonists and $PGF_{(2\alpha)}$. British Journal of Pharmacology 2000;131:562-568.
34. Colquhoun D. Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. British journal of pharmacology 1998;125:924-947.
35. Benemei S, Cortese F, Labastida-Ramírez A, et al. Triptans and CGRP blockade – impact on the cranial vasculature. The Journal of Headache and Pain 2017;18:103.
36. Chan KY, Vermeersch S, de Hoon J, Villalón CM, MaassenVanDenBrink A. Potential mechanisms of prospective antimigraine drugs: A focus on vascular (side) effects. Pharmacology & Therapeutics 2011;129:332-351.
37. Rubio-Beltran E, Labastida-Ramirez A, Villalon CM, MaassenVanDenBrink A. Is selective 5-HT_{1F} receptor agonism an entity apart from that of the triptans in antimigraine therapy? Pharmacol Ther 2018;186:88-97.
38. MaassenVanDenBrink A, van den Broek RW, de Vries R, Bogers AJ, Avezaat CJ, Saxena PR. Craniovascular selectivity of eletriptan and sumatriptan in human isolated blood vessels. Neurology 2000;55:1524-1530.
39. Chan KY, de Vries R, Leijten FPJ, et al. Functional characterization of contractions to tegaserod in human isolated proximal and distal coronary arteries. European Journal of Pharmacology 2009;619:61-67.
40. MacIntyre PD, Bhargava B, Hogg KJ, Gemmill JD, Hillis WS. Effect of subcutaneous sumatriptan, a selective $5HT_1$ agonist, on the systemic, pulmonary, and coronary circulation. Circulation 1993;87:401-405.
41. Villalón CM, Terrón JA. The 5-HT_1-like receptor mediating the increase in canine external carotid blood flow: close resemblance to the 5-HT_{1D} subtype. British Journal of Pharmacology 1994;113:13-20.
42. Villalón CM, Centurión D, Rabelo G, Vries P, Saxena Pramod R, Sánchez-López A. The 5-HT_1-like receptors mediating inhibition of sympathetic vasopressor outflow in the pithed rat: operational correlation with the 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} subtypes. British Journal of Pharmacology 1998;124:1001-1011.
43. Sánchez-López A, Centurión D, Vázquez E, Arulmani U, Saxena Pramod R, Villalón Carlos M. Pharmacological profile of the 5-HT-induced inhibition of cardioaccelerator sympathetic outflow in pithed rats: correlation with 5-HT_1 and putative 5-$ht_{5A/5B}$ receptors. British Journal of Pharmacology 2003;140:725-735.
44. Sánchez-López A, Centurión D, Vázquez E, Arulmani U, Saxena PR, Villalón CM. Further characterization of the 5-HT_1 receptors mediating cardiac sympatho-inhibition in pithed rats: pharmacological correlation with the 5-HT_{1B} and 5-HT_{1D} subtypes. Naunyn-Schmiedeberg's Archives of Pharmacology 2004;369:220-227.
45. Ferrari MD, Farkkila M, Reuter U, et al. Acute treatment of migraine with the selective 5-HT_{1F} receptor agonist lasmiditan-a randomised proof-of-concept trial. Cephalalgia 2010;30:1170-1178.
46. Farkkila M, Diener HC, Geraud G, et al. Efficacy and tolerability of lasmiditan, an oral 5-$HT_{(1F)}$ receptor agonist, for the acute treatment of migraine: a phase 2 randomised, placebo-controlled, parallel-group, dose-ranging study. Lancet Neurology 2012;11:405-413.

Supplementary material

Suppl. Table 1. Reference tracers (concentration, nM), and reference competitors used for radioligand binding competition assays for the different receptors studied. *Historical pIC_{50} values obtained at Ogeda S.A. (now Epics Therapeutics S.A., Gosselies, Belgium). Values represent mean values±SEM of a stated number of averaged technical duplicates (n).

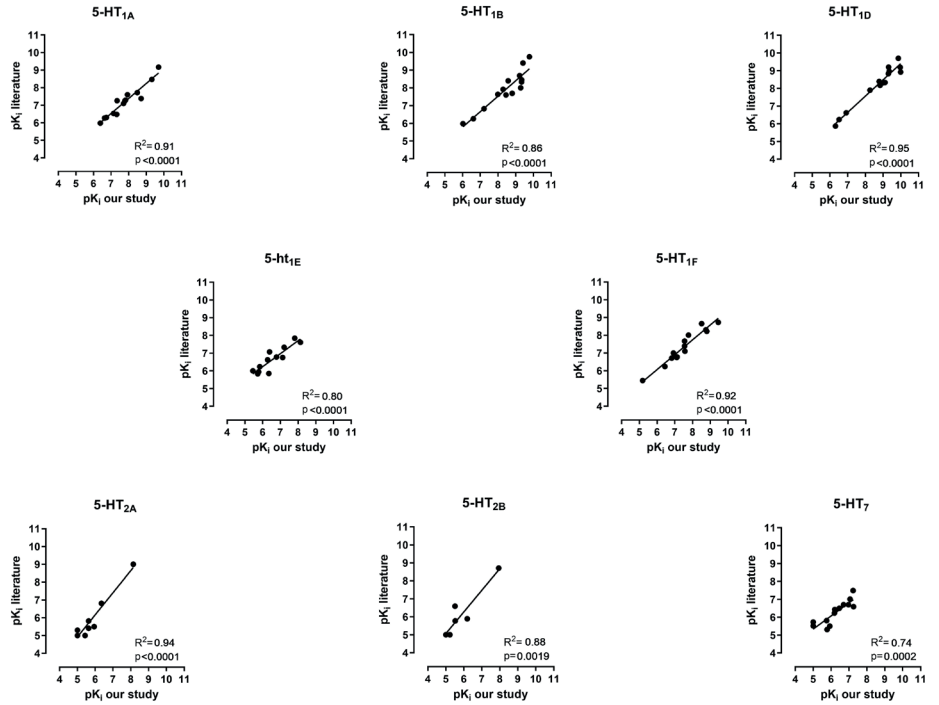
Receptor	Reference tracer	Assay concentration (nM)	Reference competitor	Historical* pIC_{50} (reference competitor)	Estimated pIC_{50} (reference competitor)	pK_i (reference competitor)
5-HT_{1A}	[^{3}H]-8-OH-DPAT	0.39	5-HT hydrochloride	8.83±0.04 (23)	9.00±0.06 (2)	9.51±0.06 (2)
5-HT_{1B}	[^{3}H]-5-CT	0.60	5-HT hydrochloride	8.42±0.10 (8)	8.61±0.19 (2)	9.09±0.19 (2)
5-HT_{1D}	[^{3}H]-5-CT	0.50	5-HT hydrochloride	8.35±0.08 (7)	8.67±0.18 (2)	9.35±0.18 (2)
5-ht_{1E}	[^{3}H]-LSD	14.0	BRL-54443	8.49±0.09 (5)	8.44±0.07 (3)	8.74±0.07 (3)
5-HT_{1F}	[^{3}H]-LSD	8.00	BRL-54443	8.59±0.17 (5)	8.54±0.12 (3)	8.96±0.12 (3)
5-HT_{2A}	[^{3}H]-Ketanserin	1.48	Ketanserin	8.22±0.18 (6)	8.17±0.04 (2)	8.69±0.04 (2)
5-HT_{2B}	[^{3}H]-Mesulergin	1.00	5-HT hydrochloride	7.67±0.09 (8)	7.68±0.05 (3)	7.89±0.05 (3)
5-HT_7	[^{3}H]-LSD	1.00	5-CT maleate	9.28±0.05 (13)	9.42±0.17 (3)	9.51±0.17 (3)

Suppl. Table 2. Reference agonists for second messenger activation assays of cAMP (5-$HT_{1A/B/E/F}$ and 5-HT_7), GTPγS (5-HT_{1D}) and IP (5-$HT_{2A/B}$). *Historical pEC_{50} values obtained at Ogeda S.A. (now Epics Therapeutics S.A., Gosselies, Belgium). Values represent mean values±SEM of a stated number of averaged technical duplicates (n).

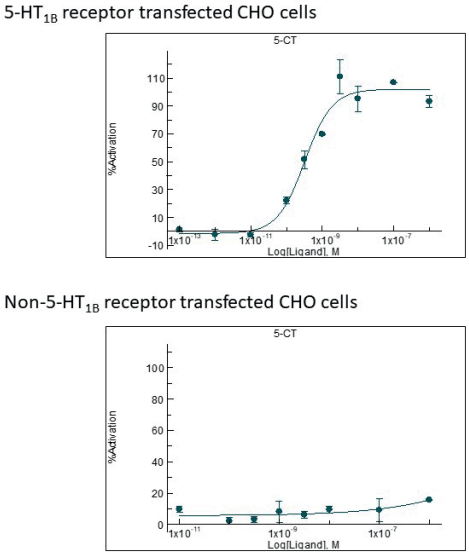
Receptor	Reference agonist	Historical* pEC_{50} cAMP/IP/ GTPγS	Estimated pEC_{50} cAMP/ IP/ GTPγS
5-HT_{1A}	5-CT maleate	9.18±0.05 (35)	9.02±0.17 (4)
5-HT_{1B}	5-CT maleate	8.78±0.07 (23)	8.80±0.01 (2)
5-HT_{1D}	5-CT maleate	9.30±0.05 (21)	9.26±0.13 (3)
5-ht_{1E}	5-HT hydrochloride	9.00±0.16 (2)	8.61±0.05 (3)
5-HT_{1F}	5-HT hydrochloride	8.94±0.15 (7)	8.69±0.16 (3)
5-HT_{2A}	α-Me-5-HT	8.68±0.05 (31)	8.33±0.06 (2)
5-HT_{2B}	α-Me-5-HT	9.70±0.09 (28)	9.63±0.08 (2)
5-HT_7	5-CT maleate	9.59±0.03 (45)	9.62±0.01 (3)

Supplementary Table 3. Summary of pEC_{50} values of vasoconstriction of the human coronary artery. These values represent the negative logarithm of the molar concentration of these compounds at which 50% of their maximal response was exerted. When a compound was devoid of vasoconstrictor activity, a pEC_{50} of 5 was set.

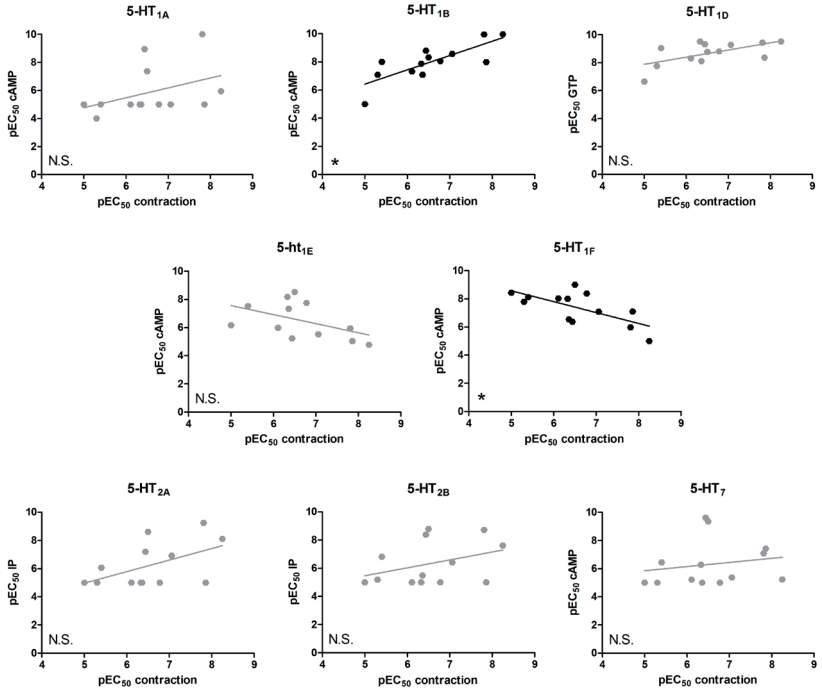
Agonist	pEC_{50}	Reference
5-HT hydrochloride	6.50	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998; Parsons et al., 1998)
5-CT maleate	6.44	(MaassenVanDenBrink, Reekers, Bax & Saxena, 2000)
Ergotamine tartrate	7.81	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Sumatriptan succinate	6.11	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Zolmitriptan	6.33	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Naratriptan hydrochloride	6.78	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Rizatriptan benzoate	6.36	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998)
Eletriptan hydrobromide	5.54	(van den Broek et al., 2000)
Frovatriptan Racemate	7.86	(Parsons et al., 1998)
Donitriptan hydrochloride	8.25	(van den Broek et al., 2002)
Avitriptan fumarate	7.06	(MaassenVanDenBrink, Reekers, Bax, Ferrari & Saxena, 1998; Saxena et al., 1997)
Lasmiditan hemisuccinate	5.00	



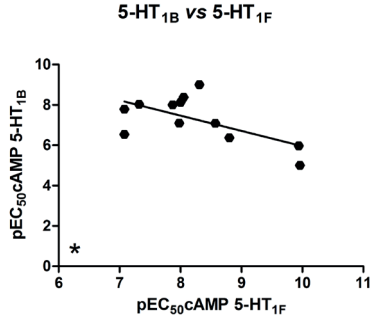
Suppl. Fig. 1. Correlation between the pK_i values obtained from literature and the pK_i values obtained in our study for lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, alniditan, 5-HT, 5-carboxamidotryptamine). For references see Suppl. Table 5.



Suppl. Fig. 2. Functional responses (cAMP assay) to 5-CT in CHO cells transfected with $5\text{-}HT_{1B}$ receptor (upper) and in CHO cells transfected with an unrelated G protein-coupled receptor.



Suppl. Fig. 3. Correlation between second messenger activation (*i.e.* cAMP, IP) and the contractile potency of lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, 5HT, 5-CT) in human isolated coronary arteries; N.S., non-significant; *P<0.05.



Suppl. Fig. 4. Correlation between the second messenger activation of 5-HT_{1B} receptor *vs* 5-HT_{1F} receptor by lasmiditan, triptans (sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, eletriptan, frovatriptan, donitriptan, avitriptan) and other 5-HT receptors ligands (ergotamine, 5HT, 5-carboxamidotryptamine) in human isolated coronary arteries; *P<0.05.

Chapter V.

Characterization of the calcitonin gene-related peptide receptor antagonists ubrogepant and atogepant in human isolated coronary, cerebral and middle meningeal arteries

Based on: ***E Rubio-Beltran****, KY Chan, AHJ Danser, A MaassenVanDenBrink*, L Edvinsson*. Accepted with minor revision.*

**Both authors contributed equally*

Abstract

Background: Migraine has been associated with dysfunctional activation of the trigeminovascular system. Calcitonin gene-related peptide (CGRP), a neuropeptide released from the trigeminal nerve fibres, has an important role in the pathophysiology of migraine and is a current therapeutic target for migraine treatment.

Methods: We examined the effects of two novel CGRP receptor antagonists, ubrogepant and atogepant, on the relaxations induced by αCGRP in human isolated middle meningeal (HMMA), cerebral (HCxA) and coronary (HCA) arteries. Furthermore, the contractile responses to atogepant and ubrogepant *per se* were studied and compared to the responses elicited by zolmitriptan in proximal and distal HCA.

Results: In intracranial arteries, both blockers antagonized the CGRP-induced relaxations more potently when compared to the inhibition observed in distal HCA, with atogepant showing a higher potency. When analyzing their antagonistic profile in HCA, ubrogepant showed a competitive antagonist profile, while atogepant showed a non-competitive one. Neither of the gepants had vasoconstrictor effect at any of the concentrations studied in HCA, whereas zolmitriptan elicited concentration-dependent contractions.

Conclusion: ubrogepant and atogepant differentially inhibit the CGRP-dependent vasodilatory responses in intracranial arteries when compared to distal HCA. Also, both gepants are devoid of vasoconstrictive properties in HCA.

Introduction

The pathophysiology of migraine is becoming increasingly well understood, particularly regarding the role of the trigeminovascular system. A (dysfunctional) activation of the trigeminovascular system has been described[1,2], followed by the activation of the trigeminovascular reflex characterized by the release of calcitonin gene-related peptide (CGRP) from the perivascular trigeminal sensory fibres, causing vasodilation of cerebral and dural vessels[3,4]. In accordance with this, our group has previously demonstrated the expression and co-localization of the CGRP receptor components in the smooth muscle cells of intracranial arteries[5]. Moreover, patients during migraine attacks show elevated release of CGRP in the cranial venous outflow[6], while intravenous infusion of CGRP in migraine patients has been shown to provoke migraine-like attacks[7].

Due to the important role of CGRP in migraine, CGRP receptor antagonists (gepants) were developed for the acute treatment of migraine. Olcegepant and telcagepant were the first gepants and both showed to be effective in clinical trials[8,9]; unfortunately, due to pharmacokinetic and/or hepatotoxic limitations, neither of these gepants reached the market[10], and the synthesis of novel gepants was temporarily halted. In recent years, two novel orally available gepants have been developed for the acute (ubrogepant) and preventive (atogepant) treatment of migraine (Fig. 1). Phase II trials with ubrogepant (NCT01613248 and NCT01657370) showed promising results for the acute treatment of migraine[11,12] and phase III trials (NCT02828020, NCT02867709 and NCT02873221) have been completed with positive results. A phase II/III trial (NCT02848326) with atogepant for the preventive treatment of episodic migraine also demonstrated positive findings[12].

As mentioned above, CGRP plays an important role in the pathophysiology of migraine[13]; however, this peptide has also been related to several physiological processes[14,15], and expression of CGRP receptors has not only been described in middle meningeal arteries, but also in cerebral and coronary arteries. This raises the possibility that CGRP receptor antagonists could affect

vascular tone in different parts of the circulation[16,17]. Therefore, the aim of this study is to assess the functional responses to human αCGRP in isolated human middle meningeal (HMMA), cerebral (HCxA) and coronary arteries (HCA), and to study the effects of ubrogepant and atogepant on the CGRP-mediated vasodilatory responses. Furthermore, we studied the *per se* effect of both gepants in HCA, and compared them to the contractile responses to zolmitriptan.

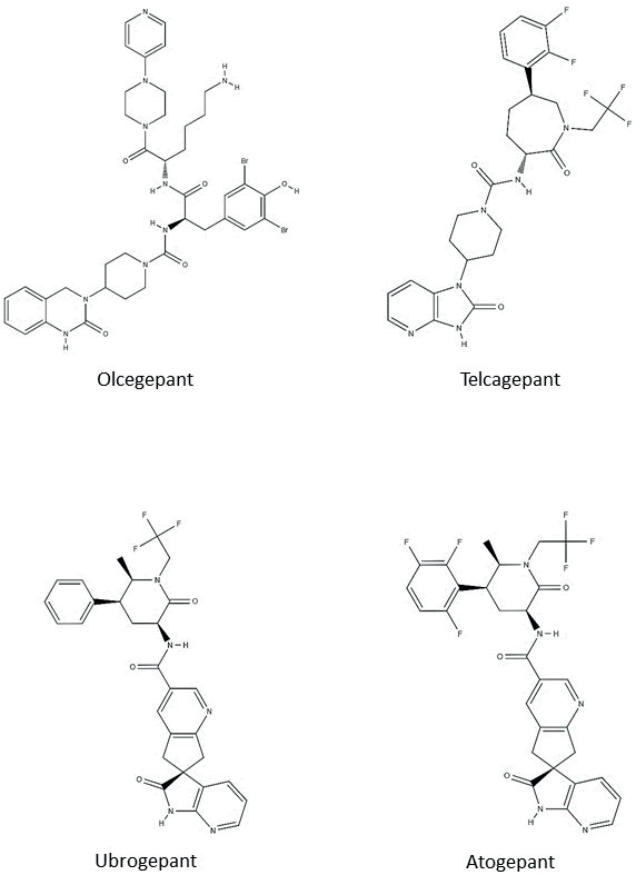


Fig. 1. Chemical structures of olcegepant, telcagepant, ubrogepant and atogepant. Modified from[23,24].

Materials and methods

Human isolated arteries

Human middle meningeal arteries (HMMA; 1 male, 5 females; aged 45±3 years, range 35-53) and cerebral (cortex) arteries (HCxA; 3 males, 4 females; aged 60±4 years, range 43-73) were removed peri-operatively from patients undergoing neurosurgical procedures at Lund University Hospital, Sweden. All vessels were placed in buffer solution, for cerebral arteries, composition (in mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 1.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; glucose, 5.5; at a pH=7.4; for meningeal arteries, composition (in mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 1.25; $MgSO_4$, 1.2; KH_2PO_4,1.2; $NaHCO_3$, 25 and glucose, 11.1; at a pH=7.4, and were kept aerated with carbogen and transported to the laboratory immediately. Human coronary arteries were obtained from eight "heart beating" organ donors (4

males, 4 females; aged 41±3 years, range 25-56). The hearts were provided post-mortem by the Heart Valve Bank, Rotterdam (currently ETB-BISLIFE Tissue Bank, Beverwijk), after donor mediation via Bio Implant Services/Eurotransplant Foundation (Leiden, The Netherlands), following removal of the aortic and pulmonary valves for homograft valve transplantation and were stored at 0-4°C in a sterile organ-protecting solution immediately after circulatory arrest. After arrival at the laboratory, proximal (internal diameter 3-5 mm) and distal (internal diameter 0.5-1 mm) portions of the right HCA were dissected and placed in a cold, oxygenated buffer solution composed as follows (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 8.3, at a pH=7.4 and were kept aerated with 5% CO_2 in O_2 (carbogen).

The Swedish part of the study was approved by Lund University Ethics Committee (LU99) and had the individual patients' approval, while the ethics committee dealing with human experimentations at Erasmus Medical Centre, Rotterdam, approved the Dutch part of the study.

Functional Experiments

Human arteries were cut into segments of 2-4 mm length, excluding distinct, macroscopically visible atherosclerotic lesions. The proximal HCA segments were mounted in 15-ml organ baths, while distal HCA segments and intracranial arteries were mounted on Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) between two small stainless-steel wires (Ø 40 µm) in 6-ml tissue baths. In both cases, the baths were filled with the respective oxygenated buffer solution at 37°C (see above). After equilibration for at least 30 min and a wash every 15 min, the vessel segments were stretched to a stable tension of about 15 mN for the proximal HCA (the optimal tension as determined in previous studies), or stretched to a tension normalized to 90% of I_{100} for the distal HCA segments and intracranial arteries, the diameter when transmural pressure equals 100 mm Hg[18]. Changes in tissue tension were measured using an isometric transducer (Harvard, South Nattick, MA) and recorded on a flatbed recorder (Servogor 124; Goerz, Neudorf, Austria, 15-ml organ baths) or using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK, 6-ml Mulvany myographs). After reaching equilibrium, the contractile capacity of HCxA segments was examined by exposure to a potassium-rich (60 mM) buffer solution, which had the same composition as the standard solution, except that the NaCl was exchanged for an equimolar concentration of KCl. Segments of HMMA and distal HCA were exposed to KCl (30 mM) to 'prime' the tissue for stable contractions. After washout, the tissue was exposed to KCl (100 mM) to determine the maximal contractile response to KCl.

The relaxant effect of human αCGRP was examined by cumulative application of increasing concentrations of the peptide (1 pM-1 µM, whole log steps for cranial arteries and half log steps for the coronary artery) in the absence or presence of ubrogepant (0.1 nM, 1 nM, 10 nM, 100 nM or 1 µM) or atogepant (0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM or 1 µM). Segments were precontracted with KCl (30 mM) before αCGRP was added. Each segment was exposed to a single cumulative concentration response curve and a matched pair's protocol was used, where one segment acted as control (no antagonist present) while in another segment from the same artery the agonist response was assessed following equilibration (20-30 min) with the antagonist. When a concentration of antagonist inhibited more than 80% of the CGRP-mediated vasodilatory responses, no further concentrations of antagonist were tested as estimation of pEC_{50} values was not reliable. Furthermore, in HCA the *per se* contractile responses to atogepant (1 pM-100 µM in distal, 100 pM-30 µM in proximal), ubrogepant (1 pM-100 µM in distal, 100 pM-30 µM in proximal) and vehicle were assessed and compared to the contractile responses elicited by zolmitriptan (1 pM-100 µM in distal, 100 pM-30 µM in proximal). The functional integrity of the endothelium was verified by observing relaxation to substance P (10 nM), after precontraction with thromboxane A_2 analogue U46619 (10 nM).

Compounds

Human αCGRP (PolyPeptide, Strasbourg, France, for the Rotterdam experiments; Sigma-Aldrich, St. Louis, MO, USA, for the Lund experiments), U46619 and zolmitriptan (Sigma-Aldrich, St. Louis, MO) were dissolved in saline. Ubrogepant and atogepant were received from the Medicinal Chemistry Department, Merck Laboratories, USA, in pre-weighed vials that had been marked by Merck and dissolved in dimethylsulphoxide (DMSO). All compounds were stored in aliquots at -20°C.

Analysis of data

The individual vasodilator responses were expressed relative to the contraction elicited by KCl (30 mM; 100% precontraction). For each segment the maximum vasodilator effect (apparent E_{max}, here defined as the response observed at the highest concentration of CGRP applied) was calculated. The concentration response curves for all agonists were analyzed by using nonlinear regression analysis, and the potency of agonists was expressed as pEC_{50} (*i.e.*, negative logarithm of the molar concentration of agonist inducing half-maximum response) by using Prism 5.0 (GraphPad Software Inc., San Diego, CA). The blocking potency of the antagonists was estimated by calculating EC_{50} ratios and plotting a Schild plot by using linear regression to get the slope value[19]. In case of depression of the maximum relaxation without a change in pEC_{50}, we could only show significant reduction in maximum relaxations, while it should in these cases be born in mind that E_{max} might have been increased at higher concentrations than applied in our experiments. Data are expressed as mean values±standard error of the mean (S.E.M.), and n refers to the number of subjects from whom the vessels were collected. Statistically significant differences were examined by Mann-Whitney U test.

Results

Functional responses to αCGRP in human isolated arteries

In HMMA, increasing concentrations of αCGRP caused a concentration-dependent vasorelaxation (Fig. 2; apparent E_{max}: 94±16%; pEC_{50}: 8.38±0.15; n=5). Similar responses were observed in HCxA (Fig. 3; apparent E_{max}: 97±10%; pEC_{50}: 8.65±0.16; n=7). Endothelial function analysis resulted in a mean relaxation to substance P of 57±10% of the precontraction to U46619 in HMMA and 75±5% in HCxA.

In distal HCA segments, concentration response curves to human αCGRP resulted in a concentration-dependent relaxation (Fig. 4; apparent E_{max}: 83±6%; pEC_{50}: 9.05±0.20; n=8). In proximal coronary segments, a small, often absent relaxant response to αCGRP (Fig. 5; 9±8%; n=8) was observed. Quantification of the pEC_{50} of these concentration-response curves was not feasible in a sensible manner. Endothelial function analysis resulted in a mean relaxation to substance P of 69±3% of the precontraction induced by U466619 in distal HCA and 53±4% in proximal HCA.

Effects of ubrogepant and atogepant in human isolated arteries

In HMMA (Fig. 2), a parallel shift of the concentration-response curve to CGRP was observed at all concentrations tested for ubrogepant (10 nM pEC_{50}: 6.67±0.46, apparent E_{max}: 45±11%; 100 nM pEC_{50}: 8.43±0.51, apparent E_{max}: 26±4%) and atogepant (10 nM pEC_{50}: 5.42±0.62, apparent E_{max}: 12±7%). In HCxA (Fig. 3), inhibition of the relaxant responses to CGRP was observed in the presence of ubrogepant (0.1 nM pEC_{50}: 7.89±0.30, apparent E_{max}: 60±8%; 1 nM pEC_{50}: 7.66±0.27, apparent E_{max}: 48±9%; 10 nM pEC_{50}: 7.62±0.45, apparent E_{max}: 42±10%; 100 nM pEC_{50}: 5.88±0.40, apparent E_{max}: 17±7%) and atogepant (0.01 nM pEC_{50}: 7.65±0.25, apparent E_{max}: 62±10%; 0.1 nM pEC_{50}: 6.70±0.06, apparent E_{max}: 36±2%; 1 nM pEC_{50}: 5.83±0.43, apparent E_{max}: 21±5%). No estimation of pK_b and pA_2 values was performed for HMMA and HCxA as none of the concentration-response curves reached a plateau; hence no accurate values could be calculated.

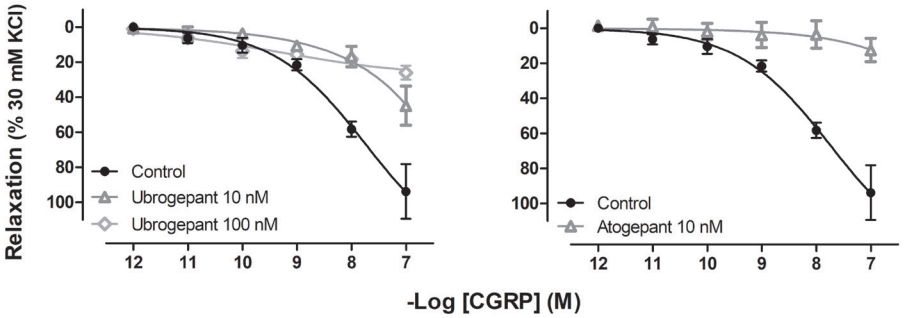


Fig. 2. Vasodilatory effect of αCGRP on human middle meningeal arteries. Concentration response curves to αCGRP in the absence or presence of ubrogepant (10-100 nM; top) or atogepant (10 nM; bottom). Values given represent mean±standard error of the mean (n=3-5 each).

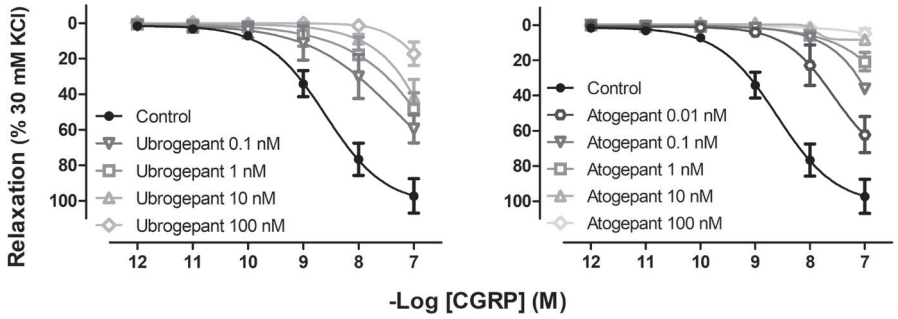


Fig. 3. Vasodilatory effect of αCGRP on human cerebral arteries. Concentration response curves to αCGRP in the absence or presence of ubrogepant (0.1-100 nM; left) or atogepant (0.01-100 nM; right). Values given represent mean±standard error of the mean (n=3-7 each).

In distal HCA (Fig. 4), a significant parallel rightward shift of the concentration-response curve to CGRP was observed with ubrogepant (10 nM: pEC_{50}: 8.20±0.23, p=0.01; 100 nM pEC_{50}: 7.15±0.36, p=0.002; 1 μM pEC_{50}: 6.97±0.34, p=0.003) and atogepant (10 nM: pEC_{50}: 7.59±0.18, p=0.003; 100 nM pEC_{50}: 6.89±0.40, p=0.001; 1 μM pEC_{50}: 6.41±0.14, p<0.001). In the proximal HCA, ubrogepant and atogepant seemed to inhibit the relaxant responses to CGRP (Fig. 5). However, as the control responses were rather small, or even absent in some cases, it was not quantifiable in a sensible manner. In distal HCA, no significant changes were observed in E_{max} in the presence of ubrogepant (10 nM E_{max}: 90±4%, p=0.22; 100 nM E_{max}: 82±11%, p=0.36; 1 μM E_{max}: 91±6%, p=0.32) or atogepant (10 nM E_{max}: 103±5%, p=0.11; 100 nM E_{max}: 91±11%, p=0.35; 1 μM E_{max}: 83±9%, p=0.50); therefore, we made an evaluation with the Schild plot (Fig. 6). For ubrogepant, the slope of the Schild plot was not significantly different from unity (0.87±0.17), thus we calculated pA_2, which amounted to 8.86±0.39 (R^2: 0.87). For atogepant, the slope of the Schild plot was significantly lower than unity (0.55±0.01), therefore we did not calculate a pA_2 value. Instead, pK_b values were calculated per individual concentration of antagonist employed (pK_b at 10 nM: 9.42±0.22; pK_b at 100 nM: 9.11±0.34; pK_b at 1 μM: 8.64±0.21).

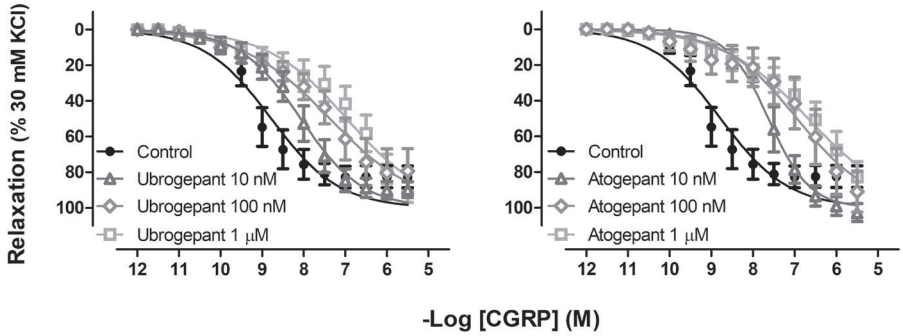


Fig. 4. Vasodilatory effect of αCGRP on human distal coronary arteries. Concentration response curves to αCGRP in the absence or presence of ubrogepant (10 nM-1 μM; top) or atogepant (10 nM-1 μM; bottom). Values given represent mean±standard error of the mean (n=7–8 each).

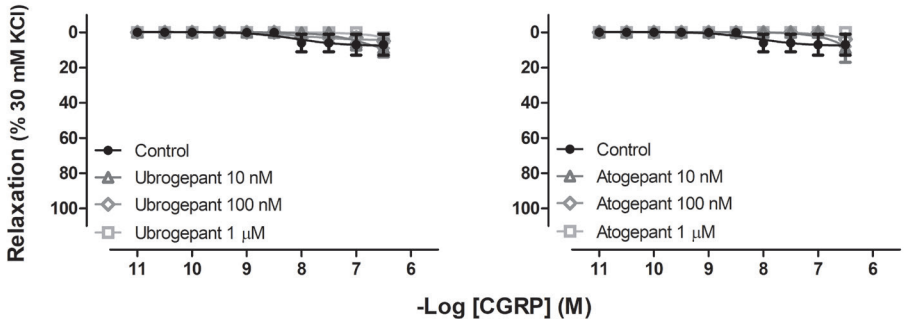


Fig. 5. Vasodilatory effect of αCGRP on human proximal coronary arteries. Concentration response curves to αCGRP in the absence or presence of ubrogepant (10 nM-1 μM; top) or atogepant (10 nM-1 μM; bottom). Values given represent mean±standard error of the mean (n=8 each).

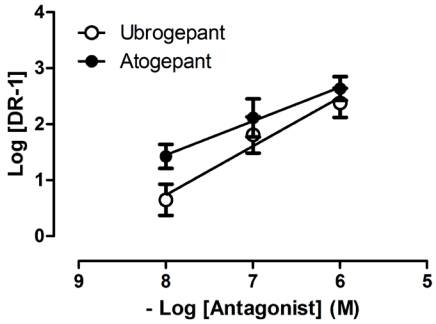


Fig. 6. Schild plot of the responses observed with atogepant and ubrogepant in distal coronary arteries. A straight line that did not differ from unity was observed for ubrogepant, but not for atogepant.

Vasoconstrictor effect of zolmitriptan, ubrogepant and atogepant in human isolated coronary arteries

Zolmitriptan caused concentration-dependent contractions in distal (pEC_{50} 7.10±0.13; E_{max}: 72±33%; n=6; Fig. 7) and proximal HCA (pEC_{50} 5.53±0.32; E_{max}: 38±12%; n=6; Fig. 8). Ubrogepant and atogepant were devoid of significant contractions in concentrations up to 100 µM in both proximal and distal segments of the HCA.

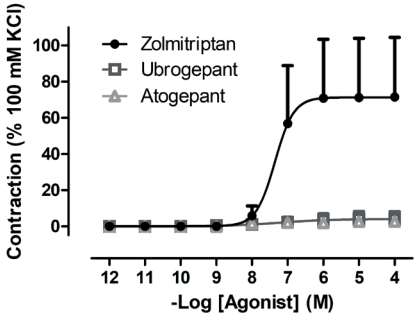


Fig. 7. Contractile responses to zolmitriptan (1 pM-100 µM), ubrogepant (1 pM-100 µM) and atogepant (1 pM-100 µM) in distal coronary arteries. Values given represent mean±standard error of the mean (n=6).

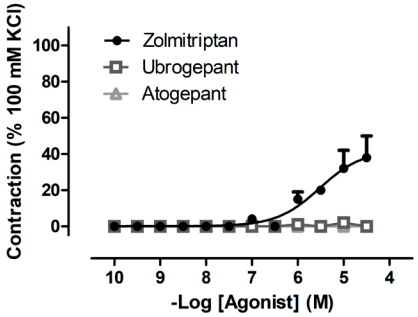


Fig. 8. Contractile responses to zolmitriptan (100 pM-30 µM), ubrogepant (100 pM-30 µM) and atogepant (100 pM-30 µM) in proximal coronary arteries. Values given represent mean±standard error of the mean (n=6-7).

Discussion

In this study, the effect of two novel gepants, ubrogepant and atogepant, on the CGRP-mediated vasodilatory responses in human isolated arteries was investigated.

First, we analyzed the inhibition of the vasorelaxant responses to CGRP in HMMA, as this is potentially one of the mechanisms of action of gepants. Our results show that both gepants effectively antagonized the vasodilatory responses to CGRP, with atogepant seeming more potent (Fig. 2). In the case of ubrogepant, developed for the acute treatment of migraine, our data suggest that during a migraine attack, reversion of the CGRP-mediated vasodilation may well be one of its mechanisms of action; whereas in the case of atogepant, currently being evaluated for the prophylactic treatment of migraine, our results suggest a prevention of the vasodilatory responses. In the latter case, it would be interesting to further asses *in vivo*, similar as for the CGRP (receptor)

antibodies that are currently in development[20], whether the chronic blockade of the CGRP receptor would result in overexpression of CGRP receptors and tolerance over time. Furthermore, if there is indeed an increase in CGRP receptor expression, physicians should be aware that withdrawal of this drug must be gradual, as migraine headaches could worsen if withdrawn abruptly[21].

CGRP has also been related to several physiological processes[14,15], such as regulation of the vascular tone[22,23], raising the concern that CGRP receptor antagonism could affect vascular tone in different parts of the circulation[17]. Considering migraine patients have increased cardiovascular risk[24-26], it is important to assess the effect of ubrogepant and atogepant in cerebral (cortex) and coronary arteries. In HCxA, atogepant was more potent than ubrogepant in antagonizing CGRP-mediated vasodilatory responses (Fig. 3). Even though the previous gepants were reported to have a limited ability to cross the blood-brain barrier[27], it is important to consider that these novel gepants are not structurally related (see Fig. 1), therefore their ability to cross the blood-brain barrier cannot be discarded, especially in the case of atogepant, as prophylactic treatment could result in high plasma levels, blood-brain barrier penetration and central therapeutic actions (and side effects). In coronary arteries, vasodilatory responses to CGRP in the proximal HCA were rather small, while in the distal portion of the HCA, these were more pronounced. This is in accordance with previous publications from our group[16,28] and shows that CGRP plays a more important role in the vasodilation of the distal portion of the coronary arteries, rather than in the proximal portion. It also reinforces the importance of cardiovascular safety studies when blocking the CGRP receptor[17,29], especially considering that migraine patients have a higher cardiovascular risk, with the grand majority of patients being female and that myocardial ischemic events in women are more common in the distal portion of the coronary artery, where CGRP plays a bigger role. Interestingly, when analyzing the effect of both gepants in the vasodilatory responses to CGRP in distal HCA, although atogepant was more potent when compared to ubrogepant, both gepants were less potent in antagonizing the vasodilatory responses in HCA than in HMMA. Though we cannot explain the different potencies of atogepant and ubrogepant in cranial versus coronary arteries, we have previously reported similar results with olcegepant[28,30] and, as it will be discussed further, it might be related to CGRP receptor heterogeneity. This property may be of clinical relevance and an advantage for these antagonists, since they would be more potent at blocking CGRP-induced vasodilation in the intracranial arteries than in the coronary arteries, where antagonism of CGRP-induced vasodilation would not be desired. Additionally, in HCA, we analyzed the *per se* vascular effects of both gepants, as current acutely acting antimigraine drugs are contraindicated in patients with coronary artery disease due to their vasocontrictive properties[31]. Ubrogepant and atogepant did not contract proximal or distal coronary arteries (Figs. 7-8), which represents an advantage for patients to whom triptans are contraindicated.

Finally, we analyzed the type of antagonism of both gepants in HCA with a Schild plot (Fig. 6). In the case of ubrogepant, the slope was not significantly different from unity, suggesting a competitive type of antagonism. In the case of atogepant, the slope was significantly smaller than unity, thus suggesting a non-competitive type of antagonism, the involvement of multiple receptors or hemi-equilibrium conditions[28,32-34]. Certainly, we have previously suggested CGRP receptor heterogeneity in the human coronary artery[28], and that this may be due to the contribution of the amylin type 1 (AMY_1) receptor in mediating the relaxations to CGRP in the distal human coronary artery[32], as described in the trigeminal ganglion[33]. Additionally, to our knowledge, no studies have assessed the selectivity of ubrogepant and atogepant over the AMY_1 (CTR/RAMP1) receptors. However, these results must be taken with caution, as our observations with the Schild plot should be further confirmed with more rigorous experiments, which fall beyond the scope of the current study.

Similarly, the mechanism behind the different potency of both antagonists in the cranial arteries when compared to the responses in coronary arteries should be addressed in future studies, as it may shed light on possible targets devoid of coronary side-effects potential.

Conclusion

Taken together, these results show that both atogepant and ubrogepant effectively inhibit the CGRP-vasodilatory responses in human meningeal, cerebral, and coronary arteries, and are both devoid of vasoconstrictive properties in HCA.

Key findings

Ubrogepant and atogepant inhibit the vasodilatory responses to CGRP in HCxA, HMMA and HCA.
The inhibition of the CGRP-mediated vasodilatory responses is more potent in the cranial arteries than in the coronary arteries.
Ubrogepant and atogepant are devoid of vasoconstrictive properties in human coronary arteries.

References

1. Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders, 3rd edition. Cephalalgia 2018;38:1-211.
2. GBD 2016 Disease and Injury Incidence and Prevalence. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet 2017;390:1211-1259.
3. Steiner TJ, Stovner LJ, Birbeck GL. Migraine: the seventh disabler. The journal of headache and pain 2013;14:1.
4. Ruiz de Velasco I, González N, Etxeberria Y, Garcia-Monco JC. Quality of Life in Migraine Patients: A Qualitative Study. Cephalalgia 2003;23:892-900.
5. Gustavsson A, Svensson M, Jacobi F, et al. Cost of disorders of the brain in Europe 2010. European Neuropsychopharmacology 2011;21:718-779.
6. Edvinsson L. The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine. Headache 2017;57 Suppl 2:47-55.
7. Edvinsson L, Uddman R. Neurobiology in primary headaches. Brain Research Brain research reviews 2005;48:438-456.
8. McCulloch J, Uddman R, Kingman TA, Edvinsson L. Calcitonin gene-related peptide: functional role in cerebrovascular regulation. Proceedings of the National Academy of Sciences 1986;83:5731-5735.
9. Goadsby PJ, Lipton RB, Ferrari MD. Migraine — Current Understanding and Treatment. New England Journal of Medicine 2002;346:257-270.
10. Edvinsson L, Chan KY, Eftekhari S, et al. Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries. Cephalalgia 2010;30:1233-1240.
11. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Annals of neurology 1990;28:183-187.
12. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22:54-61.
13. Olesen J, Diener H-C, Husstedt IW, et al. Calcitonin Gene–Related Peptide Receptor Antagonist BIBN 4096 BS for the Acute Treatment of Migraine. New England Journal of Medicine 2004;350:1104-1110.
14. Ho TW, Mannix LK, Fan X, et al. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. Neurology 2008;70:1304-1312.
15. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
16. Voss T, Lipton RB, Dodick DW, et al. A phase IIb randomized, double-blind, placebo-controlled trial of ubrogepant for the acute treatment of migraine. Cephalalgia 2016;36:887-898.
17. Tepper SJ. Anti-Calcitonin Gene-Related Peptide (CGRP) Therapies: Update on a Previous Review After the American Headache Society 60th Scientific Meeting, San Francisco, June 2018. Headache: The Journal of Head and Face Pain 2018;58:276-290.
18. Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies — successful translation from bench to clinic. Nature Reviews Neurology 2018;14:338-350.

19. Deen M, Correnti E, Kamm K, et al. Blocking CGRP in migraine patients - a review of pros and cons. J Headache Pain 2017;18:96.
20. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. Physiological Reviews 2014;94:1099.
21. Chan KY, Edvinsson L, Eftekhari S, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. J Pharmacol Exp Ther 2010;334:746-752.
22. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
23. Edvinsson L, Ho TW. CGRP receptor antagonism and migraine. Neurotherapeutics 2010;7:164-175.
24. Edvinsson L. CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. British journal of clinical pharmacology 2015;80:193-199.
25. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 1977;41:19-26.
26. Arunlakshana O, Schild HO. Some quantitative usess of drug antagonists. British Journal of Pharmacology and Chemotherapy 1959;14:48-58.
27. MaassenVanDenBrink A, Terwindt GM, van den Maagdenberg AMJM. Calcitonin gene-related peptide (receptor) antibodies: an exciting avenue for migraine treatment. Genome medicine 2018;10:10-10.
28. Aarons RD, Nies AS, Gal J, Hegstrand LR, Molinoff PB. Elevation of beta-adrenergic receptor density in human lymphocytes after propranolol administration. The Journal of Clinical Investigation 1980;65:949-957.
29. Mai TH, Wu J, Diedrich A, Garland EM, Robertson D. Calcitonin gene-related peptide (CGRP) in autonomic cardiovascular regulation and vascular structure. Journal of the American Society of Hypertension 2014;8:286-296.
30. Gennari C, Fischer JA. Cardiovascular action of calcitonin gene-related peptide in humans. Calcified Tissue International 1985;37:581-584.
31. Sacco S, Kurth T. Migraine and the risk for stroke and cardiovascular disease. Curr Cardiol Rep 2014;16:524.
32. Sacco S, Ornello R, Ripa P, Pistoia F, Carolei A. Migraine and hemorrhagic stroke: a meta-analysis. Stroke 2013;44:3032-3038.
33. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.
34. Gupta S, Mehrotra S, Villalon CM, et al. Characterisation of CGRP receptors in human and porcine isolated coronary arteries: evidence for CGRP receptor heterogeneity. Eur J Pharmacol 2006;530:107-116.
35. Maassen van den Brink A, Rubio-Beltrán E, Duncker D, Villalón CM. Is CGRP Receptor Blockade Cardiovascularly Safe? Appropriate Studies Are Needed. Headache: The Journal of Head and Face Pain 2018;58:1257-1258.
36. Gupta S, Mehrotra S, Avezaat CJJ, Villalón CM, Saxena PR, MaassenVanDenBrink A. Characterisation of CGRP receptors in the human isolated middle meningeal artery. Life Sciences 2006;79:265-271.
37. Chan KY, Labruijere S, Ramirez Rosas MB, et al. Cranioselectivity of sumatriptan revisited: pronounced contractions to sumatriptan in small human isolated coronary artery. CNS Drugs 2014;28:273-278.
38. Haanes KA, Chan KY, MaassenVanDenBrink A. Comment on "A second trigeminal CGRP receptor: function and expression of the AMY1 receptor". Annals of clinical and translational neurology 2016;3:307-308.
39. Walker CS, Eftekhari S, Bower RL, et al. A second trigeminal CGRP receptor: function and expression of the AMY1 receptor. Annals of Clinical and Translational Neurology 2015;2:595-608.
40. Kenakin T, Jenkinson S, Watson C. Determining the Potency and Molecular Mechanism of Action of Insurmountable Antagonists. Journal of Pharmacology and Experimental Therapeutics 2006;319:710-723.

PART III:

Prophylactic treatment of migraine

Chapter VI.

Blocking CGRP in migraine patients – a review of pros and cons

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Abstract

Migraine is the most prevalent neurological disorder worldwide and it has immense socioeconomic impact. Currently, preventative treatment options for migraine include drugs developed for diseases other than migraine such as hypertension, depression and epilepsy. During the last decade, however, blocking calcitonin gene-related peptide (CGRP) has emerged as a possible mechanism for prevention of migraine attacks. CGRP has been shown to be released during migraine attacks and it may play a causative role in induction of migraine attacks. Here, we review the pros and cons of blocking CGRP in migraine patients. To date, two different classes of drugs blocking CGRP have been developed: small molecule CGRP receptor antagonists (gepants), and monoclonal antibodies, targeting either CGRP or the CGRP receptor. Several trials have been conducted to test the efficacy and safety of these drugs. In general, a superior efficacy compared to placebo has been shown, especially with regards to the antibodies. In addition, the efficacy is in line with other currently used prophylactic treatments. The drugs have also been well tolerated, except for some of the gepants, which induced a transient increase in transaminases. Thus, blocking CGRP in migraine patients is seemingly both efficient and well tolerated. However, CGRP and its receptor are abundantly present in both the vasculature, and in the peripheral and central nervous system, and are involved in several physiological processes. Therefore, blocking CGRP may pose a risk in subjects with comorbidities such as cardiovascular diseases. In addition, long-term effects are still unknown. Evidence from animal studies suggests that blocking CGRP may induce constipation, affect the homeostatic functions of the pituitary hormones or attenuate wound healing. However, these effects have so far not been reported in human studies. In conclusion, this review suggests that, based on current knowledge, the pros of blocking CGRP in migraine patients exceed the cons.

Introduction

Migraine is a highly prevalent and disabling disorder for which treatment options are still inadequate. The underlying pathophysiology is largely unknown, but calcitonin gene-related peptide (CGRP) most likely plays an important role. The first time CGRP was hypothesized to be involved in migraine was in 1985[1]. This hypothesis was later supported by the finding of CGRP release during acute migraine attacks and the subsequent demonstration of normalization of CGRP levels in migraine patients after efficacious sumatriptan treatment[2]. In animal studies, triptans also inhibit the release of CGRP[3]. Evidence for a causative role of CGRP in migraine came from a study showing that intravenous provocation with CGRP induces migraine-like attacks in migraine patients[4]. This led to focus on this peptide and its receptor as a possible target for new migraine therapies.

CGRP and its receptor are expressed in both the peripheral and the central nervous system (CNS), including the trigeminovascular pathways. More than 30 years ago CGRP was demonstrated in trigeminal ganglion (TG) pseudounipolar neurons[5]. These neurons connect cranial structures to the central nervous system at the lower brainstem, caudal part of the trigeminal nucleus caudalis and upper spinal cord at C1-C2[6]. In the peripheral trigeminovascular system, as well as in the TG, CGRP is located in about 50% of the neurons and in unmyelinated Cfibers, whereas the CGRP receptor elements are expressed in about 40% of the TG neurons and in myelinated Aδ-fibers, which connect the PNS with the CNS[7,8]. In humans, CGRP is present in two isoforms, αCGRP and CGRP, where αCGRP is most abundantly found in primary spinal afferent from sensory ganglia, whereas βCGRP is mainly found in the enteric nervous system[6]. The CGRP receptor consists of three subunits: receptor activity-modifying protein 1 (RAMP1), calcitonin-like receptor (CLR) and receptor component protein (RCP)[9]. As well as playing a role in cranial nociception[10], CGRP is a potent general arterial vasodilator. At peripheral synapses, CGRP is released from trigeminal terminals results in vasodilation via CGRP

receptors on the smooth muscle cells of meningeal and cerebral blood vessels[8,11]. CGRP and its receptor are also located in the cardiovascular system where they are assumed to exert a protective role[9,12].

The first designer drug able to competitively block the effect of CGRP was olcegepant[13]. This nonpeptide CGRP-receptor antagonist showed high efficacy but had a low oral bioavailability[14]. This led, however, to the synthesis of several other small molecule CGRP receptor antagonists. This class was later called the gepants. Though promising with regards to efficacy, further development of some of the gepants was discontinued due to liver toxicity upon repeated exposure[15]. Encouraged by the efficacy of blocking CGRP for the treatment of migraine, monoclonal antibodies able to block either CGRP or its receptor were developed and tested in several preclinical modalities[16,17]. The antibodies are designer drugs that are highly specific for the target but about 500 times the size of gepants or triptans[6]. They have been designed for prophylactic use in frequent episodic and chronic migraine. In this review, we will discuss the pros and cons of blocking CGRP in migraine patients. We will review the efficacy and safety of already tested drugs and compare it to the efficacy and safety of topiramate, a widely-used migraine prophylactic. Additionally, we will review the possible consequences of blocking CGRP based on findings from animal studies. Lastly, we will discuss other concerns such as long-term use and cost of the treatment.

Efficacy of CGRP (receptor) blockade: Evidence from double-blind, placebo-controlled trials

In 2004, a proof-of-concept study showed that intravenous olcegepant was effective in the acute treatment of migraine[18]. Since then, five other gepants have been tested for the acute treatment of migraine[19-31]. Fig. 1 provides an overview of the efficacy data for these agents. All gepants were significantly better than placebo at achieving their primary outcome at adequate doses: pain freedom or relief at 2 hours. Only one study, a study on safety in coronary patients, could not demonstrate difference in pain freedom at 2 hours after telcagepant; however, only 165 of the planned 400 patients were included, reducing the statistical power of this study[27].

Five of these studies also included a comparison to a triptan[19,21,26,29,30]. In one of these studies, telcagepant showed a numerically higher efficacy than rizatriptan with regards to sustained pain relief[29]. In other trials, the efficacy of telcagepant, BI44370 and rimegepant showed no significant difference to that of zolmitriptan (5 mg), eletriptan (40 mg) and sumatriptan (100 mg), respectively[21,26,30]. In one large study, assessing the long-term safety of telcagepant, 19820 attacks were treated with telcagepant and 10981 attacks with rizatriptan. For two endpoints, pain freedom and pain relief at 2 hours, rizatriptan was superior compared to telcagepant (OR < 1 in favor of rizatriptan. OR (95% CI): 0.58 (0.45, 0.75) and 0.70 (0.55, 0.89), respectively). For all other pre-specified efficacy outcome measurements, no difference was found between the efficacy of telcagepant and rizatriptan at 2 hours[19].

Telcagepant has also been tested as prophylactic treatment of episodic migraine[25,28]. The first of these studies was terminated early due to adverse events and the pre-specified analyses could not be performed. However, post-hoc analysis showed telcagepant to be effective at four weeks in reducing migraine days[25]. In the second study, in a population of patients with perimenstrual migraine, administration of telcagepant in the perimenstrual period did not result in a significant reduction in mean monthly headache days, which was the primary endpoint[28]. There was a reduction of mean monthly on-drug headache days, but the reliability of this analysis is questionable, since no correction for multiple comparisons was done.

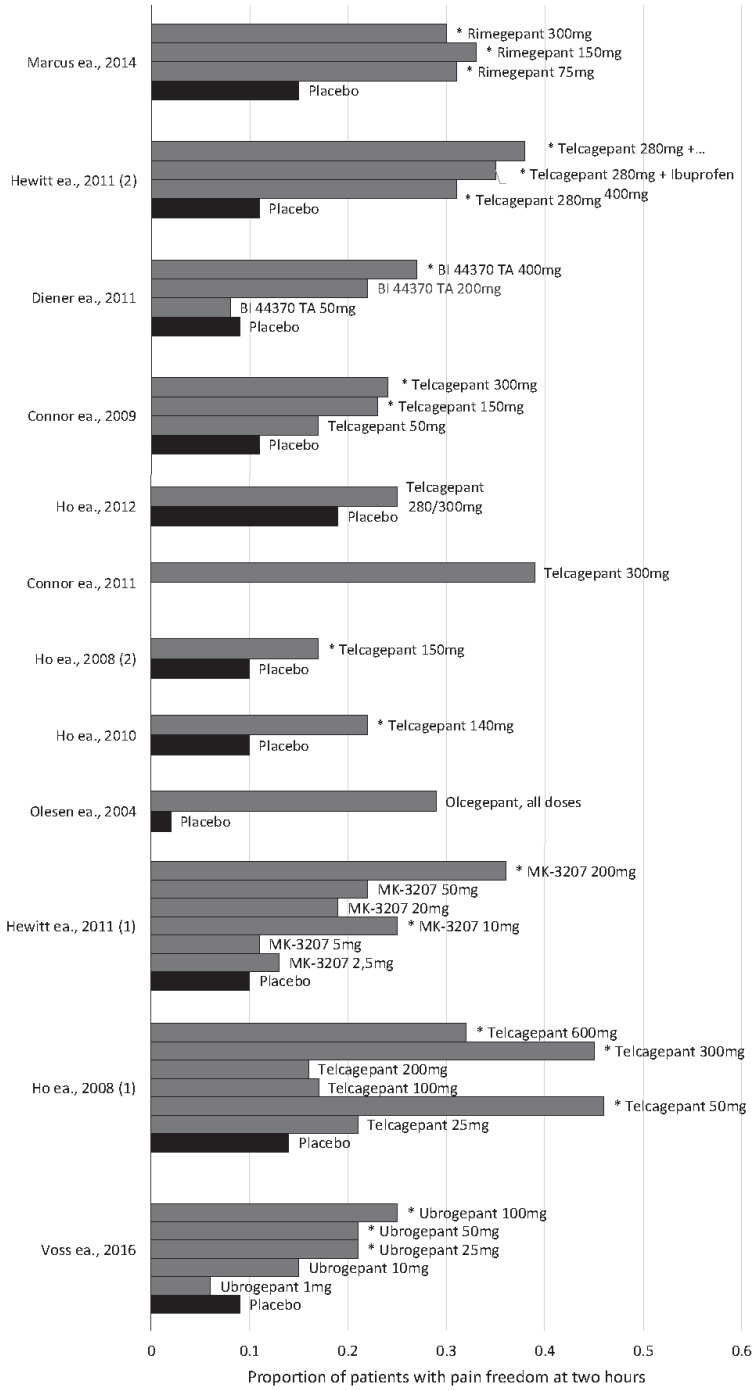

Fig. 1. Efficacy of gepants in the acute treatment of migraine. Bars indicated with * represents statistically significant values compared to placebo ($p<0.05$).

Antibodies against CGRP or the CGRP receptor have been tested as prophylactic treatment of episodic and chronic migraine. To date, four agents have been studied in six clinical studies[32-37]. Fig. 2 provides an overview of the efficacy data of the studies where reduction in migraine days was the primary endpoint. All monoclonal antibodies showed a significant reduction in their primary endpoint, either mean change from baseline in monthly migraine days (5 studies) or mean change in headache hours from baseline (1 study). These agents had an additional reduction over placebo of between 1 and 2.8 migraine days per month. In the study in chronic migraine, where change in headache hours was the primary outcome (data not included in the Fig.), the additional reduction over placebo was 22.7 and 30.4 hours per month for the two doses tested[37]. Interestingly, in one study, 11 of the 67 (16%) patients who had 5 to 14 migraine days per month at baseline, experienced no migraine days during the 12 week study period, versus no patients in the placebo group[34]. In another study, 31 of 98 (32%) patients reporting 4 to 15 migraine days at baseline had a 100% response (defined as a 28-day migraine free period over the 12-week treatment period). In the placebo group, only 18 of 104 (17%) of placebo patients had a 100% response[35]. No other studies reported on the 100% responder rate. Although these data seem interesting, they come from post-hoc analyses and so their significance remains unclear. The data from these 20 studies provides robust and consistent evidence for a crucial role of CGRP in migraine pathophysiology and a high efficacy of blocking CGRP as a prophylactic treatment.

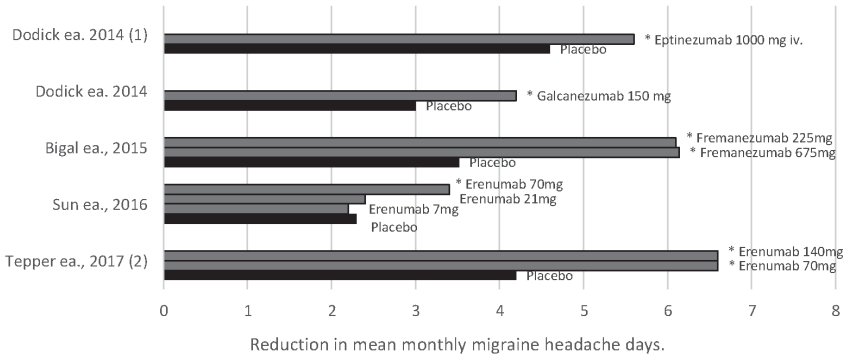

Fig. 2. Efficacy of monoclonal antibodies in the preventive treatment of migraine. Bars indicated with * represents statistically significant values compared to placebo ($p < 0.05$). (1) had change in mean monthly migraine days from baseline to weeks 5-8 as the primary endpoint. All other studies had change in monthly migraine days from baseline to weeks 9-12 of the 12-week double-blind treatment phase as the primary endpoint. In (1) the drug/placebo was administered intravenously. In all other studies, the drug/placebo were given subcutaneously. (2) is on chronic migraine patients. All other studies are on episodic migraine patients.

Is blocking CGRP as or more efficient than current preventative treatments?

Current preventative treatment options for migraine include antihypertensive drugs, antidepressants and antiepileptic medication. In contrast to CGRP (receptor) blockers, these have all been developed for diseases other than migraine and it is estimated that less than 50% of patients on prophylactics experience a 50% reduction in their monthly attack frequency[38].

Topiramate was proven efficient as a preventative treatment of episodic migraine after positive results from three randomized, multi-center, placebo-controlled studies. Thus, topiramate is currently recommended as a level A medication for prevention of episodic migraine with established efficacy (≥ 2 Class I trials) in the 2012 AHS/AAN guidelines[38]. Here, we review so far

published data from Phase III studies of the monoclonal antibodies[39-42] in relation to pivotal studies on topiramate[43-47] in episodic and chronic migraine. In three phase III studies, including over 1500 patients, topiramate 100 mg/d significantly reduced the number of monthly migraine days compared to placebo (reduction of monthly migraine days about -1.8 to -2.6 for topiramate vs. -1.0 to -1.3 for placebo). The ≥50% responder rates were also significantly higher for topiramate (37-54% vs. 22-23%, respectively)[44-46]. In the so far available data from Phase III studies of CGRP (receptor) antibodies, blocking of CGRP showed a similar efficacy with a reduction of monthly migraine days from baseline of -2.9 (verum) vs. -1.8 (placebo) for erenumab (AMG 334)[42]; -4.3 (300mg)/-3.9(100mg) vs. -3.2 (placebo) for eptinezumab (ALD-403)[40]; -4.0 (120mg)/-3.8 (240mg) vs. -2.15 (placebo) for galcanezumab (LY2951742)[39] and -3.7 (225mg monthly)/-3.4 (675mg quarterly) vs. -2.2 (placebo) for fremanezumab (TEV-48125)[41]. The ≥50% responder rates were also significantly higher than for placebo and similar, albeit a little higher, to those of topiramate, ranging from 56.3% to 62.3% (≥50% responder rates: eptinezumab: 56.3% (300mg)/49.8% (100mg) vs. 37.4% (placebo)[40]; galcanezumab: 62.3% (120mg)/60.9% (120mg) vs. 38.6% (placebo)[39]).

Topiramate has also proven efficacious in patients with chronic migraine[47]. In two randomized, placebo-controlled, double-blinded studies with 387 subjects with a daily dose of 100mg or 50-200mg topiramate showed a significant reduction in monthly migraine days compared to placebo (-6.4±5.8 vs. -4.7±6.1 and -3.5±6.3 vs. 0.2±4.7). The ≥50% responder rate was also significantly higher for topiramate (22% vs. 0% for placebo)[47]. In line with this, blocking of CGRP significantly reduced the number of monthly headache migraine days in 1113 chronic migraine patients with an average of 19.4 headache days (-4.8(120mg)/-4.6(240mg) vs. -2.7(placebo)). Likewise, the ≥50% responder rate was significantly higher for the active drug compared to placebo (27.6% (120mg)/27.5% (240mg vs. 15.4%)[48].

Another important aspect of medication is the incidence and severity of adverse events. Compared to topiramate, adverse events reported from CGRP trials were generally mild and less frequent. Upper respiratory tract infection/nasopharyngitis, and injection-site pain have so far been the most frequent reported adverse events[39-42] (see below for more details). In contrast, reported adverse events of topiramate, such as taste disturbance, weight loss, anorexia, fatigue, memory problems and paresthesia were more common in the active groups than in the placebo groups.

Safety issues regarding blocking of CGRP – are there any?

Evidence from clinical studies

Even though the knowledge of the presence and function of CGRP in the CNS is sparse, the function in both the peripheral and enteric nervous system is well established and CGRP is expressed widely throughout both systems. Thus, a wide variety of possible adverse events could be anticipated when blocking CGRP. However, reported adverse events after blocking of CGRP have in general been mild to moderate and the incidences have been low.

Among the first CGRP receptor antagonists under trial, intravenous olcegepant caused mild to moderate adverse events such as paresthesia, nausea, headache, dry mouth and unspecific vision disturbances in a minority of patients[18]. However, more serious adverse events were reported with telcagepant and MK-3207, which caused liver toxicity with transient increase of transaminases in a small group of included subjects (n=13 for telcagepant) upon repeated doses. This lead to discontinuation of the trial program for these molecules[15,25]. Other non-peptide CGRP receptor antagonists such as BI44370TA, BMS-927711, and, most recently, MK-1602 have also been tested. For all three molecules adverse events were mild to moderate and the incidence was low and similar to the placebo group[21,30,31]. No liver toxicity was reported for these drugs, and the gepant program is thus still ongoing.

More recently, great attention has been given to the development and testing of monoclonal antibodies (mABs) targeting circulating CGRP or its receptors. Most importantly, none of these drugs show liver toxicity. This is in line with the theoretical probability of mABs causing liver toxicity, which is very low, since metabolism of mABs do not result in production of toxic metabolites[49]. In addition, despite the potentially harmful inhibition of vasodilation due to CGRP inhibition, no cardiovascular concerns have been disclosed with any of these drugs[50]. In trials, eptinezumab, galcanezumab and fremanezumab, monoclonal antibodies which all target CGRP, showed variable percentages of adverse events, which in line with the gepants, were mild to moderate (e.g. upper respiratory or urinary tract infection, fatigue, back pain, arthralgia, nausea and vomiting). Erenumab, which binds to the CGRP receptor, was also safe and well tolerated in a phase 2 trial[32].

In line with the poor chance of both the non-peptide CGRP receptor antagonists and the antibodies crossing the blood-brain barrier (BBB)[51], no central side effects have been reported so far. Therefore, although crossing of the BBB is likely to occur to some extent, telcagepant has been detected in primates cerebrospinal fluid, suggesting its presence in the CNS[52], a central effect, and side effect, of these drugs seems unlikely.

Do preclinical studies give reason to be concerned about side effects?

CGRP is an ubiquitous peptide that is not only involved in migraine, but also in several physiological processes[12] and in homeostatic responses during pathophysiological conditions (Fig. 3)[9,12]. As such, it is vital to consider the possible side effects caused by the non-selective blockade of α and βCGRP with the CGRP (receptor)-antibodies. As discussed in the previous section, the adverse events of the Phase II trials were mild[32-37], but it should be noted that the duration of these trials is not sufficient to see the long-term effects of continuingly blocking CGRP or its receptor.

In the cardiovascular system, CGRP is present in nerve fibers that innervate blood vessels[53] and the heart[54,55], and participates in the regulation of blood pressure[12,55-57]. Furthermore, it has also been described to have a role in the maintenance of (cardio)vascular homeostasis during ischemic events[9] and in tissue remodeling in pulmonary hypertension[58]. This protective role raises a concern, since migraine patients present an increased cardiovascular risk[59,60]. This topic was recently reviewed elsewhere[9]. Hence, it is important to consider preexisting cardiovascular risk factors in patients (i.e. family history, tobacco exposure, obesity) to prevent a possible cardiovascular event.

Although CGRP participates in inflammatory processes[61-63], it has also been associated with facilitation of wound healing[64]. This is thought to be mediated through its ability to promote keratinocytes proliferation[65], enhance revascularization[66], reduce expression of TNF-α and attenuate macrophage infiltration[67]. A consequence of blocking CGRP could thus be alterations in wound healing and increased inflammatory responses in skin injuries at the site of injection for the antibodies. However, this is a theoretical risk which has so far not been observed in clinical trials.

The antibodies against CGRP are not selective for αCGRP but also block βCGRP. The gastrointestinal tract is highly innervated by βCGRPergic fibers from the enteric nervous system[68,69]. In fact, animal studies with antibodies against CGRP showed extensive mucosal damage[70,71], suggesting a role of CGRP in maintaining the mucosal integrity of the gastrointestinal tract. Blocking this could thus contribute to inflammatory bowel disease. Gastrointestinal motility is also considered to be modulated by CGRP, and administration of this peptide induces a dose-dependent biphasic response[72], which could lead to episodes of diarrhea or constipation. Furthermore, studies with CGRP KO mice have suggested CGRP agonists as a possible treatment for ulcer healing[73]; therefore, monitoring of gastrointestinal complications (i.e. ulcers, constipation) is recommended, even though 12 week studies have not reported these.

Finally, since, as mentioned, it is unlikely that the antibodies cross the BBB, and that the BBB penetration is changed during migraine attacks[74,75], it is important to consider the structures from CNS that are not protected by the BBB. Recent studies have demonstrated that the TG, together with the pituitary, are outside the BBB[76]. An effect on the TG could thus, partly, explain the therapeutic effect of the antibodies. However, CGRP and its receptor are also expressed in the anterior pituitary, suggesting a possible involvement in the regulation of hypothalamo-pituitary tract functions[77]. The exact involvement is still unknown, and further studies are needed to determine the long-term effects of blocking CGRP on the homeostatic functions of the pituitary hormones.

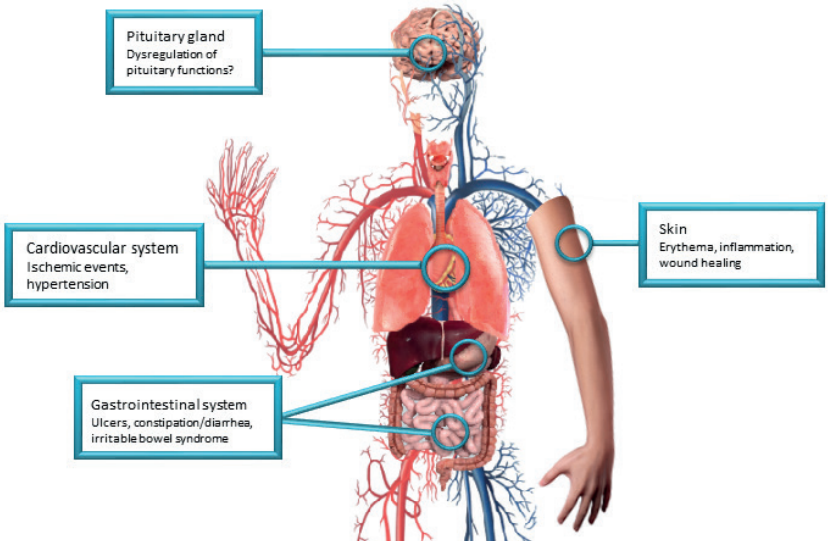

Fig. 3. Possible side effects after long-term exposure to CGRP (receptor)-antibodies. An overview of the organ systems where CGRP and the receptor are present and possible side effects that could be caused by the non-selective blockade of α- and β-CGRP with the CGRP (receptor)-antibodies

Other considerations

Even though blocking of CGRP seems to be an efficacious and safe preventative treatment of migraine, there are many other aspects to consider with regards to the pros and cons of blocking CGRP in migraine patients.

Firstly, the administration of the newly developed monoclonal antibodies is either intravenous or subcutaneous. This could potentially cause complications at the injection site, and common adverse events in those treated with fremanezumab, galcanezumab and erenumab were indeed mild injection-site pain, pruritus and erythema[78]. A disadvantage of the intravenous administration route is the need of it being administered by a medical doctor. This not only increases the placebo response in clinical trials, but does also require for the patient to spend time visiting the clinic – increasing the risk of pathologization of the patient. However, the monthly administration, which is feasible due to the long half-lives of the medication, could improve adherence and compliance to medication, which is a common problem in treating migraine[79,80]. Additionally, the CGRP antibodies seem to show a low risk for drug-drug interactions and hepatotoxicity since they are metabolized by degradation into peptides and single amino acids[81], which could be important for patients using multiple medications.

Secondly, as mentioned, the long-term risks of blocking CGRP are still unknown. Even though the absence of liver toxicity or other abnormalities in routine blood testing is in support of no or low long-term risks[78], studies testing the cardiovascular safety of the long-term blockade are warranted in order to answer the numerous questions on the possibility of higher risk in cardio- and cerebrovascular compromised patients. For example, it is unknown whether blocking CGRP could potentially transform transient mild cerebral ischemia into a full-blown brain infarct[9] and whether these risks are higher in women[9,82]. To investigate these aspects, future studies should include patients with preexisting cardiovascular conditions.

Thirdly, the exact site of action of blocking CGRP is still partly unknown and CGRP could exert its effects on receptors distinct from the CGRP receptor[9]. Recently it was put forward that CGRP may act on the amylin receptor in TG[83] as well as in human coronary arteries[84]. If this is the case, this could pose an additional – unknown – potential risk of wiping out CGRP. We can also only guess whether patients not benefitting from receptor blockade would benefit from blockage of the peptide itself. Future studies should investigate how to differentiate responders from non-responders.

Lastly, a disadvantage when using antibodies is the risk of development of antibodies against the drug[15]. Indeed, antidrug antibodies were detected with all four antibodies[78], but these did not seem to affect efficacy[32]. However, long-term studies are needed to investigate whether, at long term, neutralizing antidrug antibodies will pose a problem for efficacy and safety of blocking CGRP with monoclonal antibodies. Finally, it is well known that antibody treatment is costly and the price of the drugs has to be taken into consideration when deciding whether to use CGRP antibodies as a prophylactic treatment and which patient groups to treat.

Conclusion

Here, we have reviewed the pros and cons of blocking CGRP in migraine patients. In favor of using blocking of CGRP as a treatment of migraine, is that, based on evidence from clinical trials, whether using small molecule receptor antagonists or antibodies, the treatment is efficacious. Additionally, the liver toxicity induced by some of the gepants is not present with the antibodies, which are well tolerated. Lastly, in contrast to current prophylactic treatments, the drugs are developed specifically for migraine, based on findings from human migraine studies. Thus, the drugs may exert a more direct effect on migraine specific pathways than previously used prophylactic drugs. In addition, this provides hope and encouragement for further research into the pathophysiological mechanisms of migraine and potentially the discovery of other migraine specific therapeutic targets.

Speaking against chronically blocking CGRP, the long-term effects, particularly regarding the cardiovascular risks, are still unknown as well as the exact mode of action of the antibodies. In addition, development of neutralizing antidrug antibodies may, with time, affect the efficacy of the antibodies. Lastly, as with all antibody therapies, CGRP antibodies have the problem of being costly. However, taking into consideration the enormous socioeconomically burden that migraine is[85], the price may be well payed off.

In conclusion, based on current knowledge, we believe that the benefits of blocking CGRP, including the perspectives of improving the lives of those suffering from frequent headaches, seems to be greater than the disadvantages.

References

1. Edvinsson L. Functional role of perivascular peptides in the control of cerebral circulation. Trends in Neurosciences 1985;8:126-131.
2. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: Studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of Neurology 1993;33:48-56.

3. Goadsby PJ, Edvinsson L. Peripheral and Central Trigeminovascular Activation in Cat is Blocked by the Serotonin (5HT)-$_{1D}$ Receptor Agonist 311C90. Headache: The Journal of Head and Face Pain 1994;34:394-399.
4. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22:54-61.
5. Uddman R, Edvinsson L, Ekman R, Kingman T, McCulloch J. Innervation of the feline cerebral vasculature by nerve fibers containing calcitonin gene-related peptide: Trigeminal origin and co-existence with substance P. Neuroscience Letters 1985;62:131-136.
6. Edvinsson L. CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. British journal of clinical pharmacology 2015;80:193-199.
7. Eftekhari S, Warfvinge K, Blixt FW, Edvinsson L. Differentiation of Nerve Fibers Storing CGRP and CGRP Receptors in the Peripheral Trigeminovascular System. The Journal of Pain 2013;14:1289-1303.
8. Eftekhari S, Edvinsson L. Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. Therapeutic advances in neurological disorders 2010;3:369-378.
9. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
10. Schou WS, Ashina S, Amin FM, Goadsby PJ, Ashina M. Calcitonin gene-related peptide and pain: a systematic review. The Journal of Headache and Pain 2017;18:34.
11. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet 2010;376:645-655.
12. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. Physiological Reviews 2014;94:1099.
13. Doods H, Hallermayer G, Wu D, et al. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. British Journal of Pharmacology 2000;129:420-423.
14. Vécsei L, Szok D, Csáti A, Tajti J. CGRP antagonists and antibodies for the treatment of migraine. Expert Opinion on Investigational Drugs 2015;24:31-41.
15. Bigal ME, Walter S, Rapoport AM. Calcitonin Gene-Related Peptide (CGRP) and Migraine Current Understanding and State of Development. Headache: The Journal of Head and Face Pain 2013;53:1230-1244.
16. Juhl L, Edvinsson L, Olesen J, Jansen-Olesen I. Effect of two novel CGRP-binding compounds in a closed cranial window rat model. European Journal of Pharmacology 2007;567:117-124.
17. Edvinsson L, Nilsson E, Jansen-Olesen I. Inhibitory effect of BIBN4096BS, CGRP8–37, a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery. British Journal of Pharmacology 2007;150:633-640.
18. Olesen J, Diener H-C, Husstedt IW, et al. Calcitonin Gene–Related Peptide Receptor Antagonist BIBN 4096 BS for the Acute Treatment of Migraine. New England Journal of Medicine 2004;350:1104-1110.
19. Connor KM, Aurora SK, Loeys T, et al. Long-Term Tolerability of Telcagepant for Acute Treatment of Migraine in a Randomized Trial. Headache: The Journal of Head and Face Pain 2011;51:73-84.
20. Connor KM, Shapiro RE, Diener HC, et al. Randomized, controlled trial of telcagepant for the acute treatment of migraine. Neurology 2009;73:970-977.
21. Diener H-C, Barbanti P, Dahlöf C, Reuter U, Habeck J, Podhorna J. BI 44370 TA, an oral CGRP antagonist for the treatment of acute migraine attacks: Results from a phase II study. Cephalalgia 2011;31:573-584.
22. Hewitt DJ, Aurora SK, Dodick DW, et al. Randomized controlled trial of the CGRP receptor antagonist MK-3207 in the acute treatment of migraine. Cephalalgia 2011;31:712-722.
23. Hewitt DJ, Martin V, Lipton RB, et al. Randomized Controlled Study of Telcagepant Plus Ibuprofen or Acetaminophen in Migraine. Headache: The Journal of Head and Face Pain 2011;51:533-543.
24. Ho AP, Dahlöf CGH, Silberstein SD, et al. Randomized, controlled trial of telcagepant over four migraine attacks. Cephalalgia 2010;30:1443-1457.
25. Ho TW, Connor KM, Zhang Y, et al. Randomized controlled trial of the CGRP receptor antagonist telcagepant for migraine prevention. Neurology 2014;83:958-966.
26. Ho TW, Ferrari MD, Dodick DW, et al. 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. The Lancet 2008;372:2115-2123.
27. Ho TW, Ho AP, Chaitman BR, et al. Randomized, Controlled Study of Telcagepant in Patients With Migraine and Coronary Artery Disease. Headache: The Journal of Head and Face Pain 2012;52:224-235.
28. Ho TW, Ho A, Ge Y, et al. Randomized controlled trial of the CGRP receptor antagonist telcagepant for prevention of headache in women with perimenstrual migraine. Cephalalgia 2015;36:148-161.

29. Ho TW, Mannix LK, Fan X, et al. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. Neurology 2008;70:1304-1312.
30. Marcus R, Goadsby PJ, Dodick D, Stock D, Manos G, Fischer TZ. BMS-927711 for the acute treatment of migraine: A double-blind, randomized, placebo controlled, dose-ranging trial. Cephalalgia 2013;34:114-125.
31. Voss T, Lipton RB, Dodick DW, et al. A phase IIb randomized, double-blind, placebo-controlled trial of ubrogepant for the acute treatment of migraine. Cephalalgia 2016;36:887-898.
32. Sun H, Dodick DW, Silberstein S, et al. Safety and efficacy of AMG 334 for prevention of episodic migraine: a randomised, double-blind, placebo-controlled, phase 2 trial. The Lancet Neurology 2016;15:382-390.
33. Tepper S, Ashina M, Reuter U, et al. Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. Lancet Neurol 2017.
34. Dodick DW, Goadsby PJ, Silberstein SD, et al. 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. The Lancet Neurology 2014;13:1100-1107.
35. Dodick DW, Goadsby PJ, Spierings ELH, Scherer JC, Sweeney SP, Grayzel DS. 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. The Lancet Neurology 2014;13:885-892.
36. Bigal ME, Dodick DW, Rapoport AM, et al. 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. The Lancet Neurology 2015;14:1081-1090.
37. Bigal ME, Edvinsson L, Rapoport AM, et al. Safety, tolerability, and efficacy of TEV-48125 for preventive treatment of chronic migraine: a multicentre, randomised, double-blind, placebo-controlled, phase 2b study. The Lancet Neurology 2015;14:1091-1100.
38. D'Amico D, Tepper SJ. Prophylaxis of migraine: general principles and patient acceptance. Neuropsychiatric disease and treatment 2008;4:1155-1167.
39. Lilly's Galcanezumab Significantly Reduces Number of Migraine Headache Days for Patients with Migraine: New Results Presented at AHS [online]. https://investor.lilly.com/releasedetail.cfm?releaseid=1029791. Accessed 21 Aug
40. Alder BioPharmaceuticals Announces Positive Eptinezumab Phase 3 Results for Prevention of Frequent Episodic Migraine. [online]. http://investor.alderbio.com/releasedetail.cfm?releaseid=1031418. Accessed 21 Aug.
41. Teva's Fremanezumab Meets all Primary & Secondary Endpoints Across Both Monthly and Quarterly Dosing Regimens in Phase III Study in Episodic Migraine Prevention [online]. Available at: https://www.tevapharm.com/news/teva_s_fremanezumab_meets_all_primary_secondary_endpoints_across_both_monthly_and_quarterly_dosing_regimens_in_phase_iii_study_in_episodic_migraine_prevention_06_17.aspx. Accessed 21 Aug.
42. Novartis announces Phase III study shows AMG 334 significantly reduces monthly migraine days in people with episodic migraine [online]. Available at: https://www.novartis.com/news/media-releases/novartis-announces-phase-iii-study-shows-amg-334-significantly-reduces-monthly. Accessed 21 Aug.
43. Brandes JL, Saper JR, Diamond M, et al. Topiramate for Migraine PreventionA Randomized Controlled Trial. Jama 2004;291:965-973.
44. Diener H-C, Tfelt-Hansen P, Dahlöf C, et al. Topiramate in migraine prophylaxis. Journal of Neurology 2004;251:943-950.
45. Silberstein SD, Neto W, Schmitt J, Jacobs D, for the M-SG. Topiramate in Migraine Prevention: Results of a Large Controlled Trial. Archives of Neurology 2004;61:490-495.
46. Silberstein SD, Lipton RB, Dodick DW, et al. Efficacy and Safety of Topiramate for the Treatment of Chronic Migraine: A Randomized, Double-Blind, Placebo-Controlled Trial. Headache: The Journal of Head and Face Pain 2007;47:170-180.
47. Diener HC, Bussone G, Oene JCV, Lahaye M, Schwalen S, Goadsby PJ. Topiramate Reduces Headache Days in Chronic Migraine: A Randomized, Double-Blind, Placebo-Controlled Study. Cephalalgia 2007;27:814-823.
48. Wang W, Wang EQ, Balthasar JP. Monoclonal Antibody Pharmacokinetics and Pharmacodynamics. Clinical Pharmacology & Therapeutics 2008;84:548-558.
49. Bigal ME, Walter S, Rapoport AM. Therapeutic antibodies against CGRP or its receptor. British Journal of Clinical Pharmacology 2015;79:886-895.
50. Iyengar S, Ossipov MH, Johnson KW. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. Pain 2017;158:543-559.
51. Sur C, Hargreaves R, Bell I, et al. CSF levels and binding pattern of novel CGRP receptor antagonists in rhesus monkey and human central nervous system: toward the development of a PET tracer. Cephalalgia; 2009: 136-137.

52. Uddman R, Edvinsson L, Ekblad E, Håkanson R, Sundler F. Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regulatory Peptides 1986;15:1-23.
53. Wimalawansa SJ, MacIntyre I. Calcitonin gene-related peptide and its specific binding sites in the cardiovascular system of rat. Int J Cardiol 1988;20:29-37.
54. Opgaard OS, Gulbenkian S, Bergdahl A, et al. Innervation of human epicardial coronary veins: immunohistochemistry and vasomotility. Cardiovascular Research 1995;29:463-468.
55. Lindstedt IH, Edvinsson ML, Evinsson L. Reduced responsiveness of cutaneous microcirculation in essential hypertension – A pilot study. Blood Pressure 2006;15:275-280.
56. McCulloch J, Uddman R, Kingman TA, Edvinsson L. Calcitonin gene-related peptide: functional role in cerebrovascular regulation. Proceedings of the National Academy of Sciences 1986;83:5731-5735.
57. Edvinsson L, Mulder H, Goadsby PJ, Uddman R. Calcitonin gene-related peptide and nitric oxide in the trigeminal ganglion: Cerebral vasodilatation from trigeminal nerve stimulation involves mainly calcitonin gene-related peptide. Journal of the Autonomic Nervous System 1998;70:15-22.
58. Keith IM, Tjen-A-Looi S, Kraiczi H, Ekman R. Three-week neonatal hypoxia reduces blood CGRP and causes persistent pulmonary hypertension in rats. American Journal of Physiology - Heart and Circulatory Physiology 2000;279:H1571-H1578.
59. Sacco S, Kurth T. Migraine and the risk for stroke and cardiovascular disease. Curr Cardiol Rep 2014;16:524.
60. Kurth T, Winter AC, Eliassen AH, et al. Migraine and risk of cardiovascular disease in women: prospective cohort study. BMJ 2016;353:i2610.
61. Salmon A-M, Damaj MI, Marubio LM, Epping-Jordan MP, Merlo-Pich E, Changeux J-P. Altered neuroadaptation in opiate dependence and neurogenic inflammatory nociception in αCGRP-deficient mice. Nature Neuroscience 2001;4:357.
62. Zhang L, Hoff AO, Wimalawansa SJ, Cote GJ, Gagel RF, Westlund KN. Arthritic calcitonin/α calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain 2001;89:265-273.
63. Benschop RJ, Collins EC, Darling RJ, et al. Development of a novel antibody to calcitonin gene-related peptide for the treatment of osteoarthritis-related pain. Osteoarthritis and Cartilage 2014;22:578-585.
64. Khalil Z, Helme R. Sensory peptides as neuromodulators of wound healing in aged rats. J Gerontol A Biol Sci Med Sci 1996;51:B354-361.
65. Roggenkamp D, Köpnick S, Stäb F, Wenck H, Schmelz M, Neufang G. Epidermal Nerve Fibers Modulate Keratinocyte Growth via Neuropeptide Signaling in an Innervated Skin Model. Journal of Investigative Dermatology 2013;133:1620-1628.
66. Mishima T, Ito Y, Hosono K, et al. Calcitonin gene-related peptide facilitates revascularization during hindlimb ischemia in mice. American Journal of Physiology - Heart and Circulatory Physiology 2011;300:H431-H439.
67. Zhang X, Zhuang J, Wu H, et al. Inhibitory Effects of Calcitonin Gene-Related Peptides on Experimental Vein Graft Disease. The Annals of Thoracic Surgery 2010;90:117-123.
68. Mulderry PK, Ghatei MA, Spokes RA, et al. Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience 1988;25:195-205.
69. Sternini C. Enteric and visceral afferent CGRP neurons. Targets of innervation and differential expression patterns. Ann N Y Acad Sci 1992;657:170-186.
70. Peskar BM, Wong HC, Walsh JH, Holzer P. A monoclonal antibody to calcitonin gene-related peptide abolishes capsaicin-induced gastroprotection. European Journal of Pharmacology 1993;250:201-203.
71. Reinshagen M, Flamig G, Ernst S, et al. Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. J Pharmacol Exp Ther 1998;286:657-661.
72. Bartho L, Koczan G, Maggi CA. Studies on the mechanism of the contractile action of rat calcitonin gene-related peptide and of capsaicin on the guinea-pig ileum: effect of hCGRP (8-37) and CGRP tachyphylaxis. Neuropeptides 1993;25:325-329.
73. Ohno T, Hattori Y, Komine R, et al. Roles of calcitonin gene-related peptide in maintenance of gastric mucosal integrity and in enhancement of ulcer healing and angiogenesis. Gastroenterology 2008;134:215-225.
74. Schankin CJ, Maniyar FH, Seo Y, et al. Ictal lack of binding to brain parenchyma suggests integrity of the blood-brain barrier for 11C-dihydroergotamine during glyceryl trinitrate-induced migraine. Brain 2016;139:1994-2001.
75. Hougaard A, Amin FM, Christensen CE, et al. Increased brainstem perfusion, but no blood-brain barrier disruption, during attacks of migraine with aura. Brain 2017;140:1633-1642.
76. Eftekhari S, Salvatore CA, Johansson S, Chen TB, Zeng Z, Edvinsson L. Localization of CGRP, CGRP receptor, PACAP and glutamate in trigeminal ganglion. Relation to the blood-brain barrier. Brain Research 2015;1600:93-109.

77. Wimalawansa SJ, el-Kholy AA. Comparative study of distribution and biochemical characterization of brain calcitonin gene-related peptide receptors in five different species. Neuroscience 1993;54:513-519.
78. Mitsikostas DD, Reuter U. Calcitonin gene-related peptide monoclonal antibodies for migraine prevention: comparisons across randomized controlled studies. Current Opinion in Neurology 2017;30:272-280.
79. Hepp Z, Bloudek LM, Varon SF. Systematic Review of Migraine Prophylaxis Adherence and Persistence. Journal of Managed Care Pharmacy 2014;20:22-33.
80. Hepp Z, Dodick DW, Varon SF, Gillard P, Hansen RN, Devine EB. Adherence to oral migraine-preventive medications among patients with chronic migraine. Cephalalgia 2015;35:478-488.
81. Zhou H, Mascelli MA. Mechanisms of Monoclonal Antibody–Drug Interactions. Annual Review of Pharmacology and Toxicology 2011;51:359-372.
82. Gangula PRR, Wimalawansa SJ, Yallampalli C. Sex Steroid Hormones Enhance Hypotensive Effects of Calcitonin Gene-Related Peptide in Aged Female Rats1. Biology of Reproduction 2002;67:1881-1887.
83. Walker CS, Eftekhari S, Bower RL, et al. A second trigeminal CGRP receptor: function and expression of the AMY_1 receptor. Annals of Clinical and Translational Neurology 2015;2:595-608.
84. Haanes KA, Chan KY, MaassenVanDenBrink A. Comment on "A second trigeminal CGRP receptor: function and expression of the AMY_1 receptor". Annals of clinical and translational neurology 2016;3:307-308.
85. Estemalik E, Tepper S. Preventive treatment in migraine and the new US guidelines. Neuropsychiatric disease and treatment 2013;9:709-720.

Chapter VII.

Is CGRP receptor blockade cardiovascularly safe? Appropriate studies are needed

*Based on: A Maassen van den Brink, **E Rubio-Beltrán**, D Duncker, CM Villalón (2018) Headache; 58:11257-1258*

With great interest, we read the publication by Depre and colleagues[1] describing that inhibition of the canonical CGRP receptor does not seem to worsen myocardial ischemia contrary to theoretical concerns[2]. In a randomized, double-blind, placebo-controlled study the authors did not find evidence for an adverse effect of the CGRP receptor antibody erenumab on exercise time during a treadmill test in patients with stable angina. Although we certainly appreciate the endeavor to address this important issue, we are concerned that the study population, the study design, and the interpretation of the results do not allow for such a reassuring conclusion.

While the authors rightly indicate that it is currently not clear to which extent CGRP is relevant in maintaining blood flow in case of myocardial and cerebral ischemia, we do not agree with their argument stating that "the concentrations of exogenous CGRP required to increase total exercise time or protect against myocardial ischemia far exceed the endogenous physiological levels of CGRP that are released during a response to ischemia". This is because it is not the systemic plasma concentration that is relevant in this perspective, but the actual concentration of CGRP at the *neuro-vascular junction*, where CGRP is released. Obviously, the plasma concentration is likely to be several log units lower than the junctional concentration due to dilution and hydrolysis. Further, we feel that the argument that erenumab does not contract the human isolated coronary artery per se[3] does not add to the introduction, since the question that should be answered here is whether inhibition of the actions of CGRP is potentially harmful in myocardial ischemia.

More importantly, the patients included in this study suffered from stable angina pectoris, which often is caused by a stenosis of the epicardial conducting portions of the coronary artery. As we pointed out earlier[2], the importance of CGRP in the proximal, epicardial portions of the coronary artery bed seems limited, while CGRP is a highly effective vasodilator in the intramyocardial, smaller (distal) sections of the coronary artery bed. Thus, it is unfortunate that a patient population with, most likely, mainly diseased proximal coronary arteries, was chosen for this study, despite the advantage of a clear-cut definition of these patients. Although stable angina pectoris due to epicardial stenosis may occur in both men and women, this is typically considered a "male" form of cardiac pathology[4], as illustrated by the fact that 78% of patients included in the current study were male. In contrast, in females, who are the majority of migraine sufferers and thus also the majority of the population likely to use erenumab or related drugs in future, coronary artery disease often presents as diffuse atherosclerosis, without an angiographically detectable stenosis[4-7]. These observations indicate that coronary microvascular dysfunction plays a more important role in angina pectoris in female patients and that blocking the effects of CGRP in female patients may have different effects than in male patients.

An essential concern of this study is based on pharmacokinetic and pharmacodynamic considerations. The authors rightly indicate that plasma concentrations obtained 30 minutes after intravenous infusion of 140-mg erenumab (the time interval until the start of the treadmill test) will provide "a substantial margin over concentrations achieved by subcutaneous administration of 140 mg". In contrast, their claim that "the use of 140 mg intravenous dose of erenumab ensured rapid and robust blockade of the CGRP receptor" is not substantiated by any evidence. It should be taken into account that, before a receptor blocking antibody can effectively occupy the receptor where it is binding to, the antibody should first have access to the receptor biophase. In this case, erenumab was infused intravenously and thus reached the blood vessel wall from the luminal side. The CGRP receptor is located in the smooth muscle wall[5], thus it may take several hours before the receptor was reached by erenumab at sufficiently high concentrations to induce an effective blockade of the CGRP receptor, especially given the large molecular size (150 kDa) of erenumab. This is well beyond the time the treadmill test had finished. A way to verify whether blockade has been achieved (at

least in skin blood vessels) is by assessing blockade of capsaicin-induced increases in dermal blood flow. The earliest time point for such measurements that has been published, to the best of our knowledge, for erenumab is 2 days after intravenous administration[8], which is about 100-fold longer than the period applied in the current study. Thus, we feel that evidence for significant blockade of the canonical CGRP receptor should have been provided to substantiate the statement that CGRP receptor blockade was reached during the treadmill study, since otherwise the interpretation of the current study is questionable.

Taken together, we would politely urge the authors to provide evidence for the fact that vascular CGRP receptor blockade has been achieved 30 minutes after intravenous infusion of erenumab, since this is a crucial part of the study. Further, we plead for cardiovascular safety studies on patients and/or experimental animals with microvascular disease, as such a group may better represent the patients at cardiovascular risk after the use of erenumab. Even if the antibodies against CGRP or its receptor would not increase the risk for cardiovascular ischemia, there will be cases of patients with ischemic complaints, even without a causal relationship. Appropriate studies in relevant subjects may avoid sudden distress, such as happened with the triptans in the past.

References

1. Depre C, Antalik L, Starling A, et al. 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; 2018;58:715-723.
2. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
3. Rubio-Beltran E, Labastida A, de Vries R, et al. Effects of AMG 334 on human isolated coronary artery. Cephalalgia; 2016: 41-41.
4. Humphries KH, Pu A, Gao M, Carere RG, Pilote L. Angina with "normal" coronary arteries: Sex differences in outcomes. American Heart Journal 2008;155:375-381.
5. Maas AHEM, Stam HCG, Eizema K, et al. Red alert for women's heart: the urgent need for more research and knowledge on cardiovascular disease in women: Proceedings of the Workshop held in Brussels on Gender Differences in Cardiovascular disease, 29 September 2010. European Heart Journal 2011;32:1362-1368.
6. Duncker DJ, Koller A, Merkus D, Canty JM. Regulation of Coronary Blood Flow in Health and Ischemic Heart Disease. Progress in Cardiovascular Diseases 2015;57:409-422.
7. Chan KY, Edvinsson L, Eftekhari S, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. J Pharmacol Exp Ther 2010;334:746-752.
8. Vu T, Ma P, Chen JS, et al. Pharmacokinetic-Pharmacodynamic Relationship of Erenumab (AMG 334) and Capsaicin-Induced Dermal Blood Flow in Healthy and Migraine Subjects. Pharmaceutical Research 2017;34:1784-1795.

Chapter VIII.

Characterization of vasodilatory responses in the presence of the CGRP receptor antibody erenumab in human isolated arteries

Based on: ***E Rubio-Beltrán****, A Labastida-Ramírez, KA Haanes, A vd Bogaerdt, AJJC Bogers, C Dirven, AHJ Danser, C Xu, J Snellman and A MaassenVanDenBrink (2019) Cephalalgia; In Press.*

Abstract

Background: Migraine is associated with activation of the trigeminovascular system, release of calcitonin gene-related peptide (CGRP) and dilation of dural arteries. Novel treatments target CGRP or its receptor, which are present in all vascular beds, raising cardiovascular concerns. Erenumab is a human CGRP-receptor antibody approved for the prophylactic treatment of migraine.

Methods: We characterized the relaxant responses to CGRP in the absence and presence of erenumab (1 μM) in isolated human middle meningeal (HMMA), internal mammary (HIMA) and (proximal and distal) coronary arteries (HCA). Furthermore, in HIMA from cardiovascularly-compromised patients, we assessed the pharmacological specificity of erenumab by investigating whether the vasodilatory responses to acetylcholine, sodium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) and nicardipine, along with the vasoconstrictor responses to dihydroergotamine, were modified by erenumab.

Results: CGRP induced concentration-dependent vasodilatory responses in all vessels studied that were significantly antagonized by erenumab. In HIMA from cardiovascularly-compromised patients, the responses to acetylcholine, sodium nitroprusside, PACAP, VIP, nicardipine and dihydroergotamine were unaffected by erenumab.

Conclusion: Erenumab inhibits CGRP-induced vasodilatory responses in HMMA, HIMA and HCA. Moreover, erenumab shows functional specificity as no interaction was observed with the relaxant responses to several vasodilators, nor the dihydroergotamine-dependent vasoconstrictor responses.

Introduction

Migraine is a highly disabling neurovascular disorder and its pathophysiology remains elusive. However, it has been associated with an activation of the trigeminovascular system, release of calcitonin gene-related peptide (CGRP) and increase in middle meningeal artery circumference specific to the head pain side[1]. Based on the involvement of CGRP in the pain signalling pathway of migraine, small molecule-CGRP receptor antagonists (gepants) were developed for the treatment of migraine. The first gepants did not reach the market, due to hepatotoxicity cases and pharmacokinetic problems[2]. Although novel gepants are currently being developed, with no reported toxicity so far[3], the most recent approach for CGRP blockade consists of the antibodies against CGRP (eptinezumab, fremanezumab, galcanezumab) or its receptor (erenumab). They have all shown to be effective for the prophylactic treatment of migraine and are either approved or likely soon to be approved for commercialization[4].

While the development of antibodies directed against the CGRP pathway (*i.e.,* antibodies against CGRP or the CGRP receptor) represents a milestone in migraine treatment, it is important to also consider the implications of peripheral CGRP receptor blockade. To begin with, CGRP fibres innervate blood vessels, and are thought to contribute in the homeostatic responses to ischemic events[5-7]. This raises some concerns, especially as migraine patients present increased cardiovascular risk[8,9]. Indeed, all the antibodies have been reported to be well tolerated even in subjects exposed longer that one year, with no cardiovascular events reported in the clinical trials that were considered to be related to CGRP pathway blockade[10]. Moreover, a study explored the effect of erenumab on exercise time during a treadmill test in mainly male patients with stable angina, and no changes were observed[11], although no evidence was provided for CGRP receptor blockade to be established already at the time of the treadmill test[12]. In addition, previous studies have also shown that the vasodilatory role of CGRP in the coronary arteries is more prominent in the distal portion when compared to the proximal portion[13]. While males are more prone than females to present ischemic events in the proximal portion of the coronary artery, females are more prone than men to present

myocardial ischemic events in the distal portion of the coronary artery[14]. As the vast majority of migraine patients are female, it is important to study the effects of erenumab in the distal portion of the coronary arteries. Also, for migraine patients with established cardiovascular disease, it is important to investigate whether blockade of the CGRP pathway could worsen their disease[6]. Thus, appropriate *in vitro* and *in vivo* studies are needed to assess the vascular safety of blocking the CGRP pathway.

The aim of this study was to investigate the inhibition of the vasodilatory responses to CGRP by erenumab in human isolated meningeal artery (HMMA), one of the proposed sites of therapeutic action. But also, in view of theoretical cardiovascular safety concerns, in human isolated proximal and distal coronary arteries (HCA), and in internal mammary arteries (HIMA) from cardiovascularly compromised patients undergoing coronary artery bypass grafting surgery. Furthermore, in HIMA, we studied the functional specificity of erenumab by comparing the relaxant responses to several vasodilators in the absence and presence of erenumab, namely: (i) acetylcholine, coupled to endothelium-dependent, nitric oxide-cGMP signalling; (ii) sodium nitroprusside, coupled to endothelium-independent, nitric oxide-cGMP signalling; (iii) pituitary adenylate cyclase activating peptide-38 (PACAP) and vasoactive intestinal peptide (VIP), peptides of interest for migraine pathophysiology that are coupled to an adenylate cyclase-cAMP signalling pathway; and (iv) nicardipine, a calcium channel blocker, prescribed for the treatment of hypertension that may also be used in migraine. Finally, as migraine patients under prophylactic treatment could still use acute antimigraine medication, the vasoconstrictor responses to dihydroergotamine (DHE) in absence and presence of erenumab were also analyzed, to discard a possible augmentation of the contractile responses due to the inhibition of the CGRP-mediated vasodilation.

Methods

Human isolated arteries collection

Middle meningeal arteries

Segments of HMMA (internal diameter 0.5–1.5 mm) were obtained from six patients (two male and four female, 49±8 years old) who underwent neurosurgical procedures requiring a trepanation of the skull. The HMMA, attached to the *dura mater*, was collected in a sterile organ-protecting solution and immediately transported to the laboratory to be dissected and subsequently placed in a cold, oxygenated Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_2$ 25 and glucose 11.1; pH 7.4.

Coronary arteries

Coronary arteries were obtained from six "heart beating" organ donors (two male and four female; 52±5 years old), who died of non-cardiac disorders. The hearts were provided by the Heart Valve Bank Beverwijk (at that time still located in Rotterdam) from Dutch post-mortem donors, after donor mediation by The Dutch Transplantation Foundation (Leiden, The Netherlands), following removal of the aortic and pulmonary valves for homograft valve transplantation. All donors gave permission for research. Immediately after circulatory arrest, the hearts were stored at 4°C in a sterile organ protecting solution and were brought to the laboratory within 24 hours of death. After arrival, the right proximal (internal diameter 3–5 mm) and distal (internal diameter 0.5–1 mm) portions of the HCA were dissected and placed in a cold, oxygenated with carbogen (95% O_2/5% CO_2) Krebs buffer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The studies on coronary arteries were approved by the Scientific Advisory Board of the Rotterdam Heart Valve Bank.

Internal mammary arteries

Segments of HIMA (internal diameter 2–3 mm) were obtained perioperatively from 10 male patients (72±2 years old) undergoing coronary artery bypass surgery. After completion of the coronary bypass procedure, the remaining segment of HIMA was immediately brought to the laboratory. Connective tissue was removed from the segment and the tissue was kept in cold Krebs solution (for composition see above), aerated with carbogen. All vessels were used on the same day or stored overnight and used the following day for functional experiments. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, approved the study protocols with regard to mammary arteries and middle meningeal arteries.

Isometric tension measurements

Proximal HCAs were cut into segments of 2–4 mm length, excluding macroscopically visible atherosclerotic lesions. Segments were mounted on stainless steel hooks in 15-mL organ baths filled with oxygenated Krebs buffer solution at 37°C. After 30 min of stabilization, the vessel segments were stretched to a tension of about 15 mN, as described earlier by our group[13]. Changes in tension were measured with an isometric force transducer (Harvard, South Natick, MA, U.S.A.) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The HMMA, distal HCA and HIMA were cut into circular 1-2 mm long segments and mounted in Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) between two parallel small stainless-steel wires (40 µm Ø). Baths were filled with oxygenated Krebs buffer (37°C) and their tension was normalized to 90% of I_{100} for all segments (the diameter when transmural pressure equals 100 mm Hg) as previously reported[15]. Data was recorded using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK).

Experimental protocols

A paired parallel set up (*i.e.* experiments with and without erenumab were performed in different segments obtained from the same artery) was used. Initially, all segments were exposed to 30 mM KCl to 'prime' the tissue for stable contractions. After washout, the tissue was exposed to 100 mM KCl to determine the reference contractile response. All segments were pre-contracted with 30 mM KCl after being incubated with vehicle or erenumab (1 µM)[16] for 15 min. After 15 min of precontraction (*i.e.*, after a total incubation time of 30 min for erenumab), a concentration response curve to human αCGRP (0.1 nM-1 µM, half logarithmic steps) was performed.

Additionally, in HIMA, after segments were pre-contracted (30 mM KCl) and incubated with vehicle or erenumab (1 µM), concentration response curves to acetylcholine (0.1 nM-3 µM, half logarithmic steps), sodium nitroprusside (1 nM-10 µM, half logarithmic steps), PACAP (0.1 nM-1 µM, whole logarithmic steps), VIP (0.1 nM-1 µM, whole logarithmic steps), or nicardipine (1 nM-30 µM, whole logarithmic steps) were performed. Also, in vessels incubated 30 min with vehicle or erenumab (1 µM), a concentration response curve to DHE (1 nM-100 µM, whole logarithmic steps) was performed.

Finally, at the end of each experiment, i.e., after construction of a concentration response curve, and washing out, the functional integrity of the endothelium was verified by observing relaxation to bradykinin (1 µM, HIMA) or substance P (10 nM, HMMA and HCA) after precontraction with the thromboxane A_2 analogue U46619 (10 nM) in every individual vessel segment.

Statistical analysis

Vasodilatory responses were expressed as percentage of the precontraction induced by 30 mM KCl. For contractile responses, the values were expressed as percentage of the contraction induced by

100 mM KCl. Curves covering the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} (negative log of the molar concentration of an agonist needed to reach half of its maximal effect) and E_{max} (maximal response) values. If E_{max} was not reached, the contraction or relaxation obtained at the highest concentration of agonist was considered as E_{max}, except for CGRP in the presence of erenumab, where the respective control E_{max} (i.e., in the absence of erenumab) was used as E_{max} for fitting. The blocking potency of erenumab in each tissue was estimated by calculating EC_{50} ratios and plotting a Schild plot [17] and constraining the slope to unity to obtain the apparent pK_b values. All data are presented as mean±S.E.M. Significant differences in pEC_{50} and E_{max} between control and erenumab groups were examined with a paired t test. Differences between tissues were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey's post hoc analysis. P values of 0.05 or less were assumed to denote significant changes.

Compounds used

Erenumab and vehicle were kindly provided by Amgen (Thousand Oaks, CA, U.S.A.). Acetylcholine chloride, sodium nitroprusside, nicardipine hydrochloride, dihydroergotamine mesylate and U46619 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human α-CGRP, PACAP and VIP were obtained from PolyPeptide (Strasbourg, France).

Results

Responses to 100 mM and 30 mM KCl resulted in a mean contraction of 19±4 mN and 17±3.1 mN, respectively.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated middle meningeal arteries

In HMMA (Fig. 1), the vasodilatory responses to CGRP were significantly shifted in the presence of 1 μM erenumab (control: pEC_{50} 8.56±0.16 *vs.* erenumab: pEC_{50} 6.51±0.19; n=6 each; t(5)=16.74, p<0.0001). The apparent pK_b value was 8.05±0.12. No significant changes were observed in the maximal responses to CGRP (control: E_{max} 64±11% *vs.* erenumab E_{max}: 56±10%; n=6 each; t(5)=2.11, p=0.088). Verification of endothelial function resulted in a mean dilation of 83±3% of the precontraction.

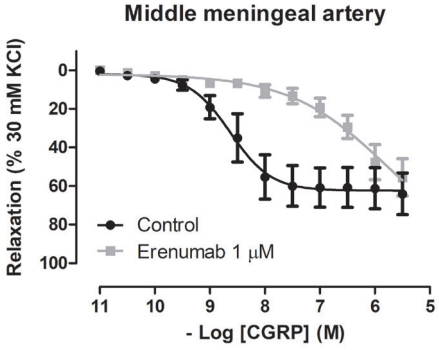

Fig. 1. Relaxant responses to CGRP in the middle meningeal artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=6.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated coronary arteries

Relaxant responses to CGRP in the proximal HCA (pEC_{50}<6.54; n=4), were inhibited in the presence of 1 μM erenumab (pEC_{50}<5.24; n=4), with no significant change in the response obtained at the highest

concentration (control: E_{max} 36±6% vs. erenumab: E_{max} 21±3%; t(3)=1.86, p=0.15). Due to the limited effect of CGRP on the proximal portion of the HCA, no apparent pK_b value was calculated. In the distal HCA, a significant shift was observed in the vasodilatory responses to CGRP (control: pEC_{50} 9.04±0.18 vs. erenumab: pEC_{50} 6.81±0.18; n=6 each; t(5)=13.46, p<0.0001), with no change in the maximal relaxation (control: E_{max} 85±5% vs. erenumab: E_{max} 83±6%; n=6 each; t(5)=0.88, p=0.42; Fig. 2) and an apparent pKb value of 8.22±0.17. A significant difference was observed between the maximal relaxation to CGRP in proximal and distal HCA (E_{max} proximal: 36±6% vs. E_{max} distal: 85±5%; t(8)=4.95, p=0.001). Endothelial function analysis resulted in a mean dilation of 12 ± 3% of the precontraction in proximal HCA and of 89 ± 4% in distal HCA.

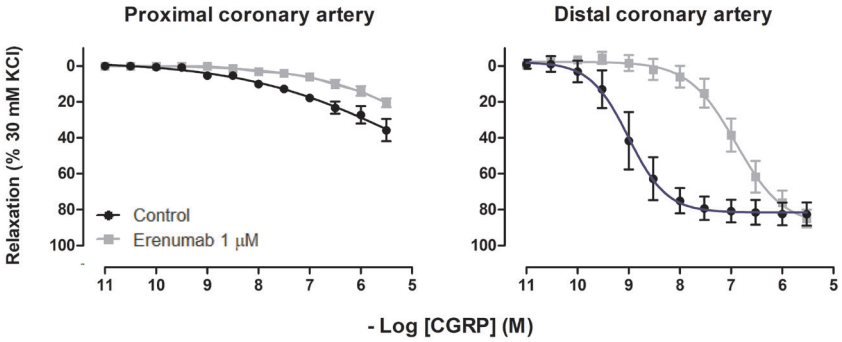

Fig. 2. Relaxant responses to CGRP in the proximal (left) and distal (right) coronary arteries in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=4-6.

Effect of erenumab on the vasodilatory responses to CGRP in human isolated internal mammary artery

In HIMA, the vasodilatory responses to CGRP were also significantly inhibited by erenumab (control: pEC_{50} 7.83±0.34 *vs.* erenumab: pEC_{50} 5.94±0.37; n=8 each; t(7)=4.18, p=0.004), with an apparent pK_b value of 7.85±0.46, and no significant change in the maximal response was observed (control: E_{max} 32±12% vs. erenumab: 24±10%; n=8 each; t(7)=1.44, p=0.19; Fig. 3). Verification of endothelium function resulted in a dilation of 6±3% of the precontraction. Precontraction with 30 mM KCl was not modified by erenumab (control: 154±47% *vs.* erenumab 141±22% of the contraction to 100 mM KCl; t(7)=0.47, p=0.66; data not shown).

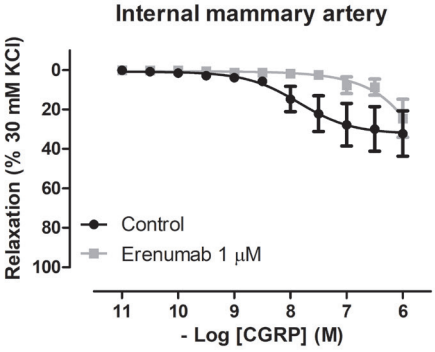

Fig. 3. Relaxant responses to CGRP in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=8.

Effect of erenumab on non-CGRP induced vasodilatory responses in human isolated mammary artery

The relaxant responses to the different vasodilators studied were not modified in the presence of erenumab (Table 1, Fig. 4)

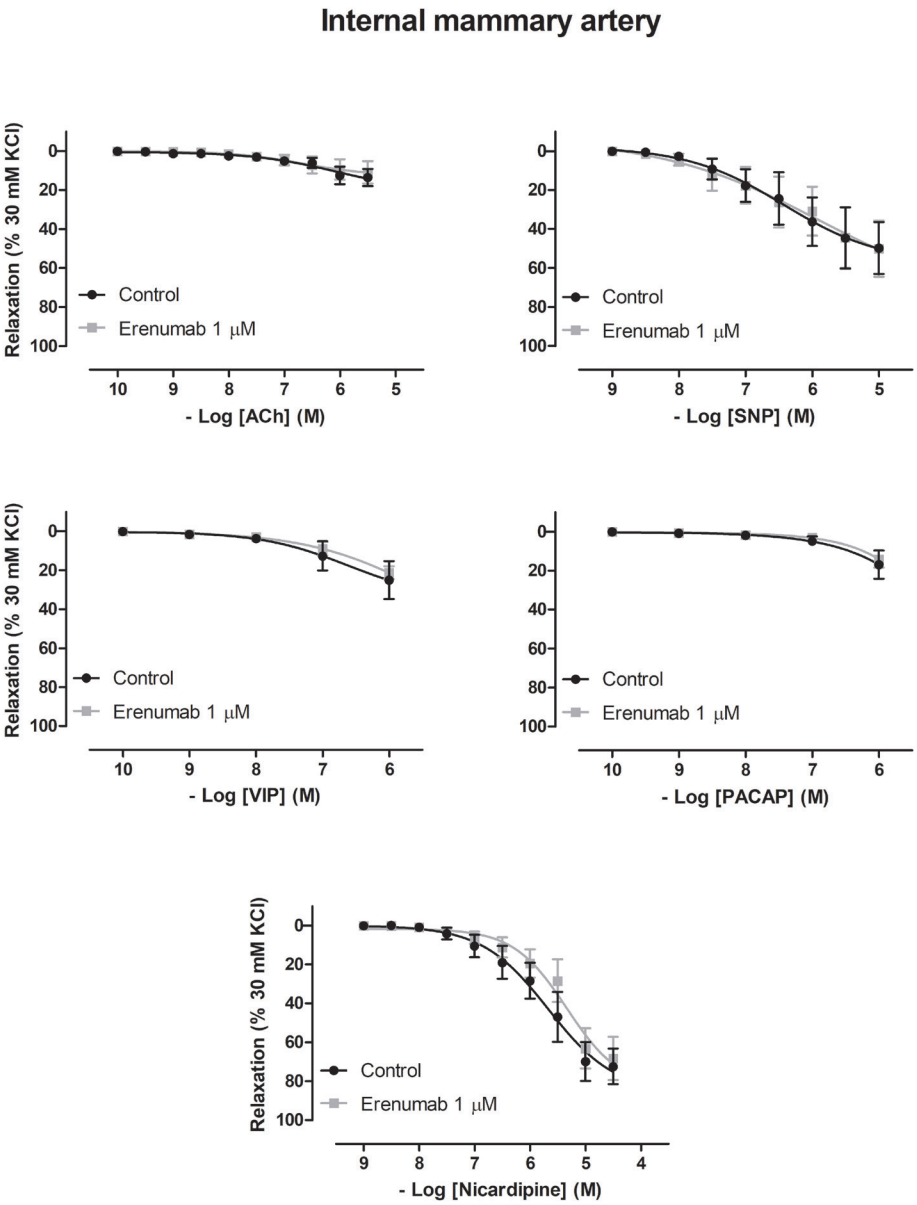

Fig. 4. Vasodilatory responses to acetylcholine, sodium nitroprusside, VIP, PACAP and nicardipine in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=7 each.

Table 1. Vasodilatory responses to acetylcholine, sodium nitroprusside, VIP, PACAP and nicardipine in the HIMA in the absence and presence of erenumab; n=7 each.

Agonist	pEC_{50}			E_{max} (%)		
	Control	Erenumab	P value	Control	Erenumab	P value
Acetylcholine	7.42±0.96	6.22±0.45	0.66	14±4	11±6	0.57
Sodium nitroprusside	6.31±0.29	6.44±0.44	0.98	50±13	50±14	0.97
VIP	6.83±0.11	7.05±0.45	0.29	25±10	21±3	0.69
PACAP	6.78±0.01	7.00±0.21	0.28	17±7	14±4	0.63
Nicardipine	5.77±0.24	5.49±0.06	0.23	72±9	68±11	0.49

E_{max}: maximal response; PACAP: pituitary adenylate cyclase activating polypeptide-38; pEC_{50}: negative log of the molar concentration of an agonist needed to reach half of its maximal effect; VIP: vasoactive intestinal peptide

Effect of erenumab on the contractile responses to dihydroergotamine

Similar to above, contractile responses to DHE (pEC_{50} control: 6.78±0.40 *vs.* pEC_{50} erenumab: 6.65±0.40, t(6)=0.41, p=0.72; E_{max} control: 11±6% *vs.* E_{max} erenumab: 17±12%, t(6)=0.66, p=0.53; n=7 each) were unaffected by the presence of erenumab (Fig. 5).

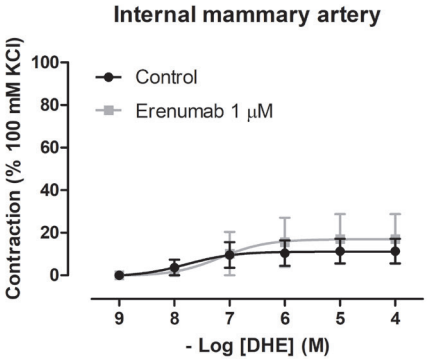

Fig. 5. Contractile responses dihydroergotamine (DHE) in the human isolated internal mammary artery in the presence of erenumab (1 μM) or the vehicle. Data are expressed as mean±SEM, n=7 each.

Comparison of the responses to CGRP in human middle meningeal, coronary and mammary arteries

The vasodilatory responses to CGRP were more potent in the distal portion of the HCA when compared to the HIMA (pEC_{50} 9.04±0.18 *vs.* pEC_{50} 7.83±0.34, respectively; F(2,17)=5.471, $p_{apparent}$=0.01). No significant differences were observed in the potency between HMMA and HIMA (pEC_{50} 8.56±0.16 *vs.* pEC_{50} 7.83±0.34, respectively; F(2,17)=5.471, $p_{apparent}$=0.15), nor between HMMA and the distal portion of the HCA (pEC_{50}: 8.56±0.16 *vs.* pEC_{50}: 9.04±0.18, respectively; F(2,17)=5.471, $p_{apparent}$=0.47).

When comparing the E_{max}, the vasodilatory responses to CGRP were more pronounced in the distal portion of the HCA, when compared to the HIMA (E_{max} distal HCA: 85±5% *vs.* E_{max} HIMA: 32±12%; F(3,20)=6.302, $p_{apparent}$=0.004). No significant differences were observed between the maximal responses to CGRP in HMMA and HIMA (E_{max} HMMA: 64±11% *vs.* E_{max} HIMA: 32±12%; F(3,20)=6.302, $p_{apparent}$=0.112) nor HMMA and distal HCA (E_{max} HMMA: 64±11% *vs.* E_{max} distal HCA: 85±5%; F(3,20)=6.302, $p_{apparent}$=0.47). Also, no significant differences were observed between the maximal vasodilatory response to CGRP in proximal HCA and HIMA (E_{max} proximal HCA: 36±6% *vs.* E_{max} HIMA: 32±12%; F(3,20) = 6.302, $p_{apparent}$=0.991) nor proximal HCA and HMMA (E_{max} proximal HCA: 36±6% *vs.* E_{max} HMMA: 64±11%; F(3,20)=6.302, $p_{apparent}$=0.311)

Finally, no significant difference was observed in the potency of erenumab to antagonize the responses to CGRP amongst the tissues studied (p=0.73; pK_b HMMA: 8.05±0.12 *vs.* pK_b HCA: 8.22±0.17, F(2,17)=0.3106, $p_{apparent=}$0.94; pK_b HMMA: 8.05±0.12 *vs.* pK_b HIMA: 7.85±0.46, F(2,17)=0.3106, $p_{apparent=}$0.91; pK_b HCA: 8.22±0.17 *vs.* pK_b HIMA: 7.85±0.46, F(2,17)=0.3106, $p_{apparent}$=0.72).

Discussion

In this study the inhibition of the CGRP-vasodilatory responses by erenumab was examined in human isolated arteries. We used a supratherapeutic concentration of erenumab (1 μM)[16] to allow a clear analysis of its effects on CGRP as well as other vasoactive substances of interest.

Firstly, we investigated the effect of erenumab on the vasorelaxant responses to CGRP in HMMA, with our results showing a significant shift of the concentration response curve to CGRP in the presence of erenumab. As antibodies are considered to have a BBB permeability of < 0.1%[18] and erenumab has been shown to be effective for the prophylactic treatment of migraine[19,20], it is considered that the mechanisms of action of erenumab are peripheral, with one of them being possibly inhibition of the CGRP-mediated vasodilation of the dural arteries[1]. While erenumab has no vasoconstrictive properties *per se*[21], its success as prophylactic treatment may well be (partly) by effectively preventing the vasodilatory responses to CGRP in the HMMA, associated with the onset of migraine attacks. In accordance with this, human provocation studies have shown dilation of the HMMA on the headache side at migraine onset, and headache relief after vasoconstriction of the HMMA by sumatriptan[1,22]. Certainly, these studies have been performed during exogenously provoked migraine-like attacks and a magnetic resonance angiography study of the intracranial and extracranial arteries in patients with spontaneous migraine attacks failed to show extracranial arterial dilatation[23]. However, as previously addressed by our group[24], in the latter study authors could not exclude dilatation of dural branches of the HMMA, as those small branches could not be analyzed due to technical limitations.

Due to the theoretical cardiovascular concerns of blocking the actions of CGRP[6,25], especially since migraine patients have an increased cardiovascular risk[8,9], we further studied the effect of erenumab in proximal and distal HCA (Fig. 2). Although it has been shown that erenumab does not contract the HCA[21], it is important to consider the risks of the blockade of the cardioprotective vasodilation by CGRP[7]. In our study, and in accordance with previous work[13,26,27], the vasodilatory responses to CGRP in the distal portion of the HCA were significantly more pronounced than in the proximal portion of the HCA and HIMA. Moreover, in the presence of erenumab, a significant shift was observed in both portions of the HCA, that seemed to be more pronounced in the distal portion. This reinforces the importance of appropriate vascular safety studies in migraine patients, with especial emphasis in female patients that are more prone to present ischemic events in the distal portion of the coronary arterial bed, where the role of CGRP in cardioprotection seems to be more significant[7,12,14].

Furthermore, we analyzed the effect of erenumab on isolated arteries from cardiovascularly compromised patients. For this, we obtained HIMA peri-operatively from coronary artery bypass surgery patients, most of them suffering from atherosclerotic disease that is usually more prominent in the proximal HCA and more common in men as previously mentioned, thus all our experiments were performed in HIMAs obtained from male subjects. However, while the responses to CGRP are more pronounced in the distal coronary artery (where women are more prone to present ischemic events), as previously mentioned, when a patient undergoes coronary bypass surgery, a portion of HIMA is grafted and thus gets incorporated in the proximal coronary arterial bed, making it a relevant tissue to study the characteristics of the CGRP-mediated vasodilatory responses and the effects of erenumab. Based on the limited relaxation to bradykinin that was analyzed in every individual HIMA segment, and in accordance with the small vasodilatory responses to acetylcholine (Fig. 4), functional endothelial quality was limited in these coronary artery bypass grafts, probably associated with the endothelial dysfunction associated with cardiovascular disease[28]. When analyzing the responses to CGRP, a concentration-dependent vasodilation was observed, which was significantly antagonized in the presence of erenumab, with no change in the maximal response. The E_{max} of CGRP in the HIMA was significantly lower when compared to the distal portion of the HCA and not significantly different when compared to the proximal HCA (Figs. 2-3), suggesting a similar role for CGRP in HIMA and proximal HCA. The vasodilatory peptides VIP and PACAP, currently considered possible therapeutic targets for migraine[29], do not seem to play an important role in HIMA vasodilation as their maximal response was rather low. Most importantly, erenumab did not modify the responses to acetylcholine and sodium nitroprusside (nitric oxide-cGMP signalling), nor the vasorelaxant responses to PACAP and VIP (adenylate cyclase-cAMP signalling). Even though the cardiovascular safety concerns are theoretical and trials have not reported cardiovascular events that were considered to be related to inhibition of the CGRP pathway[10], the vasodilatory responses to acetylcholine, VIP and PACAP in our study were limited. Therefore, further studies should address the vasodilatory pathways involved in ischemic conditions after long-term blockade of the CGRP pathway. Nonetheless, erenumab did not modify the vasodilatory responses to nicardipine, an antihypertensive given to cardiovascularly compromised patients (Fig. 4), and did not augment the vasoconstrictor responses to 30 mM KCl or DHE, an acute acting antimigraine drug that could be taken concomitantly with erenumab (Fig. 5). Similar results have previously been reported in HCA with sumatriptan[21], which is of great importance for patients under ergot (or triptan) treatment.

Finally, as the efficacy and potency of the CGRP-dependent vasodilatory responses differ amongst arteries[13], CGRP receptor blockade by erenumab could also present differential responses depending on the vessel studied; however, erenumab had a similar potency (pK_b) across the distal HCA (8.22±0.17), HMMA (8.05±0.12) and HIMA (7.85±0.46). When comparing these results to the gepants, similar pK_b values were obtained previously by our group for telcagepant in distal HCA and HMMA (8.43±0.24 and 8.03±0.16, respectively)[13,26]. Interestingly, olcegepant was more potent in HMMA (pK_b: 10.59±0.54) than in the distal portion of the HCA, with pK_b values ranging from 8.41±0.26 to 9.29±0.34, depending on the concentration studied[30,31]. While we do not know the reason for this discrepancy, it may well be caused by an underlying heterogeneity in CGRP receptors that could be targeted by olcegepant[30,32,33], whereas erenumab only acts at the canonical CGRP receptor[34], thus, receptors other than the CGRP receptor to which CGRP may still bind (*e.g.* amylin 1 receptor), may compensate for blockade of the CGRP receptor. Conversely, in the case of the antibodies directed against CGRP, peptides other than CGRP that may also bind to the CGRP receptor, may exert compensatory effects. Further studies should address whether there are clinically relevant differences (*i.e.* in efficacy or cardiovascular safety), between the prophylactic treatment with the antibodies directed against CGRP and against the CGRP receptor.

Our results, taken together, show a differential response profile to CGRP in human isolated arteries, being more potent in the distal portion of the HCA, when compared to the proximal portion and the HMMA and HIMA. Moreover, erenumab significantly inhibits CGRP-mediated vasodilation *in vitro* and does not interact with responses to other vasodilatory or contractile agents of interest.

Conclusion

In conclusion, erenumab is a potent inhibitor of the vasodilatory responses to CGRP in HMMA, HCA and HIMA. While the prominent role of CGRP in distal coronary artery warrants further safety studies, particularly in women, it is important to point out that erenumab does not interact with vasodilatory responses to other vasodilators, nor with the contractions to DHE.

References

1. Khan S, Amin FM, Christensen CE, et al. Meningeal contribution to migraine pain: a magnetic resonance angiography study. Brain 2018;142:93-102.
2. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
3. Tepper SJ. History and Review of anti-Calcitonin Gene-Related Peptide (CGRP) Therapies: From Translational Research to Treatment. Headache: The Journal of Head and Face Pain 2018;0.
4. Russo AF. CGRP-Based Migraine Therapeutics: How Might They Work, Why So Safe, and What Next? ACS Pharmacology & Translational Science 2018.
5. Chai W, Mehrotra S, Jan Danser AH, Schoemaker RG. The role of calcitonin gene-related peptide (CGRP) in ischemic preconditioning in isolated rat hearts. European Journal of Pharmacology 2006;531:246-253.
6. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
7. Homma S, Kimura T, Sakai S, et al. Calcitonin gene-related peptide protects the myocardium from ischemia induced by endothelin-1: Intravital microscopic observation and 31P-MR spectroscopic studies. Life Sciences 2014;118:248-254.
8. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.
9. Sacco S, Ornello R, Ripa P, Pistoia F, Carolei A. Migraine and hemorrhagic stroke: a meta-analysis. Stroke 2013;44:3032-3038.
10. Mitsikostas DD, Reuter U. Calcitonin gene-related peptide monoclonal antibodies for migraine prevention: comparisons across randomized controlled studies. Current Opinion in Neurology 2017;30:272-280.
11. Depre C, Antalik L, Starling A, et al. 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: The Journal of Head and Face Pain 2018;58:715-723.
12. Maassen van den Brink A, Rubio-Beltrán E, Duncker D, Villalón CM. Is CGRP Receptor Blockade Cardiovascularly Safe? Appropriate Studies Are Needed. Headache: The Journal of Head and Face Pain 2018;58:1257-1258.
13. Chan KY, Edvinsson L, Eftekhari S, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. J Pharmacol Exp Ther 2010;334:746-752.
14. Humphries KH, Pu A, Gao M, Carere RG, Pilote L. Angina with "normal" coronary arteries: Sex differences in outcomes. American Heart Journal 2008;155:375-381.
15. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res 1977;41:19-26.
16. Vu T, Ma P, Chen JS, et al. Pharmacokinetic-Pharmacodynamic Relationship of Erenumab (AMG 334) and Capsaicin-Induced Dermal Blood Flow in Healthy and Migraine Subjects. Pharmaceutical Research 2017;34:1784-1795.
17. Arunlakshana O, Schild HO. Some quantitative usess of drug antagonists. British Journal of Pharmacology and Chemotherapy 1959;14:48-58.
18. Poduslo JF, Curran GL, Berg CT. Macromolecular permeability across the blood-nerve and blood-brain barriers. Proceedings of the National Academy of Sciences 1994;91:5705-5709.

19. Dodick DW, Ashina M, Brandes JL, et al. ARISE: A Phase 3 randomized trial of erenumab for episodic migraine. Cephalalgia 2018;38:1026-1037.
20. Ashina M, Tepper S, Brandes JL, et al. Efficacy and safety of erenumab (AMG334) in chronic migraine patients with prior preventive treatment failure: A subgroup analysis of a randomized, double-blind, placebo-controlled study. Cephalalgia 2018;38:1611-1621.
21. Bussiere JL, Davies R, Dean C, et al. Nonclinical safety evaluation of erenumab, a CGRP receptor inhibitor for the prevention of migraine. Regulatory Toxicology and Pharmacology 2019.
22. Asghar MS, Hansen AE, Amin FM, et al. Evidence for a vascular factor in migraine. Annals of Neurology 2011;69:635-645.
23. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. The Lancet Neurology 2013;12:454-461.
24. MaassenVanDenBrink A, Ibrahimi K, Edvinsson L. Intracranial and extracranial arteries in migraine. The Lancet Neurology 2013;12:847-848.
25. Deen M, Correnti E, Kamm K, et al. Blocking CGRP in migraine patients - a review of pros and cons. J Headache Pain 2017;18:96.
26. Edvinsson L, Chan KY, Eftekhari S, et al. Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries. Cephalalgia 2010;30:1233-1240.
27. Gulbenkian S, Opgaard OS, Ekman R, et al. Peptidergic innervation of human epicardial coronary arteries. Circulation Research 1993;73:579-588.
28. Dharmashankar K, Widlansky ME. Vascular endothelial function and hypertension: insights and directions. Current hypertension reports 2010;12:448-455.
29. Rubio-Beltran E, Correnti E, Deen M, et al. PACAP38 and PAC1 receptor blockade: a new target for headache? J Headache Pain 2018;19:64.
30. Gupta S, Mehrotra S, Villalon CM, et al. Characterisation of CGRP receptors in human and porcine isolated coronary arteries: evidence for CGRP receptor heterogeneity. Eur J Pharmacol 2006;530:107-116.
31. Gupta S, Mehrotra S, Avezaat CJJ, Villalón CM, Saxena PR, MaassenVanDenBrink A. Characterisation of CGRP receptors in the human isolated middle meningeal artery. Life Sciences 2006;79:265-271.
32. Walker CS, Eftekhari S, Bower RL, et al. A second trigeminal CGRP receptor: function and expression of the AMY1 receptor. Annals of Clinical and Translational Neurology 2015;2:595-608.
33. Haanes KA, Chan KY, MaassenVanDenBrink A. Comment on "A second trigeminal CGRP receptor: function and expression of the AMY1 receptor". Annals of clinical and translational neurology 2016;3:307-308.
34. Shi L, Lehto SG, Zhu DXD, et al. Pharmacologic Characterization of AMG 334, a Potent and Selective Human Monoclonal Antibody against the Calcitonin Gene-Related Peptide Receptor. Journal of Pharmacology and Experimental Therapeutics 2016;356:223-231.

Chapter IX.

Propranolol in men and women; differential role for sex steroids in the trigeminovascular system

Based on: ***E Rubio-Beltrán****, RM Schoon, J vd Berg, CCM Schuiling-Veninga, BCP Koch, CM Villalón, J Versmissen, AHJ Danser, AH vd Meiracker, K Ibrahimi, A MaassenVanDenBrink. Submitted.*

Abstract

Objective: To assess whether propranolol has an inhibitory effect on the trigeminovascular system.
Methods: We investigated the effect of propranolol (80 mg, 90 min after oral administration, corresponding to the T_{max}) on the rise of dermal blood flow (DBF) of the forehead skin (innervated by the trigeminal nerve) by capsaicin application (0.6 mg/ml) and electrical stimulation (0.2-1.0 mA) before and after placebo (grapefruit juice) and propranolol (oral solution diluted in grapefruit juice) in a randomized, double-blind, placebo-controlled cross-over study, including healthy males (n=10, age±SD: 29±11 years) and females on contraceptives (n=11, 25±3 years). Additionally, we correlated our results with data from a Dutch prescription database by analyzing the change in triptan use after propranolol prescription in a population similar to our DBF study subjects (males and females, 20-39 years old).
Results: DBF responses to capsaicin were attenuated after propranolol but not after placebo. When stratifying by sex, no changes in the DBF responses to capsaicin were observed in females after propranolol, whereas the decrease remained significant in males. DBF responses to electrical stimulation were not modified in any of the cases. When comparing the change in triptan use after propranolol, a more pronounced decrease was observed in male patients than in female patients on contraceptives.
Conclusion: Propranolol (80 mg) inhibits capsaicin-induced increases in DBF in a sex-dependent manner; also, a more pronounced decrease in triptan use observed in male patients. These results taken together suggest an interaction between propranolol and sex steroids in the modulation of the trigeminovascular system.

Introduction

Migraine is a highly disabling neurovascular disorder[1]; it is estimated that around 16% of the world population suffers of migraine, with 70% being women[2]. The pathophysiology of migraine remains largely unknown, however, it is considered to involve a dysfunctional activation of the trigeminovascular system and vasodilation of the trigeminal innervated vessels (*e.g.* middle meningeal artery), mainly mediated by the release of calcitonin gene-related peptide (CGRP), a neuropeptide present in perivascular sensory fibers[3,4].

Migraine treatment can be either acute, aimed to reverse the attack once it has begun, or prophylactic, designed to reduce the frequency and severity of migraine attacks. In case of the former, drugs have been developed specifically for the treatment of migraine based on the current knowledge of the pathophysiological mechanisms underlying this neurovascular disorder (*e.g.* triptans)[5-8]. However, for decades, migraine prophylactic treatment was not developed specifically for this disorder, but rather created for other disorders and later discovered to reduce migraine attack frequency in some patients, as is the case for propranolol. In the 1960's, Rabkin and colleagues[9] noted that one patient under propranolol treatment for *angina pectoris* showed a marked improvement of his "vascular headaches". Further similar cases[10,11] resulted in the first clinical trial[12] on the efficacy of propranolol for the treatment of migraine, with positive results. Nowadays, propranolol is still widely prescribed for the preventative treatment of migraine but its mechanism of action is not clear, as not all β-blockers are effective as prophylactic treatment and the lack of efficacy does not correlate with their β-adrenoceptor selectivity (*i.e.* β or β_1-adrenoceptor)[13]. This suggests that their mechanism of action may well not be related to their antagonistic properties on β-adrenoceptors and/or their antihypertensive properties but to other unknown mechanism(s).

Currently, one of the main targets for migraine treatment is blocking the CGRP pathway, either with antagonists of the CGRP receptor (gepants)[14], with antibodies against CGRP or its receptor[14,15],

or, combined with other pharmacological actions, by inhibiting CGRP release[8]. Our group has developed a non-invasive, reproducible human model that can be used to evaluate trigeminal-nerve induced vasodilation mediated by CGRP. For this, the capsaicin-induced changes on the rise of dermal blood flow (DBF) of the forehead (innervated by the trigeminal nerve) are measured[16]. We have previously shown with this model that sumatriptan modulates the human trigeminovascular system[17], and also demonstrated that CGRP-induced trigeminovascular increases in dermal blood flow depend on the menstrual cycle[18]. Considering the importance of the trigeminovascular system and CGRP in the pathophysiology of migraine and the fact that the mechanism of action of propranolol remains largely unknown, the aim of this study was to investigate the effect of propranolol on the modulation of the trigeminovascular system.

Methods

Standard protocol approvals, registrations and patient consents

The study protocol was reviewed and approved by the independent Ethics Committee of Erasmus MC, Rotterdam, the Netherlands, (MEC 2016-196), and was registered at the Netherlands Trial Register (ID: NTR6007). All participants gave written informed consent after explanation of the study, which was conducted in accordance with local laws, the ethical principles of the Declaration of Helsinki, as well as the principles of Good Clinical Practice.

Data Availability Statement

Anonymized data will be shared by request from any qualified investigator.

Design and procedures

This was a randomized, double-blind, placebo-controlled, crossover study. Healthy, non-smoking male and female individuals, aged 18–64, body mass index (BMI) 20–28 kg/m^2, without history of migraine, cardiovascular disease or use of medication, were eligible. Subjects with systolic blood pressure (SBP) values lower than 110 mm Hg or heart rate (HR) lower than 60 bpm were excluded from the study due to safety reasons in view of the cardiovascular effects of propranolol. Females were using oral contraceptives and continued their use without the 'stop week' to avoid the confounding influence of varying steroid levels[19,20].

Experiments were performed in a quiet, temperature-controlled room. All individuals had two visits, scheduled with a one-week washout in between and during the same time of day. Participants were not allowed to use vasoactive drugs (including nonsteroidal anti-inflammatory drugs) for >48 hours. Furthermore, they could not consume alcohol, caffeine-containing beverages and chocolate for >12 hours prior to the start of experiments. For experiments in the morning, a light breakfast three hours before the start of the experiment was allowed and for experiments in the afternoon a light lunch three hours for the start of an experiment was allowed.

After arriving, all the subjects underwent a weight and height measurement. Female subjects had to take a pregnancy test. Furthermore, an electrocardiogram was performed in order to identify and exclude subjects with heart problems, especially conduction disorders. Before each measurement, a light meal was provided, that was the same for all subjects. Measurements were performed before and 90 min after either placebo or propranolol (corresponding to propranolol peak plasma concentration[21,22]) during the two research visits. During the experiment the subjects rested supine on a bed and were not allowed to speak. Measurements before administration of propranolol or placebo were always performed on the right side of the forehead, while measurements after administration of propranolol or placebo were performed on the left side of the forehead. We have previously shown that responses do not differ between sides[16,17]. Capsaicin solution (2 mM,

corresponding to 0.6 mg/ml, diluted in a mixture of ethanol 100%, Tween 20 and distilled water; 3:3:4) and physiological saline (0.9% NaCl, 0.5 ml) were placed in reservoirs specifically designed for this purpose (drug delivery electrodes, Perimed AB, Järfälla, Sweden). For the capsaicin solution, this electrode was merely used as a reservoir, since no iontophoresis was used for capsaicin, which was simply applied to the skin. Furthermore, a ground electrode (Perimed AB, Järfälla, Sweden) was placed in the neck region 15 cm apart from the other electrodes with the negative lead of the iontophoresis device (Periont 382b, Perimed, Sweden) connected to the electrode containing saline (used for electrical stimulation, ES) and the positive lead connected to the electrode in the neck. Capsaicin- and ES-induced dermal forehead vasodilatation were measured with a laser Doppler perfusion imager as previously described[16-18]. Briefly, after 15 min of supine rest, DBF at the site of the capsaicin electrode was continuously measured with the PIM 3 laser Doppler flow device for 40 min. After baseline measurement for 2 min, iontophoresis of saline was applied at 0.2 mA for 1 min. DBF was subsequently measured for 6 min. This process was repeated with increasing currents (0.4 mA, 0.6 mA, 0.8 mA) up to 1.0 mA.

After the first measurement, subjects were given propranolol (80 mg, Syprol® 50 mg/5 ml) dissolved in 150 ml of grapefruit juice, or placebo (grapefruit juice only). Grapefruit juice was used in this study because its bitter taste masked the taste of the propranolol solution, which had a tangerine taste. Grapefruit juice does not affect the metabolism of propranolol. The DBF measurements described above were then repeated after 90 min corresponding to the approximate peak plasma concentration of propranolol[21,22]. After the DBF measurements two vials (6 ml each) of blood were collected to determine the plasma levels of propranolol. A questionnaire was given to all subjects to ask about the side effects they experienced during the experiments.

In order to exclude that changes in DBF were due to the hemodynamic changes induced by propranolol, HR, SBP and diastolic (DBP) blood pressure values were measured at the beginning and at the end of each measurement in triplicate, and later correlated to the changes in DBF. As changes in HR, SBP and DBP could unblind the researcher, experiments were performed always by two researchers: one that always performed the blood pressure measurements and a second that performed the DBF measurements and was never aware of the blood pressure values.

Propranolol levels

Blood was collected via the cubital vein. Propranolol levels were determined by the Laboratory of Clinical Pharmacology of Erasmus Medical Center with a Thermo Vantage LC-MS/MS (Thermo Fisher Scientific, Massachussetts, USA). The lower limit of quantification was 10 µg/l, with a range of quantification of 10-2500 µg/l. Samples were measured in 2 blinded batches.

Retrospective prescription database study

For our descriptive, retrospective study on the use of drugs we used data from a Dutch prescription database (www.IADB.nl) from Groningen University. The database contains information on drugs, namely, delivery date, anatomical therapeutic chemical-code, delivered quantity, dose per day and the number of delivered defined daily doses (DDD) of 60 public pharmacies in the north-east of The Netherlands, including about 600,000 patients. Sex and date of birth are also registered[23].

Patients enter the database once a drug is delivered to them via one of the pharmacies that are registered at IADB.nl. The database contains prescriptions disregarding the prescriber or the medical insurance. Information about medication that was used during hospital admission or 'over-the-counter' medication is not included in the database. The database does not contain information on the indication of a drug, nor does it contain information on ethnicity, social-economic status or life style factors. The database has been validated and is representative for the Dutch population[23].

Research population and inclusion period of the retrospective prescription database study
The research population included all men and women (20-39 years old, roughly representative for the population in our function study) that started propranolol treatment in the period between 1995 and 2017. For inclusion in this study, the patients should be registered in the database for at least 12 months before until 12 months after the start of propranolol and should have received at least two prescriptions for triptans in the 12 months before the start of propranolol. Further, the use of hormonal contraceptives was registered.

Statistical analysis
For the DBF measurements, sample size was based on previous studies from our group[17], at 5% significance (two-tailed) with 80% power. Baseline and maximal DBF responses (expressed in arbitrary units, a.u.) to capsaicin and ES were calculated before and after propranolol or placebo. Differences in DBF responses to capsaicin (our primary endpoint) and ES during propranolol and placebo were calculated for each participant in a blinded manner. Responses to propranolol and placebo were compared within participants using Student's paired t-test. Current-response curves of the ES sequence (0.2 mA–1.0 mA) were analyzed with repeated-measures analysis of variance (ANOVA).

For the retrospective prescription database study, triptan use, before and after the start of propranolol was determined based on the number of DDD and the number of prescriptions during 12 months, both mean and median were calculated. The change in triptan use was defined as the difference in delivered DDD's in the 12 months after the start of propranolol use, relative to the 12 months before. The results were stratified to sex, age and the use of hormonal contraceptives. A log transformation was performed to correct for the skewness of the data. Change in triptan use was then compared within subjects using a paired t-test. To compare the absolute change in DDD's (*i.e.* DDD's after minus DDD's before) between males and females, an unpaired t-test was used. A $p < 0.05$ was considered to indicate significance. Group values are provided as mean values and SEM, unless stated otherwise.

Results
Twenty-one healthy volunteers (10 males), aged 27±2 years (range 18-57) participated (Fig. 1). Demographics are described in Table 1.

Table 1. Demographics of the DBF study population.

	Male	Female	Both
Population (n)	10	11	21
Age, years	29 (11)	25 (3)	27 (8)
BMI, kg/m²	24.1 (2.9)	23.4 (2.3)	23.7 (2.6)
Height	1.80 (0.07)	1.64 (0.06)	1.72 (0.11)
Weight	78.4 (10.5)	62.8 (4.9)	70.3 (11.2)
BP, mm Hg			
Systolic	123 (10)	122 (12)	122 (11)
Diastolic	74 (9)	74 (8)	74 (8)
HR, bpm	67 (7)	79 (9)	73 (11)

Abbreviations: BMI=body mass index; BP=blood pressure; HR=heart rate. Data are counts or mean (SD)

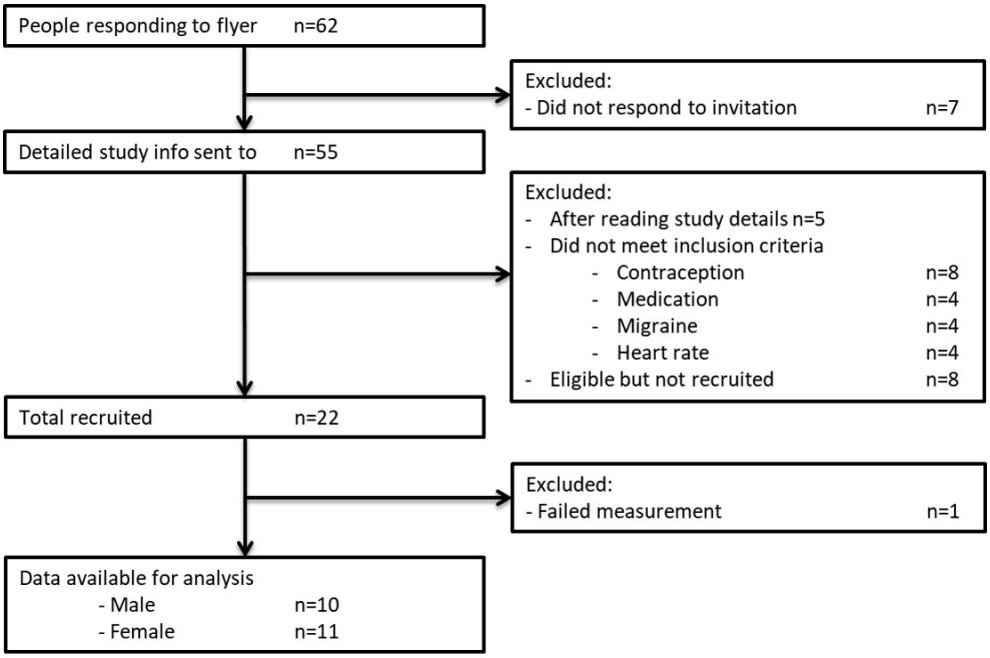

Fig. 1. Recruitment flow diagram.

Hemodynamic changes

After propranolol, SBP, DBP and HR were significantly decreased, but not after placebo (Table 2).

Table 2. Hemodynamic changes after placebo and propranolol.

	Males			Females			Both		
	Before	**After**	**p value**	**Before**	**After**	**p value**	**Before**	**After**	**p value**
Placebo									
BP, mm Hg									
Systolic	109 (4)	111 (5)	0.237	109 (7)	109 (8)	0.891	109 (6)	110 (7)	0.369
Diastolic	64 (5)	65 (6)	0.470	63 (4)	64 (5)	0.570	64 (4)	65 (5)	0.301
HR, bpm	64 (8)	62 (8)	**0.015**	69 (7)	70 (8)	0.360	66 (8)	66 (9)	0.692
Propranolol									
BP, mm Hg									
Systolic	111 (6)	108 (7)	**0.009**	107 (7)	103 (8)	**0.004**	109 (7)	105 (8)	**<0.0001**
Diastolic	65 (4)	64 (5)	0.152	62 (4)	60 (6)	0.120	64 (4)	62 (6)	**0.029**
HR, bpm	61 (8)	54 (8)	**<0.0001**	69 (8)	61 (7)	**<0.0001**	65 (9)	58 (8)	**<0.0001**

Abbreviations: BP=blood pressure; HR=heart rate. Data are mean (SD)

Forehead DBF responses to capsaicin or electrical stimulation

Changes in forehead DBF to capsaicin were not significantly correlated to the changes in SBP ($p=0.403$, $r^2=0.037$), DBP ($p=0.678$, $r^2=0.009$) or HR ($p=0.963$, $r^2=0.0001$) after propranolol (Fig. 2).

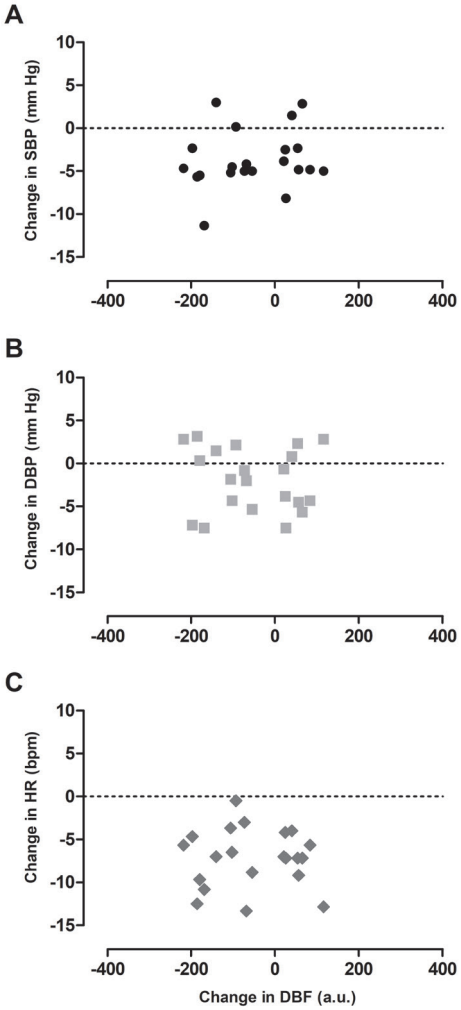

Fig. 2. Correlation of the changes in DBF and the hemodynamic variables after propranolol intake. No correlation was observed between the changes in DBF and SBP (A), DBP (B), nor HR (C). DBF: dermal blood flow; DBP: diastolic blood pressure; HR: heart rate; SBP: systolic blood pressure.

Changes in DBF to capsaicin were not significantly different before and after placebo (before: 504±31 a. u. *vs.* after: 505±41 a. u., respectively; p=0.966). In contrast, DBF responses to capsaicin were significantly attenuated after propranolol (before: 513±33 a.u. *vs.* after: 466±35 a.u.; p=0.042; Fig. 3). Stratification of the data by sex showed that the DBF responses to capsaicin were significantly decreased in males (478±60 a.u. *vs*. 389±56 a.u.; p=0.005), but not in female subjects (544±32 a.u. *vs.* 536±33 a.u.; p=0.788) after propranolol (Fig. 3). No significant difference was observed after placebo in males (484±49 a.u. *vs*. 459±75 a.u.; p=0.662), nor in females (521±40 a.u. *vs*. 546±37 a.u.).

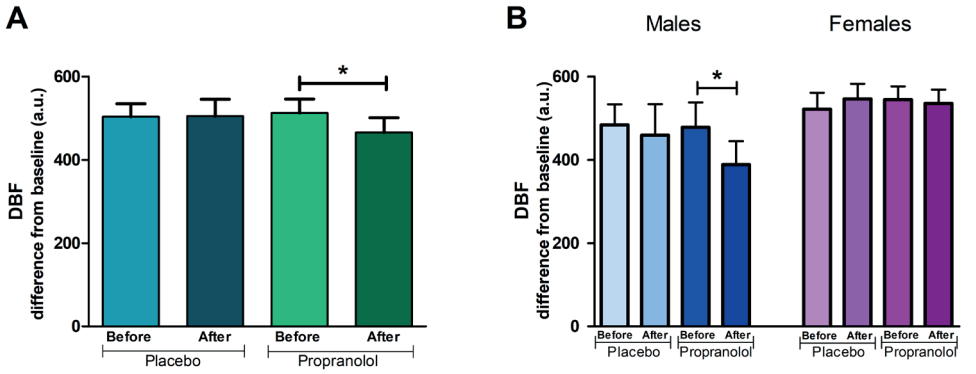

Fig. 3. Forehead DBF responses to capsaicin. DBF response to capsaicin before and after placebo or propranolol as change from baseline (in a.u.) in (A) both sexes and (B) divided by sex (males, left; females, right). Data are presented as mean±SEM. *Significant decrease in DBF response to capsaicin after propranolol. DBF: dermal blood flow.

The DBF responses to ES were not affected by either propranolol (p=0.744) or placebo (p=0.400, Fig. 4). When sub-grouping by sex, DBF responses in males were also not significantly modified after placebo (p=0.514), nor after propranolol (p=0.404); similarly, in female subjects, no significant difference was observed after placebo (p=0.372) or propranolol (p=0.556; data not shown).

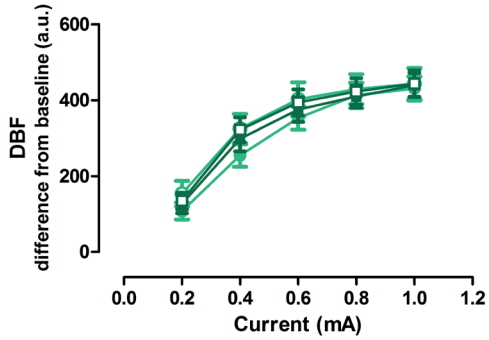

Fig. 5. Forehead DBF responses to ES. Maximal DBF response to ES with an increasing stimulation current before placebo (light green circle) or propranolol (dark green square) and after placebo (light green open circle) or propranolol (dark green open square) as change from baseline (in a.u.). Data are presented as mean±SEM. DBF: dermal blood flow; ES: electrical stimulation.

Pain in electrode site was the most reported side effect (5/21), followed by tingling in electrode site (2/21), weird sensation (1/21 after placebo vs. 1/21 after propranolol) and drowsiness (1/21 after placebo vs. 1/21 after propranolol). Fatigue and weakness were reported after propranolol (1/21 each), but not after placebo. One subject reported neck stiffness due to uncomfortable pillow and two subjects reported nausea and flickering lights after placebo.

Propranolol levels

Mean plasma levels of propranolol were 63.40±12 µg/l (Fig. 5, therapeutic levels between 20-300, NVZA toxicologie.org info). When stratifying by sex, propranolol levels were significantly higher in women when compared to men (90.49±21 µg/l *vs*. 36.31±7 µg/l, respectively; p=0.023), also when adjusted for body weight (data not shown).

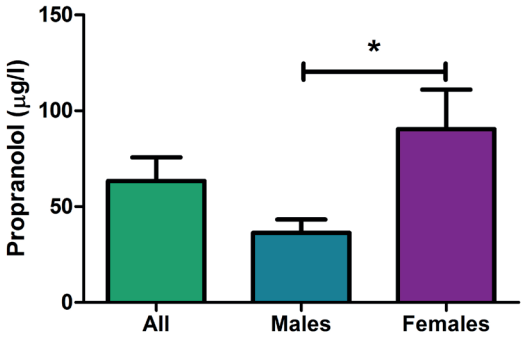

Fig. 6. Propranolol plasma levels. Propranolol plasma levels (in µg/l) pooled, and divided by sex. *Significant difference in propranolol levels between male and female subjects.

Retrospective prescription database study

A total of 291 subjects were included in our analysis (31±7 years old, range 20-39) . The average DDD of triptans in the 12 months before propranolol prescription was 61.2±3.6, which was slightly decreased in the 12 months after propranolol prescription to 59.1±4.3 DDD ($p<0.0001$), representing a decrease of 3%.

When stratifying by sex, 47 males (16%, 31±6 years old, range 20-39) and 244 females (84%, 31±6 years old, range 20-39) were part of the study. When further stratifying female subjects by use of contraceptives, 151 women on contraceptives were included in our analysis (52%, 30±6 years old, range 20-39). Male subjects had a lower DDD of triptans in the 12 months before propranolol than females (42.2±6.0 *vs.* 64.8±4.1, respectively). Furthermore, in males the average DDD of triptans in 12 months after propranolol, was decreased to 35.8±5.8 DDD, representing a reduction of 15% ($p=0.002$) whereas in females, there was a modest decrease to 63.6±4.9 DDD (-2%; $p<0.0001$). When further stratifying to women on contraceptives, an average of 63.0±4.7 DDD of triptans in 12 months before propranolol was observed, with a reduction to 58.4±5.4 DDD in 12 months (-7%; $p<0.0001$). Percentage of change between males and females was significantly different (-15% *vs.* -2%; $p=0.0218$). Similarly, the percentage of change between females on contraceptives and males was significantly different (-7 % vs. -15 %; $p=0.0464$).

Discussion

Since Rabkin and colleagues[9] reported in 1966 that propranolol could be effective for the treatment of "vascular headaches", it has become one of the most widely prescribed drugs for the prophylactic treatment of migraine; however, its exact mechanism of action is not yet known. On this basis, in this study we investigated the effect of propranolol on the modulation of the trigeminovascular system, currently one of the main targets for migraine treatment.

Our results show that a single dose of propranolol (80 mg) significantly inhibits the trigeminal nerve-mediated vasodilation, more specifically, the capsaicin-dependent vasodilation, as the DBF responses to ES were not significantly modified (Figs. 3 and 5). Previous results from our group have shown in the same model that the acutely acting antimigraine drug sumatriptan is able to inhibit the DBF responses to capsaicin to approximately 75% of the control response[17], whereas in the present study we observed a considerably smaller reduction of the DBF responses to capsaicin. This less pronounced inhibition may explain why propranolol is not effective as an acutely acting antimigraine drug, but rather a prophylactic antimigraine agent. Regarding this point, it has been

shown that after continuous administration, elimination of propranolol becomes saturated and it accumulates to a greater extent than predicted by its half-life, due to a decrease in presystemic extraction from 78% after the first dose to 66% following 80 mg[24]. In fact, after chronic administration of the same oral dose to different patients, a 20-fold variation in plasma levels has been described[25]. Moreover, it is important to consider that in the present study we chose the lowest effective dose of propranolol used in clinical trials for migraine treatment[13, 26] as acute intake of higher doses was not ethically feasible. Thus, it is reasonable to assume that higher propranolol doses might have exerted a more pronounced inhibition of the capsaicin-induced increases in DBF. More importantly, the study took place at T_{max} after a single dose, while in clinical practice propranolol levels would be in steady state. However, since no mechanistic information is available thus far, this was an elegant way to study the effect without exposing healthy subjects to a long period of propranolol treatment. Other groups have shown in murine models of cortical spreading depression (CSD) (considered the underlying cause of migraine with aura), that propranolol prevents the changes in behavior (*e.g.* freezing) and cerebral blood flow changes induced by CSD[27], with studies even showing prevention of CSD onset and migration[28,29]. Additionally, it has been shown that chronic administration of l-propranolol, but not of d-propranolol, inhibits the CSD migration and/or onset[28]. Nevertheless, the role of CSD in the pathophysiology of migraine remains debatable[30], and both enantiomers have been proven to be effective for the prophylactic treatment of migraine[31]. Therefore, inhibition of the CSD-related changes seems unlikely to be the only mechanism behind the efficacy of propranolol. Moreover, it has been shown that microiontophoretic ejection of propranolol inhibits thalamocortical activity in response to superior sagittal sinus stimulation and L-glutamate-evoked neuronal activation[32] in a murine model of central trigeminovascular activation. However, not all β-blockers cross the blood brain barrier, such as is the case of atenolol; therefore, their efficacy cannot be only mediated via central mechanisms. In the present study, we showed that a single dose of propranolol modulates the *human peripheral* trigeminovascular system, which broadens the therapeutic implications of our results.

A question that remains open in this study is the exact nature of the receptor(s)/mechanism(s) involved in the modulation of the trigeminovascular system by propranolol. Indeed, (presynaptic/prejunctional) β-adrenoceptors would seem to be the most likely candidates, as it has been shown in rat cerebral cortex and rostral ventrolateral medulla that propranolol inhibits glutamate release via presynaptic receptors[33,34]. Moreover, the inhibition by propranolol of thalamocortical activity in response to dural stimulation was shown to be mediated via β_1-adrenoceptors[32]. However, not all β-blockers are effective for the prophylactic treatment of migraine, and their efficacy does not depend on their selectivity, as the β_1-adrenoceptor antagonists atenolol and metoprolol are effective for the prophylactic treatment of migraine, whereas acebutolol is not[13]. Interestingly, while all the effective β-blockers (*e.g.* propranolol, metoprolol, atenolol) are β-adrenoceptor antagonists, all the non-effective ones (*e.g.* pindolol, acebutolol, labetalol) possess intrinsic sympathomimetic activity, which means that those "β-blockers" behave in fact as partial agonists of the β-adrenoceptors[35]. This could suggest that total blockade of β-adrenoceptors is needed for an effective therapeutic response in migraine treatment. In addition, propranolol has been shown to not only act as a β-adrenoceptor antagonist, but also as a mixed agonist/antagonist of 5-HT receptors in a tissue-dependent manner[36-46]. Furthermore, a single point mutation increases the affinity of β-adrenoceptor antagonists for the 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors[47], all of which have been described to be involved in the therapeutic actions of the triptans[8]. Of special interest, activation of prejunctional 5-HT_{1D} and 5-HT_{1F} receptors is thought to inhibit the release of CGRP from the trigeminal fibers that innervate the dura[8] and the forehead skin, which is in accordance with our

previous observations in our DBF forehead model with sumatriptan[17]. As really high concentrations of propranolol are needed in order to observe an effective therapeutic response in migraine patients[13], it would seem that not only is propranolol acting as a β-adrenoceptor antagonist, but also as an agonist of prejunctional receptors present on trigeminal fibers, possibly 5-HT_{1D} and/or 5-HT_{1F} receptors. This is reinforced by the lack of correlation between the changes in DBF and in SBP, DBP and HR observed after propranolol in the present study (Fig. 2). However, if the inhibition of the capsaicin-induced DBF responses after propranolol were mediated only via prejunctional 5-$HT_{1D/1F}$ receptors, then the inhibition of the capsaicin-induced DBF responses by sumatriptan (a 5-$HT_{1B/1D/1F}$ receptor agonist[8]) previously reported by our group[17], would have presented the same sex-dependent profile.

As mentioned above, in the present study we observed that after stratifying by sex, the inhibition of the DBF responses to capsaicin was sex-dependent, as a pronounced (and significant) inhibition remained present in men, but not in women (Fig. 3). Interestingly, the plasma levels of propranolol in females were significantly higher than in males (Fig. 5), thus excluding a pharmacokinetic cause for the sex-dependent effects. We have previously shown in our forehead model that the trigeminal nerve-induced vasodilatory responses and hormone levels are altered in female migraine patients[18] whereas other studies have demonstrated that ovarian steroid hormones modulate the activation of the trigeminovascular system[19]. Nonetheless, the exact mechanisms involved remain thus far unknown. Moreover, a recent study in rodents showed that application of CGRP to the cranial meninges results in behavioral responses consistent with headache in preclinical models in a female-specific manner[48], reinforcing the interaction between sex-hormones and CGRPergic fibers in the modulation of the trigeminovascular system. Additionally, in a murine model of addiction, a sex-dependent response to propranolol was observed, involving 5-HT and β-adrenergic signaling[49]. Considering that women represent around 70% of migraine patients[2], it is important to address whether our results can be translated into the clinical practice. With this goal in mind, we performed a retrospective study where we correlated our results with data from the Dutch IADB.nl prescription database, and analyzed the change in triptan use after propranolol prescription in a population similar to our DBF study subjects. Our results show that after propranolol, both sexes reduced their use of triptans, with a more pronounced decrease in male subjects when compared to females on contraceptives. Certainly, these results must be interpreted with caution, as it was a retrospective study with a rather small population size and the exact indication for propranolol was not stated. More studies that fall beyond the scope of our current study, should assess whether propranolol shows a sex-dependent efficacy, and the possible receptors/mechanisms involved.

In conclusion, our study shows that an acute dose of propranolol (80 mg) inhibits the capsaicin-induced increases in DBF in a sex-dependent manner. Additionally, in a retrospective study, a more pronounced decrease in triptan use was observed in male patients when compared to females on contraceptives. These results, taken together suggest an interaction between propranolol and sex steroids in the modulation of the trigeminovascular system and support the usefulness of our human model to evaluate trigeminovascular modulation by current and prospective antimigraine drugs.

References

1. Stovner LJ, Nichols E, Steiner TJ, et al. Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurology 2018;17:954-976.
2. Buse DC, Loder EW, Gorman JA, et al. Sex Differences in the Prevalence, Symptoms, and Associated Features of Migraine, Probable Migraine and Other Severe Headache: Results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache: The Journal of Head and Face Pain 2013;53:1278-1299.

3. Goadsby PJ, Lipton RB, Ferrari MD. Migraine - Current Understanding and Treatment. New England Journal of Medicine 2002;346:257-270.
4. Edvinsson L. The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine. Headache 2017;57 Suppl 2:47-55.
5. Feniuk W, Humphrey PPA. The development of a highly selective 5-HT_1 receptor agonist, sumatriptan, for the treatment of migraine. Drug Development Research 1992;26:235-240.
6. The Subcutaneous Sumatriptan International Study Group Treatment of migraine attacks with sumatriptan. New England Journal of Medicine 1991;325:316-321.
7. Deleu D, Hanssens Y. Current and Emerging Second-Generation Triptans in Acute Migraine Therapy: A Comparative Review. The Journal of Clinical Pharmacology 2000;40:687-700.
8. Rubio-Beltran E, Labastida-Ramirez A, Villalon CM, MaassenVanDenBrink A. Is selective 5-HT_{1F} receptor agonism an entity apart from that of the triptans in antimigraine therapy? Pharmacol Ther 2018.
9. Rabkin R, Stables DP, Levin NW, Suzman MM. The prophylactic value of propranolol in angina pectoris. American Journal of Cardiology 1966;18:370-380.
10. Wykes P. The treatment of angina pectoris with coexistent migraine. The Practitioner 1968;200:702.
11. Békés M, Matos L, Rausch J, Török E. Treatment of migraine with propranolol. The Lancet 1968;292:980.
12. Weber RB, Reinmuth OM. The treatment of migraine with propranolol. Neurology 1972;22:366-366.
13. Jackson JL, Cogbill E, Santana-Davila R, et al. A Comparative Effectiveness Meta-Analysis of Drugs for the Prophylaxis of Migraine Headache. PloS one 2015;10:e0130733-e0130733.
14. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
15. Edvinsson L. CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. British journal of clinical pharmacology 2015;80:193-199.
16. Ibrahimi K, Vermeersch S, Danser A, et al. Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead. Cephalalgia 2014;34:514-522.
17. Ibrahimi K, Danser AHJ, Terwindt GM, van den Meiracker AH, MaassenVanDenBrink A. A human trigeminovascular biomarker for antimigraine drugs: A randomised, double-blind, placebo-controlled, crossover trial with sumatriptan. Cephalalgia 2016;37:94-98.
18. Ibrahimi K, van Oosterhout WPJ, van Dorp W, et al. Reduced trigeminovascular cyclicity in patients with menstrually related migraine. Neurology 2015;84:125-131.
19. Labastida-Ramirez A, Rubio-Beltran E, Villalon CM, MaassenVanDenBrink A. Gender aspects of CGRP in migraine. Cephalalgia 2019;39:435-444.
20. Ibrahimi K, Vermeersch S, Frederiks P, et al. The influence of migraine and female hormones on capsaicin-induced dermal blood flow. Cephalalgia 2017;37:1164-1172.
21. Eldon MA, Kinkel AW, Daniel JE, Latts JR. Bioavailability of propranolol hydrochloride tablet formulations: Application of multiple dose crossover studies. Biopharmaceutics & Drug Disposition 1989;10:69-76.
22. Clinical pharmacology and biopharmaceutics reviews. Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Application number: 205410Orig1s000. [online]. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2014/205410Orig1s000ClinPharmR.pdf. Accessed 29-04-2019.
23. Visser ST, Schuiling-Veninga CCM, Bos JHJ, de Jong-van den Berg LTW, Postma MJ. The population-based prescription database IADB.nl: its development, usefulness in outcomes research and challenges. Expert Review of Pharmacoeconomics & Outcomes Research 2013;13:285-292.
24. Wood AJ, Carr K, Vestal RE, Belcher S, Wilkinson GR, Shand DG. Direct measurement of propranolol bioavailability during accumulation to steady-state. British journal of clinical pharmacology 1978;6:345-350.
25. Shand DG. Pharmacokinetics of propranolol: a review. Postgrad Med J 1976;52 Suppl 4:22-25.
26. Linde K, Rossnagel K. Propranolol for migraine prophylaxis. Cochrane Database Syst Rev 2004:CD003225.
27. Kurauchi Y, Haruta M, Tanaka R, et al. Propranolol prevents cerebral blood flow changes and pain-related behaviors in migraine model mice. Biochemical and Biophysical Research Communications 2019;508:445-450.
28. Ayata C, Jin H, Kudo C, Dalkara T, Moskowitz MA. Suppression of cortical spreading depression in migraine prophylaxis. Annals of Neurology 2006;59:652-661.
29. Richter F, Mikulik O, Ebersberger A, Schaible H-G. Noradrenergic Agonists and Antagonists Influence Migration of Cortical Spreading Depression in Rat—a Possible Mechanism of Migraine Prophylaxis and Prevention of Postischemic Neuronal Damage. Journal of Cerebral Blood Flow & Metabolism 2005;25:1225-1235.
30. Charles AC, Baca SM. Cortical spreading depression and migraine. Nature Reviews Neurology 2013;9:637.
31. Stensrud P, Sjaastad O. Short-term clinical trial of propranolol in racemic form (INDERAL), d-propranolol and placebo in migraine. Acta Neurologica Scandinavica 1976;53:229-232.

32. Shields KG, Goadsby PJ. Propranolol modulates trigeminovascular responses in thalamic ventroposteromedial nucleus: a role in migraine? Brain 2004;128:86-97.
33. Okuda N, Kohara K, Mikami H, et al. Effect of propranolol on central neurotransmitter release in wistar rats analysed by brain microdialysis. Clinical and Experimental Pharmacology and Physiology 1999;26:220-224.
34. Wang S-J, Coutinho V, Sihra TS. Presynaptic cross-talk of β-adrenoreceptor and 5-hydroxytryptamine receptor signalling in the modulation of glutamate release from cerebrocortical nerve terminals. British Journal of Pharmacology 2002;137:1371-1379.
35. Olsson JE, Behring HC, Forssman B, et al. Metoprolol and propranolol in migraine prophylaxis: a double-blind multicentre study. Acta Neurologica Scandinavica 1984;70:160-168.
36. Schoeffter P, Pfeilschifter J, Bobirnac I. 5-Hydroxytryptamine 5-HT_{1B} receptors inhibiting cyclic AMP accumulation in rat renal mesangial cells. Naunyn-Schmiedeberg's Archives of Pharmacology 1995;351:35-39.
37. Pauwels PJ, Palmier C. Inhibition by 5-HT of forskolin-induced cAMP Formation in the renal opossum Epithelial cell line OK: Mediation by a 5-HT1B like receptor and antagonism by methiothepin. Neuropharmacology 1994;33:67-75.
38. Glennon RA, Pierson ME, McKenney JD. Stimulus generalization of 1-(3-trifluoromethylphenyl) piperazine (TFMPP) to propranolol, pindolol and mesulergine. Pharmacology Biochemistry and Behavior 1988;29:197-199.
39. Hjorth S, Carlsson A. Is pindolol a mixed agonist-antagonist at central serotonin (5-HT) receptors? European Journal of Pharmacology 1986;129:131-138.
40. Maura G, Ulivi M, Raiteri M. (–)-Propranolol and (±)-cyanopindolol are mixed agonists-antagonists at serotonin autoreceptors in the hippocampus of the rat brain. Neuropharmacology 1987;26:713-717.
41. Tsuchihashi H, Nakashima Y, Kinami J, Nagatomo T. Characteristics of ^{125}I-Iodocyanopindolol Binding to b-Adrenergic and Serotonin-1B Receptors of Rat Brain: Selectivity of b-Adrenergic Agents. The Japanese Journal of Pharmacology 1990;52:195-200.
42. Hoyer D, Clarke DE, Fozard JR, et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev 1994;46:157-203.
43. Murphy TJ, Bylund DB. Characterization of serotonin-1B receptors negatively coupled to adenylate cyclase in OK cells, a renal epithelial cell line from the opossum. Journal of Pharmacology and Experimental Therapeutics 1989;249:535-543.
44. Nishimura Y. Characterization of 5-hydroxytryptamine receptors mediating contractions in basilar arteries from stroke-prone spontaneously hypertensive rats. British Journal of Pharmacology 1996;117:1325-1333.
45. Schoeffter P, Hoyer D. 5-Hydroxytryptamine 5-HT_{1B} and 5-HT_{1D} receptors mediating inhibition of adenylate cyclase activity. Naunyn-Schmiedeberg's Archives of Pharmacology 1989;340:285-292.
46. Adham N, Ellerbrock B, Hartig P, Weinshank RL, Branchek T. Receptor reserve masks partial agonist activity of drugs in a cloned rat 5-hydroxytryptamine1B receptor expression system. Molecular Pharmacology 1993;43:427-433.
47. Adham N, Tamm JA, Salon JA, Vaysse PJJ, Weinshank RL, Branchek TA. A single point mutation increases the affinity of serotonin 5-$HT_{1Dα}$, 5-$HT_{1Dβ}$, 5-HT_{1E} and 5-HT_{1F} receptors for β-adrenergic antagonists. Neuropharmacology 1994;33:387-391.
48. Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. The Journal of Neuroscience 2019;39:4323-4331.
49. Kohtz AS, Aston-Jones G. Cocaine Seeking During Initial Abstinence Is Driven by Noradrenergic and Serotonergic Signaling in Hippocampus in a Sex-Dependent Manner. Neuropsychopharmacology 2016;42:408.

PART IV:

New therapeutic targets and pathophysiology of migraine

Chapter X.

PACAP38 and PAC_1 receptor blockade: a new target for headache?

Based on: ***E Rubio-Beltrán,*** *E Correnti, M Deen, K Kamm, T Kelderman, L Papetti, S Vigneri, A MaassenVanDenBrink, L Edvinsson. On behalf of the European Headache Federation School of Advanced Studies (2018) Journal of Headache and Pain; 19:64.*

Abstract

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) is a widely distributed neuropeptide involved in neuroprotection, neurodevelopment, nociception and inflammation. Moreover, PACAP38 is a potent inducer of migraine-like attacks, but the mechanism behind this has not been fully elucidated.

Migraine is a neurovascular disorder, recognized as the second most disabling disease. Nevertheless, the antibodies targeting calcitonin gene-related peptide (CGRP) or its receptor are the only prophylactic treatment developed specifically for migraine. These antibodies have displayed positive results in clinical trials, but are not effective for all patients; therefore, new pharmacological targets need to be identified.

Due to the ability of PACAP38 to induce migraine-like attacks, its location in structures previously associated with migraine pathophysiology and the 100-fold selectivity for PAC_1 receptor when compared to VIP, new attention has been drawn to this pathway and its potential role as a novel target for migraine treatment. In accordance with this, antibodies against PACAP38 (ALD 1910) and PAC_1 receptor (AMG 301) are being developed, with AMG 301 already in Phase II clinical trials. No results have been published so far, but in preclinical studies, AMG 301 has shown responses comparable to those observed with triptans. If these antibodies prove to be effective for the treatment of migraine, several considerations should be addressed, for instance, the potential side effects of long-term blocking of the PACAP (receptor) pathway. Moreover, it is important to investigate whether these antibodies will indeed represent a therapeutic advantage for the patients that do not respond the CGRP (receptor)-antibodies.

In conclusion, the data presented in this review indicate that PACAP38 and PAC_1 receptor blockade are promising migraine therapies, but results from clinical trials are needed in order to confirm their efficacy and side effect profile.

Discovery of PACAP

The description of the pituitary adenylate cyclase activating polypeptide-38 (PACAP38) was made by Arimura and his team in 1989, following the extraction of the peptide from more than 4000 samples of ovine hypothalamus. After the isolation, its characterization showed that it was formed by 38 amino acids, with a 68% homology with the vasoactive intestinal peptide (VIP), described almost twenty years earlier[1]. Subsequently, the peptide was synthesized and shown to activate adenylyl cyclase (AC) in cultures of rat pituitary cells, thereby obtaining its name as pituitary adenylate cyclase activating polypeptide. A year later, a fragment of PACAP38 with similar AC activation profile was isolated. This was formed by 27 amino acids and thus named PACAP27[2]. That same year, cloning of cDNA from ovine PACAP38 revealed that the amino acid sequence of the mature human PACAP38 was identical to that of the ovine. In addition, later studies showed that it was identical in all mammals[3], suggesting that it has been conserved during evolution.

This review will give an overview of PACAP, its complex signaling pathway, the role PACAP and its receptors have in physiological conditions and their involvement in some disorders, with special focus on migraine. Moreover, the preclinical results of PACAP (receptor) blockade in migraine models, the side effects that could be expected in clinical trials, and the considerations that must be taken if PACAP (receptor)-antibodies are effective for migraine treatment will be discussed.

Pharmacology

PACAP belongs to a wider group of peptides called the VIP/glucagon/growth hormone releasing factor/secretin superfamily. The ADCYAP1gene, located on chromosome 18, encodes PACAP;

initially, a proprotein is expressed, and later processed to form a 38 amino acid peptide (PACAP38) with a cleavage-amidation site that can generate a 27-residue-amidated fragment (PACAP27). In mammals, the most prevalent form is PACAP38[4], therefore, in this review PACAP38 will be referred as PACAP unless stated otherwise.

Three PACAP receptors have been described: $VPAC_1$, $VPAC_2$ and PAC_1, all coupled to G-proteins (Fig. 1). $VPAC_1$ and $VPAC_2$ receptors present equal affinity for PACAP and VIP and their activation stimulates AC. On the other hand, PAC_1 receptor is 100 times more selective for PACAP and presents a complex signaling pathway[4].

Alternative splicing of the PAC_1 receptor gene results in several isoforms. These receptor variants are characterized by shorter extracellular domains (PAC_1*short*, PAC_1*veryshort*), different inserts in an intracellular loop important for G-protein interaction (PAC_1*null*, PAC_1*hip*, PAC_1*hop1*, PAC_1*hop2*, PAC_1*hiphop1*, PAC_1*hiphop2*) and/or discrete sequences located in transmembrane domains II and IV (PAC_1*TM4*)[5-8]. Of relevance, in humans, twelve homologues have been reported[7,9-11], and have been reviewed elsewhere[12,13]. For each splice variant, PACAP38 and PACAP27 present similar affinity and potency for AC and phospholipase C (PLC) stimulation, but different efficacy (i.e. maximal effect) of PLC responses[14,15]. Although in several processes the activation of AC or PLC can result in similar "stimulatory" responses, in smooth muscle cells (e.g. blood vessels), activation of AC leads to vasodilation, whereas PLC activation results in vasoconstriction. This plays an important role in disorders such as migraine, where expression of a PAC_1 receptor isoform with a lower PLC efficacy could favor AC stimulation, thus facilitating vasodilatory responses in cranial blood vessels[16,17].

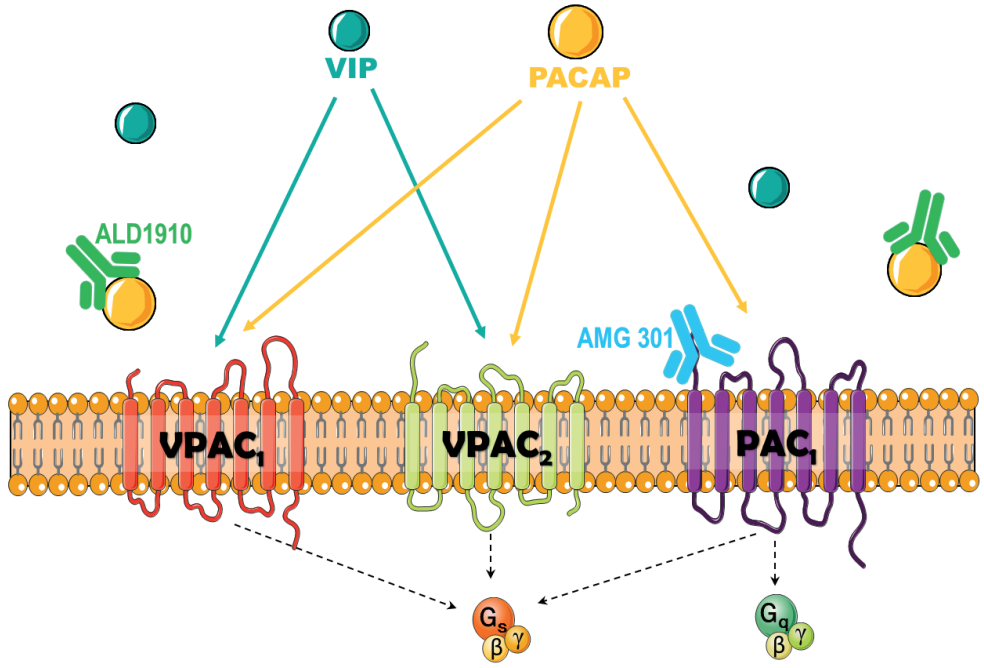

Fig. 1. PACAP receptors. Three receptors to PACAP have been described: $VPAC_1$, $VPAC_2$ and PAC_1. VIP and PACAP show similar affinity for $VPAC_1$ and $VPAC_2$, whereas PACAP is 100-fold more selective for PAC_1 receptor. The antibodies developed for prophylactic migraine treatment bind either to PACAP (PACAP38, ALD1910) or to the PAC_1 receptor (AMG 301).

To study PAC_1 receptor-mediated responses, selective agonists and antagonists are used. Currently, one selective agonist has been described, maxadilan[18,19] and three antagonists M65, Max.d.4 and PACAP6-38[20]. However, no study has investigated whether such compounds are selective for one PAC_1 receptor variant, or whether they bind to all isoforms. Moreover, PACAP6-38 also binds to the $VPAC_2$ receptor, and, together with M65, have been shown to behave as agonists of the PAC_1 receptor in certain tissues[21,22]. Hence, novel selective pharmacological tools are needed to characterize PAC_1 receptor-mediated responses. Indeed, an antibody against PAC_1 receptor, such as AMG 301, could be useful for characterization; however, it is yet not clear if this antibody is selective for one specific variant. If the antibody would be selective for one of the splice variants, this may affect its therapeutic potential, in particular if there are different splice variants expressed in different human populations. On the other hand, different splice variants might hypothetically offer the possibility of designing a drug that would selectively affect the PAC_1 receptor in the trigeminovascular system, while not affecting PAC_1 receptors at other sites in the body, thus reducing its potential side effects.

Physiological roles of PACAP and PAC_1 receptor

Preclinical studies have shown that PACAP and PAC_1 receptors are widely distributed, both centrally and peripherally. It is therefore not surprising that it is described as a (neuro)hormone, neurotransmitter, neuromodulator, neurotrophic factor and immunomodulator[13]. As the PAC_1 receptor is currently under investigation for migraine treatment, only the distribution of this receptor will be reviewed, while the distribution of $VPAC_{1/2}$ receptors is reviewed extensively elsewhere[13,23,24].

PACAP/PAC_1 receptor in the central nervous system

PACAP fibers and PAC_1 receptors are widely expressed throughout the central nervous system (CNS) with the highest density of both in the hypothalamus and supraoptic nucleus[25-31]. In accordance with this, PAC_1 receptor activation has been associated with release of vasopressin and regulation of drinking behavior[32,33], decrease of food intake[34-36], modulation of the sleep/wake cycle[37,38], clock gene expression[38], melatonin synthesis stimulation[39], sexual maturation[40,41], stress and sexual behavior[41,42], learning[43], pain processing[44] and psychomotor responsiveness[45] .

Of special interest for migraine, both PACAP fibers and the PAC_1 receptor are present in the paraventricular nucleus of the hypothalamus, the ventrolateral periaqueductal gray, the locus coeruleus, the solitary nucleus, the trigeminal nucleus caudalis (TNC) and the trigeminal ganglion (TG). These structures have all been associated with nociception and/or migraine pathophysiology[23,46-49].

PACAP/PAC_1 receptor in the periphery

Peripherally, PACAP fibers and/or cell bodies have been described in acrosome caps of primary spermatocytes, mature spermatids, in the testis, epithelial cells from epididymal tubules, the ovaries, mammary glands, in stromal stem cells and terminal placental villi, and the amount of PACAP mRNA increases with the progression of pregnancy[50-52]. Similarly, PAC_1 receptors have been described in spermatids, the penile corpus cavernosum, the ovaries, the chorionic vessels and in stromal and decidual cells of the placenta[51,53-55]. Considering the presence of PACAP and PAC_1 receptors also in hypothalamus and pituitary, an important role in modulation of the hypothalamo-pituitary-gonadal axis is suggested.

PACAP fibers and cell bodies are also found in the adrenal gland, pancreas, epithelium and smooth muscle cells of the urinary tract, the bladder, urethra, larynx, lungs, gastrointestinal smooth muscle cells, duodenal mucosa, thymus, spleen and innervating vascular smooth muscle cells[23,26,56-67]. PAC_1 receptors have been described in the adrenal medulla, pancreas, liver, lungs, enterochromaffin-like cells, thymus and vascular smooth muscle cells[47,56,62,67-70].

Due to their vast distribution peripherally, PACAP and the PAC_1 receptor are involved in a variety of physiological processes, such as regulation of adrenaline release[71], stimulation of adipocyte thermogenesis[72], lipid metabolism[73], metabolic stress adaptation[74], glucose and energy homeostasis[75], renin production[76,77] and inflammatory responses[78]. Furthermore, PACAP and the PAC_1 receptor have a crucial role in the long-term maintenance of neurogenic vasodilation in the periphery and in the homeostatic responses to cerebral, retinal, cardiac, hepatic, intestinal and renal ischemic events[79-88]. This topic has been extensively reviewed elsewhere[89].

PACAP and PAC_1 receptor in pathophysiological conditions

Besides being involved in several physiological processes, PACAP is thought to contribute to the pathophysiology of several conditions.

PACAP has been associated with regulation of inflammatory processes. In an arthritis model, $PACAP^{-/-}$ mice showed absence of arthritic hyperalgesia and reduction of joint swelling, vascular leakage and inflammatory cell accumulation. In the late phase of the disease, immune cell function and bone neoformation were increased[90]. In rheumatoid arthritis, the vasodilatory effects of PACAP through activation of the PAC_1 receptor facilitated plasma leakage, edema formation, and leukocyte migration[91,92]. Furthermore, $PACAP^{-/-}$ mice developed more severe inflammation and tumors in a model of colitis[78]. In preclinical models, upregulation of PACAP and its receptors in micturition pathways contributed to the development of urinary bladder dysfunction, including symptoms of increased voiding frequency and pelvic pain[58], suggesting a role in low urinary tract dysfunction. In the nervous system, studies demonstrated anxiogenic actions of PACAP and the possibility of blocking anxiety-related behaviors with PAC_1 receptor antagonists[93-95]. In patients with post-traumatic stress disorder (PTSD), blood levels of PACAP correlated with severity of stress-related symptoms[96], and in females, a single nucleotide polymorphism in the estrogen response element of the PAC_1 receptor gene is predictive of PTSD diagnosis[97].

Furthermore, PACAP plays a complex role in pain transmission. At the peripheral sensory nerve terminals, pro- and anti-nociceptive effects are observed; while in CNS, central sensitization, increase of neuronal excitation and induction of chronic pain have been described[98]. In an acute somatic and visceral inflammatory model, PACAP decreased pain transmission; however, after application in the spinal cord, a transient induction of analgesia was followed by long-lasting algesia[99]. Moreover, injection of PACAP into the paraventricular nucleus of hypothalamus increased the activity of the TNC, an effect which was inhibited by the PAC_1 receptor antagonist[48]. Although it has been shown that PACAP is transported through the blood-brain barrier (BBB) actively, it is rapidly degraded or returned by efflux pumps[100]. Thus, a direct central action of peripheral PACAP is unlikely.

Although the role of PACAP in pain processing remains elusive, clinical data strongly suggest the involvement of PACAP in the pathophysiology of migraine and cluster headache (CH). Recent evidence of a correlation between a genetic variant of the PAC_1 receptor gene (ADCYAP1R1) and susceptibility to CH was demonstrated[103]. Another study identified a relationship between altered PACAP levels in peripheral blood and different types of headache[104]. Further, two studies reported low interictal plasma levels of PACAP in migraine and CH when compared to controls[105,106]. Particularly, a detailed analysis of PACAP mRNA expression in peripheral blood mononuclear cells detected a significantly lower level of PACAP in migraine patients compared to healthy controls, with no significant differences revealed between the control group and tension-type headache, CH or medication overuse headache groups. Interestingly, PACAP increased ictally in jugular or cubital blood of migraine[105,107,108] and CH patients[93,106], and levels decreased as headache ameliorated after sumatriptan administration[108]. Finally, when administered to migraine patients, PACAP induced an

instant headache in 90% of patients, which was later followed by a delayed headache similar to a migraine-like attack in two thirds of the subjects[109]. This has led to study the role of PACAP in migraine pathophysiology as will be discussed in the next section.

PACAP in migraine pathophysiology

The use and development of experimental animal and human models of headache, migraine in particular, have provided invaluable insight into the pathophysiological mechanisms underlying headache disorders[110,111]. To investigate the molecular mechanisms behind the headache-inducing effects of PACAP, a number of animal studies have been conducted. Additionally, several human studies have been performed, some of these in combination with imaging techniques. In the following sections, both human and animal studies investigating the headache-related effects of PACAP will be reviewed.

Human studies

The headache-inducing effect of PACAP was first reported in a study on cerebral blood flow in healthy volunteers, where 10 out of 11 participants reported mild to moderate headache after PACAP infusion[112]. A double-blind, randomized, placebo-controlled, crossover study later showed that 12 out of 12 healthy subjects and 11 out of 12 migraine patients reported headache after intravenous infusion of PACAP, compared to two and three, respectively, after placebo[109]. Further, two healthy subjects and one migraine patient reported a migraine-like attack within 2 h after infusion, whereas six migraine patients reported a migraine-like attack within 6 h after infusion. This study also found dilation of middle cerebral artery (MCA) and the superficial temporal artery after PACAP infusion.

The role of vasodilation in PACAP induced headache was further explored in a magnetic resonance angiography (MRA) study in healthy volunteers[113]. Eight out of nine participants reported an immediate headache and 100% reported a delayed headache after PACAP infusion. Further, over a 5 h period PACAP induced a sustained dilation of the *extracranial* middle meningeal artery (MMA) but no change in intracerebral MCA. Collectively, these studies support the notion that PACAP induces headache via sustained vasodilation. In another MRA study, PACAP infusion induced headache in 91% of included migraine patients, and 73% reported migraine-like attacks compared to 82% and 18%, respectively, after VIP administration. Further, PACAP induced a long-lasting (>2 h) dilation of extracranial arteries, whereas the dilation caused by VIP normalized after 2 h. This further underlines prolonged vasodilation as the migraine inducing mechanism of PACAP[114]. Interestingly, in an *in vitro* study neither PACAP nor VIP were potent in inducing vasodilation of the human MMA[115].

In a resting-state magnetic resonance study, infusion of PACAP affected connectivity in the salience, the default mode and the sensorimotor network during migraine attacks. VIP had no effect on these networks[116]. Another study in migraine patients reproduced the induction of migraine-like attacks in 72% of patients and showed that PACAP induced premonitory symptoms in 48% of patients compared to 9% after CGRP[117], suggesting an effect on central PAC_1 receptors. However, PACAP is rapidly degraded or transported back after actively crossing the BBB[100]; therefore, the premonitory symptoms could be mediated via activation of a central structure that is not protected by the BBB. Lastly, increased blood markers of hypothalamic activation, neuronal damage and peptide release from parasympathetic and sensory perivascular nerve fibers were found during PACAP-induced migraine-like attacks[118].

The human studies point out PACAP as a key player in migraine pathophysiology[102]. As VIP does not induce migraine-like attacks, it is assumed that PACAP's actions are mediated by PAC_1 receptor activation. However, it is still too early to rule out $VPAC_{1/2}$ receptors as additional potential antimigraine targets, since no studies in humans have been performed with antagonists. Further, the short half-life of VIP (2 min[119]), could be the cause of its lack of migraine-inducing effects.

Animal studies

To characterize the exact receptor involved in PACAP-mediated actions, the vasodilatory effect of PACAP was elucidated in animal studies showing that both VIP, PACAP38 and PACAP27 induce vasodilation of the rat MMA *in vivo*[120,121]. Interestingly, this effect was blocked by $VPAC_1$ antagonists in the former[120] and $VPAC_2$ antagonists in the latter[121]. Both studies found no effect of PAC_1 antagonists on vasodilation. In contrast, an *ex vivo* study found that PAC_1 antagonists reversed the PACAP-induced vasodilation in the rat MMA[17]. As mentioned previously, PAC_1 receptor antagonists have shown agonistic behavior and affinity for $VPAC_2$ receptors. This could explain the contradictory results observed in the rat MMA vasodilation studies. Therefore, different methods must be used, in order to elucidate the receptors involved in migraine pathophysiology. For example, in a *in vivo* model of chronic migraine, induced by recurrent chemical dural stimulation, PAC_1 receptor mRNA was shown to be increased in the TG, but not in the TNC, and no significant differences were found in the expression of the $VPAC_1$ and $VPAC_2$ receptors[122]. Moreover, in an *in vivo* rat model, intravenous administration of AMG 301, the PAC_1 receptor antibody, inhibited evoked nociceptive activity in the trigemino-cervical complex, and the results were comparable to the inhibition observed with sumatriptan[123].

In addition to sustained vasodilation, mast cell degranulation has also been suggested as one of the headache inducing mechanisms of PACAP. This hypothesis is based on findings from animal studies showing that PACAP degranulates mast cells from the rat dura mater[124]. Further, PACAP-induced delayed vasodilation of the rat MMA is attenuated in mast cell depleted rats[125]. Interestingly VIP did not result in mast cell release of histamine from the dura[126].

Collectively, the animal studies confirm that PACAP induces vasodilation and suggest that this effect might be mediated through degranulation of mast cells. Also, recent results show that these effects are most likely exerted through activation of the PAC_1 receptor. Due to the contradictory results, further studies are warranted to confirm this.

PACAP (receptor) blockade as a therapeutic target

As shown above, PACAP seems to play an important role in migraine pathophysiology. Although the exact receptor involved has not yet been elucidated, some studies indicate that the PAC_1 receptor is the most important[17,48,113,117,122,123]. Therefore, both PACAP and PAC_1 receptor have been suggested as novel targets for migraine treatment and possibly a new therapeutic option for patients who do not respond to CGRP (receptor) blocking drugs. Although both neuropeptides co-localize in the trigeminal ganglion[49], and could share some biological cascades, the PACAP-induced migraine attacks indicate an independent role of PACAP in the genesis of migraine.

In this light, the interest from pharmaceutical companies for blocking the PACAP/PAC_1 receptor pathway has increased. There are two therapeutic approaches to inhibit PACAP: (i) PAC_1 receptor antagonists or antibodies directed against this receptor; or (ii) antibodies directed against the peptide PACAP[102]. Since PAC_1 receptor antagonists have been reported to act as agonists depending on the tissue (see Pharmacology), the antibodies seem a better option for blocking this receptor.

Currently, a phase 2a, randomized, double blind, placebo-controlled study is underway to evaluate the efficacy and safety of a PAC_1 receptor antibody (AMG 301) in subjects with chronic or episodic migraine (Clinical trials identifier: NCT03238781). Unfortunately, no preliminary results have been published so far. Preclinical studies are also evaluating a monoclonal antibody (ALD1910) targeting PACAP38 for its potential in the treatment of migraine patients who have an inadequate response to therapeutics directed at CGRP or its receptor[128].

Potential side effects of PACAP/PAC$_1$ receptor blockade

Indeed, the possibility of a new therapeutic target for prophylactic migraine treatment is exciting; however, it is important to consider that PACAP and PAC$_1$ receptor participate in numerous physiological processes (see Fig. 2). As antibodies are not likely to cross the BBB, only the possible side effects regarding peripheral blockade of PACAP and PAC$_1$ receptor will be discussed.

As PACAP and PAC$_1$ receptor are expressed throughout the components of the hypothalamo-pituitary-gonadal axis[50-52], and the pituitary gland is not protected by the BBB, a dysregulation of the functions of this axis could be a concern. Also, the immune system has been described to be regulated by activation of PAC$_1$ receptor[61]. This, together with its participation in the modulation of inflammatory processes, could result in alterations in the immune response and increased production of pro-inflammatory cytokines[78,129]. In accordance with this, in a mouse model of colitis, PACAP-deficient mice developed a more severe disease[78].

Blocking PACAP might also alter the response to metabolic stress. Studies with PACAP-deficient mice have shown a more profound and longer lasting insulin-induced hypoglycemia and a reduction in glucose-stimulated insulin secretion[74,75]. Moreover, PACAP-deficient mice had hepatic microvesicular steatosis, intracellular fat accumulation in muscle and skeletal muscle and depletion of subcutaneous white fat[73].

Furthermore, PACAP and PAC$_1$ receptor participate in vasodilatory responses, renin release and regulation of cardiovascular function[77,115,125]. Although the density of VPAC$_{1/2}$ and PAC$_1$ receptors in coronary artery is less than that in cranial MMA[115], arguing for a limited role in cardiac ischemia, a protective role in ischemic events has been described. Thus, considering the increased cardiovascular risk that migraine patients present[130-133], careful monitoring of patients with preexisting cardiovascular risk factors is advised. However, similar concerns have been raised with the CGRP (receptor)-antibodies[134,135], with no cardiovascular adverse events reported in the clinical trials[136].

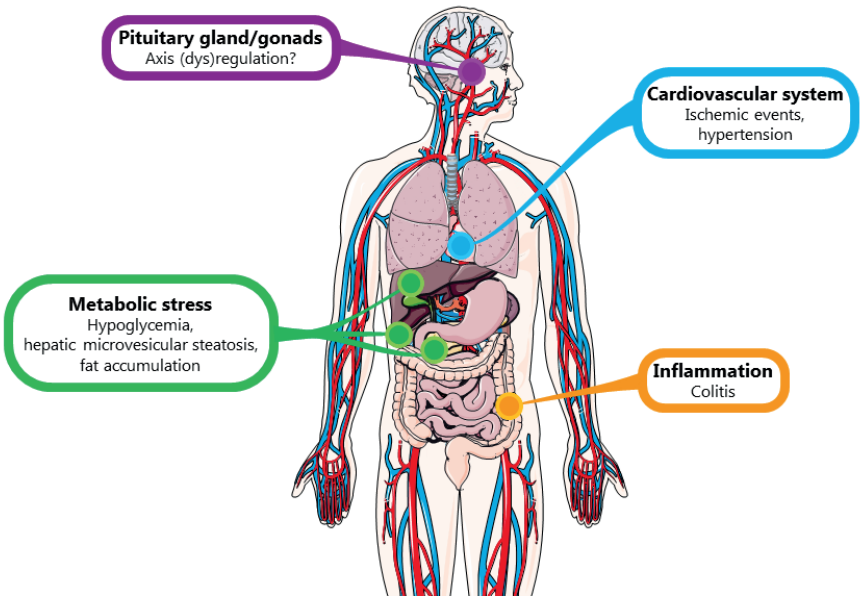

Fig. 2. Possible side effects after long-term exposure to PACAP (receptor)-antibodies. An overview of the organ systems where PACAP and PAC$_1$ receptor are present and the possible side effects that could be observed.

Further considerations

If the antibodies against the PAC_1 receptor prove to be effective for the prophylactic treatment of migraine, some concerns should be addressed. Firstly, as previously discussed, it is important to consider the possible side effects of long-term blocking of PACAP/PAC_1 receptor, with emphasis on the cardiovascular system, as migraine patients present a higher cardiovascular risk. Therefore, safety studies in patients with cardiovascular disease are needed. Moreover, the administration route of the antibody against the PAC_1 receptor is subcutaneous, thus erythema, pruritus and mild pain in the injection site could be expected, as it has been observed with the CGRP (receptor) – antibodies[137]. Nevertheless, the monthly administration represents an advantage for treatment adherence.

It will also be important to define if PAC_1 receptor antibodies will really represent a therapeutic advantage for the patients that are not responding to the CGRP (receptor)-antibodies. Since studies have shown that PACAP and CGRP co-localize in structures relevant for migraine pathophysiology (e.g. trigeminal ganglion)[49], PACAP blockade may only be effective for the same patients to whom CGRP blockade is already effective. If a distinction can be made between patient groups this would also shed light on the pathophysiology of migraine, as it could distinguish between CGRP-associated or PACAP-associated migraine patients. Moreover, the PAC_1 receptor sequence that is recognized by the antibody has not been disclosed, thus, the variants of the receptor to which the antibody binds are not known. If revealed, it would be interesting to study whether certain receptor isoforms predispose patients to present migraine, or whether the treatment will only be effective in patients with those isoforms.

Finally, as mentioned previously, it is still too early to rule out $VPAC_{1/2}$ receptors as therapeutic targets for migraine treatment. Therefore, ALD1910, the antibody against PACAP38, currently undergoing preclinical studies[128], broadens the therapeutic options for migraine treatment. However, further safety studies should be addressed, as blocking PACAP38 would inhibit the actions of three different receptors, increasing the possibilities of adverse side effects.

Conclusion

The possible role of PACAP/PAC_1 receptor blockade as migraine treatment has been reviewed. All three PACAP receptors have been described in TG, TNC and (dural) arteries, structures previously related to migraine pathophysiology[47,49]. Indeed, infusion of PACAP is able to induce migraine-like attacks[109]. Moreover, interictally, low plasma levels of PACAP have been described[105], while during a migraine attack, PACAP increases in jugular and cubital blood[105,108] and decreases as headache ameliorates after sumatriptan administration[108].

Clinical studies have shown that infusion of VIP does not induce migraine-like headaches[114], therefore, it is considered that the possible receptor involved in PACAP actions is PAC_1 receptor, as VIP has affinity for $VPAC_1$ and $VPAC_2$ receptors. Although this could be attributed to pharmacokinetic aspects (i.e. half-life), rather than pharmacodynamic. Pharmacological characterization in preclinical studies has given contradictory results, indicating a complex pharmacology of the PAC_1 receptor[21,22]. However, a recent *in vivo* study showed that intravenous infusion of PAC_1 receptor antibody, inhibited evoked nociceptive activity in the trigemino-cervical complex in rats, and these results were comparable to the inhibition observed with sumatriptan[123]. These results have led to the development of antibodies against PACAP (ALD1910) and PAC_1 receptor (AMG 301) for migraine treatment.

In conclusion, the data presented in this review indicate that PACAP and PAC_1 receptor blockade are promising migraine therapies but results from clinical trials are needed in order to confirm their efficacy and their side effects profile.

References

1. Miyata A, Arimura A, Dahl RR, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochemical and Biophysical Research Communications 1989;164:567-574.
2. Miyata A, Jiang L, Dahl RD, et al. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). Biochemical and Biophysical Research Communications 1990;170:643-648.
3. Kimura C, Ohkubo S, Ogi K, et al. A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. Biochemical and Biophysical Research Communications 1990;166:81-89.
4. Harmar AJ, Fahrenkrug J, Gozes I, et al. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR Review 1. British Journal of Pharmacology 2012;166:4-17.
5. Shinohara K, Funabashi T, Nakamura TJ, Mitsushima D, Kimura F. Differential regulation of pituitary adenylate cyclase-activating peptide receptor variants in the rat suprachiasmatic nucleus. Neuroscience 2002;110:301-308.
6. Joumot L, Waeber C, Pantaloni C, et al. Differential signal transduction by six splice variants of the pituitary adenylate cyclase-activating peptide (PACAP) receptor. Biochemical Society Transactions 1995;23:133-137.
7. Pantaloni C, Brabet P, Bilanges B, et al. Alternative Splicing in the N-terminal Extracellular Domain of the Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) Receptor Modulates Receptor Selectivity and Relative Potencies of PACAP-27 and PACAP-38 in Phospholipase C Activation. Journal of Biological Chemistry 1996;271:22146-22151.
8. Chatterjee TK, Sharma RV, Fisher RA. Molecular Cloning of a Novel Variant of the Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) Receptor That Stimulates Calcium Influx by Activation of L-type Calcium Channels. Journal of Biological Chemistry 1996;271:32226-32232.
9. Lutz EM, Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC_1 receptor in human neuroblastoma cells: Consequences for signaling by VIP and PACAP. Molecular and Cellular Neuroscience 2006;31:193-209.
10. Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL. N-Terminal Splice Variants of the Type I PACAP Receptor: Isolation, Characterization and Ligand Binding/Selectivity Determinants. Journal of Neuroendocrinology 1999;11:941-949.
11. Pisegna JR, Wank SA. Cloning and Characterization of the Signal Transduction of Four Splice Variants of the Human Pituitary Adenylate Cyclase Activating Polypeptide Receptor: Evidence for dual coupling to adenylate cyclase and phospholipase C. Journal of Biological Chemistry 1996;271:17267-17274.
12. Blechman J, Levkowitz G. Alternative Splicing of the Pituitary Adenylate Cyclase-Activating Polypeptide Receptor PAC_1: Mechanisms of Fine Tuning of Brain Activity. Frontiers in Endocrinology 2013;4.
13. Dickson L, Finlayson K. VPAC and PAC receptors: From ligands to function. Pharmacology & Therapeutics 2009;121:294-316.
14. Spengler D, Waeber C, Pantaloni C, et al. Differential signal transduction by five splice variants of the PACAP receptor. Nature 1993;365:170.
15. Braas KM, May V. Pituitary Adenylate Cyclase-activating Polypeptides Directly Stimulate Sympathetic Neuron Neuropeptide Y Release through PAC_1 Receptor Isoform Activation of Specific Intracellular Signaling Pathways. Journal of Biological Chemistry 1999;274:27702-27710.
16. Erdling A, Sheykhzade M, Maddahi A, Bari F, Edvinsson L. VIP/PACAP receptors in cerebral arteries of rat: Characterization, localization and relation to intracellular calcium. Neuropeptides 2013;47:85-92.
17. Syed AU, Koide M, Braas KM, May V, Wellman GC. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Potently Dilates Middle Meningeal Arteries: Implications for Migraine. Journal of Molecular Neuroscience 2012;48:574-583.
18. Moro O, Lerner EA. Maxadilan, the Vasodilator from Sand Flies, Is a Specific Pituitary Adenylate Cyclase Activating Peptide Type I Receptor Agonist. Journal of Biological Chemistry 1997;272:966-970.
19. Tatsuno I, Uchida D, Tanaka T, et al. Maxadilan specifically interacts with PAC1 receptor, which is a dominant form of PACAP/VIP family receptors in cultured rat cortical neurons. Brain Research 2001;889:138-148.
20. Uchida D, Tatsuno I, Tanaka T, et al. Maxadilan Is a Specific Agonist and Its Deleted Peptide (M65) Is a Specific Antagonist for PACAP Type 1 Receptor. Annals of the New York Academy of Sciences 1998;865:253-258.
21. Sághy É, Payrits M, Helyes Z, et al. Stimulatory effect of pituitary adenylate cyclase-activating polypeptide 6-38, M65 and vasoactive intestinal polypeptide 6-28 on trigeminal sensory neurons. Neuroscience 2015;308:144-156.

22. Reglodi D, Borzsei R, Bagoly T, et al. Agonistic Behavior of PACAP6-38 on Sensory Nerve Terminals and Cytotrophoblast Cells. Journal of Molecular Neuroscience 2008;36:270-278.
23. Vaudry D, Falluel-Morel A, Bourgault S, et al. Pituitary Adenylate Cyclase-Activating Polypeptide and Its Receptors: 20 Years after the Discovery. Pharmacological Reviews 2009;61:283-357.
24. Sherwood NM, Krueckl SL, McRory JE. The Origin and Function of the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)/Glucagon Superfamily*. Endocrine Reviews 2000;21:619-670.
25. Köves K, Arimura A, Görcs TG, Somogyvári-Vigh A. Comparative Distribution of Immunoreactive Pituitary Adenylate Cyclase Activating Polypeptide and Vasoactive Intestinal Polypeptide in Rat Forebrain. Neuroendocrinology 1991;54:159-169.
26. Kóves K, Arimura A, Somogyvári-Vigh A, Vigh S, Miller JIM. Immunohistochemical Demonstration of a Novel Hypothalamic Peptide, Pituitary Adenylate Cyclase-Activating Polypeptide, in the Ovine Hypothalamus*. Endocrinology 1990;127:264-271.
27. Hannibal J. Pituitary adenylate cyclase-activating peptide in the rat central nervous system: An immunohistochemical and in situ hybridization study. Journal of Comparative Neurology 2002;453:389-417.
28. Kivipelto L, Absood A, Arimura A, Sundler F, Håkanson R, Panula P. The distribution of pituitary adenylate cyclase-activating polypeptide-like immunoreactivity is distinct from helodermin- and helospectin-like immunoreactivities in the rat brain. Journal of Chemical Neuroanatomy 1992;5:85-94.
29. Joo KM, Chung YH, Kim MK, et al. Distribution of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptors ($VPAC_1$, $VPAC_2$, and PAC_1 receptor) in the rat brain. Journal of Comparative Neurology 2004;476:388-413.
30. Mikkelsen JD, Hannibal J, Larsen PJ, Fahrenkrug J. Pituitary adenylate cyclase activating peptide (PACAP) mRNA in the rat neocortex. Neuroscience Letters 1994;171:121-124.
31. Suda K, Smith DM, Ghatei MA, Murphy JK, Bloom SR. Investigation and Characterization of Receptors for Pituitary Adenylate Cyclase-Activating Polypeptide in Human Brain by Radioligand Binding and Chemical Cross-Linking. The Journal of Clinical Endocrinology & Metabolism 1991;72:958-964.
32. Kageyama K, Hanada K, Iwasaki Y, et al. Pituitary adenylate cyclase-activating polypeptide stimulates corticotropin-releasing factor, vasopressin and interleukin-6 gene transcription in hypothalamic 4B cells. Journal of Endocrinology 2007;195:199-211.
33. Nomura M, Ueta Y, Larsen PJ, et al. Water Deprivation Increases the Expression of Pituitary Adenylate Cyclase-Activating Polypeptide Gene in the Rat Subfornical Organ*. Endocrinology 1997;138:4096-4100.
34. Mizuno Y, Kondo K, Terashima Y, Arima H, Murase T, Oiso Y. Anorectic Effect of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) in Rats: Lack of Evidence for Involvement of Hypothalamic Neuropeptide Gene Expression. Journal of Neuroendocrinology 1998;10:611-616.
35. Mounien L, Bizet P, Boutelet I, et al. Pituitary adenylate cyclase-activating polypeptide directly modulates the activity of proopiomelanocortin neurons in the rat arcuate nucleus. Neuroscience 2006;143:155-163.
36. Mounien L, Do Rego J-C, Bizet P, et al. Pituitary Adenylate Cyclase-Activating Polypeptide Inhibits Food Intake in Mice Through Activation of the Hypothalamic Melanocortin System. Neuropsychopharmacology 2008;34:424.
37. Kawaguchi C, Tanaka K, Isojima Y, et al. Changes in light-induced phase shift of circadian rhythm in mice lacking PACAP. Biochemical and Biophysical Research Communications 2003;310:169-175.
38. Hannibal J, Fahrenkrug J. Target areas innervated by PACAP-immunoreactive retinal ganglion cells. Cell and Tissue Research 2004;316:99-113.
39. Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, Fahrenkrug J. Dissociation between Light-Induced Phase Shift of the Circadian Rhythm and Clock Gene Expression in Mice Lacking the Pituitary Adenylate Cyclase Activating Polypeptide Type 1 Receptor. The Journal of Neuroscience 2001;21:4883-4890.
40. Apostolakis EM, Riherd DN, O'Malley BW. PAC_1 Receptors Mediate Pituitary Adenylate Cyclase-Activating Polypeptide- and Progesterone-Facilitated Receptivity in Female Rats. Molecular Endocrinology 2005;19:2798-2811.
41. Shintani N, Mori W, Hashimoto H, et al. Defects in reproductive functions in PACAP-deficient female mice. Regulatory Peptides 2002;109:45-48.
42. Amir-Zilberstein L, Blechman J, Sztainberg Y, et al. Homeodomain Protein Otp and Activity-Dependent Splicing Modulate Neuronal Adaptation to Stress. Neuron 2012;73:279-291.
43. Otto C, Kovalchuk Y, Wolfer DP, et al. Impairment of Mossy Fiber Long-Term Potentiation and Associative Learning in Pituitary Adenylate Cyclase Activating Polypeptide Type I Receptor-Deficient Mice. The Journal of Neuroscience 2001;21:5520-5527.

44. Ohnou T, Yokai M, Kurihara T, et al. Pituitary adenylate cyclase-activating polypeptide type 1 receptor signaling evokes long-lasting nociceptive behaviors through the activation of spinal astrocytes in mice. Journal of Pharmacological Sciences 2016;130:194-203.
45. Hashimoto H, Shintani N, Tanaka K, et al. Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). Proceedings of the National Academy of Sciences 2001;98:13355-13360.
46. Tajti J, Uddman R, Edvinsson L. Neuropeptide Localization in the 'Migraine Generator' Region of the Human Brainstem. Cephalalgia 2001;21:96-101.
47. Knutsson M, Edvinsson L. Distribution of mRNA for VIP and PACAP receptors in human cerebral arteries and cranial ganglia. NeuroReport 2002;13:507-509.
48. Robert C, Bourgeais L, Arreto CD, et al. Paraventricular hypothalamic regulation of trigeminovascular mechanisms involved in headaches. Journal of Neuroscience 2013;33:8827-8840.
49. Eftekhari S, Salvatore CA, Johansson S, Chen TB, Zeng Z, Edvinsson L. Localization of CGRP, CGRP receptor, PACAP and glutamate in trigeminal ganglion. Relation to the blood-brain barrier. Brain Research 2015;1600:93-109.
50. Koh P-O, Won C-K, Noh H-S, Cho G-J, Choi W-S. Expression of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in human placenta. Journal of veterinary science 2005;6:1-5.
51. Scaldaferri ML, Modesti A, Palumbo C, et al. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and PACAP-Receptor Type 1 Expression in Rat and Human Placenta*. Endocrinology 2000;141:1158-1167.
52. Vaccari S, Latini S, Barberi M, Teti A, Stefanini M, Canipari R. Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. Journal of Endocrinology 2006;191:287-299.
53. Koh PO, Kwak SD, Kim HJ, et al. Expression patterns of pituitary adenylate cyclase activating polypeptide and its type I receptor mRNAs in the rat placenta. Molecular Reproduction and Development 2003;64:27-31.
54. Guidone G, Müller D, Vogt K, Mukhopadhyay AK. Characterization of VIP and PACAP receptors in cultured rat penis corpus cavernosum smooth muscle cells and their interaction with guanylate cyclase-B receptors. Regulatory Peptides 2002;108:63-72.
55. Li M, Funahashi H, Mbikay M, Shioda S, Arimura A. Pituitary adenylate cyclase activating polypeptide-mediated intracrine signaling in the testicular germ cells. Endocrine 2004;23:59-75.
56. Mazzocchi G, Malendowicz LK, Rebuffat P, Gottardo L, Nussdorfer GG. Expression and Function of Vasoactive Intestinal Peptide, Pituitary Adenylate Cyclase-Activating Polypeptide, and Their Receptors in the Human Adrenal Gland. The Journal of Clinical Endocrinology & Metabolism 2002;87:2575-2580.
57. Portela-Gomes GM, Lukinius A, Ljungberg O, Efendic S, Ahrén B, Abdel-Halim SM. PACAP is expressed in secretory granules of insulin and glucagon cells in human and rodent pancreas: Evidence for generation of cAMP compartments uncoupled from hormone release in diabetic islets. Regulatory Peptides 2003;113:31-39.
58. Girard BM, Tooke K, Vizzard MA. PACAP/Receptor System in Urinary Bladder Dysfunction and Pelvic Pain Following Urinary Bladder Inflammation or Stress. Frontiers in Systems Neuroscience 2017;11.
59. Luts A, Uddman R, Alm R, Basterra J, Sundler F. Peptide-Containing Nerve Fibers in Human Airways: Distribution and Coexistence Pattern. International Archives of Allergy and Immunology 1993;101:52-60.
60. Sundler F, Ekblad E, Absood A, Håkanson R, Köves K, Arimura A. Pituitary adenylate cyclase activating peptide: A novel vasoactive intestinal peptide-like neuropeptide in the gut. Neuroscience 1992;46:439-454.
61. Abad C, Martinez C, Leceta J, Juarranz MG, Delgado M, Gomariz RP. Pituitary Adenylate-Cyclase-Activating Polypeptide Expression in the Immune System. Neuroimmunomodulation 2002;10:177-186.
62. Tokuda N, Arudchelvan Y, Sawada T, et al. PACAP Receptor (PAC1-R) Expression in Rat and Rhesus Monkey Thymus. Annals of the New York Academy of Sciences 2006;1070:581-585.
63. Ny L, Larsson B, Alm P, et al. Distribution and effects of pituitary adenylate cyclase activating peptide in cat and human lower oesophageal sphincter. British Journal of Pharmacology 1995;116:2873-2880.
64. Cardell Lars O, Hjert O, Uddman R. The induction of nitric oxide-mediated relaxation of human isolated pulmonary arteries by PACAP. British Journal of Pharmacology 1997;120:1096-1100.
65. Martin F, Baeres M, Møller M. Origin of PACAP-immunoreactive Nerve Fibers Innervating the Subarachnoidal Blood Vessels of the Rat Brain. Journal of Cerebral Blood Flow & Metabolism 2004;24:628-635.
66. Filipsson K, Tornøe K, Holst J, Ahrén B. Pituitary Adenylate Cyclase-Activating Polypeptide Stimulates Insulin and Glucagon Secretion in Humans*. The Journal of Clinical Endocrinology & Metabolism 1997;82:3093-3098.

67. Borboni P, Porzio O, Pierucci D, et al. Molecular and Functional Characterization of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP-38)/Vasoactive Intestinal Polypeptide Receptors in Pancreatic β-Cells and Effects of PACAP-38 on Components of the Insulin Secretory System1. Endocrinology 1999;140:5530-5537.
68. Gottschall PE, Tatsuno I, Miyata A, Arimura A. Characterization and Distribution of Binding Sites for the Hypothalamic Peptide, Pituitary Adenylate Cyclase-Activating Polypeptide*. Endocrinology 1990;127:272-277.
69. Busto R, Prieto JC, Bodega G, Zapatero J, Carrero I. Immunohistochemical localization and distribution of VIP/PACAP receptors in human lung. Peptides 2000;21:265-269.
70. Zeng N, Kang TAO, Lyu RM, et al. The Pituitary Adenylate Cyclase Activating Polypeptide Type 1 Receptor (PAC1-R) Is Expressed on Gastric ECL Cells: Evidence by Immunocytochemistry and RT-PCR. Annals of the New York Academy of Sciences 1998;865:147-156.
71. Fukushima Y, Hikichi H, Mizukami K, et al. Role of endogenous PACAP in catecholamine secretion from the rat adrenal gland. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2001;281:R1562-R1567.
72. Diané A, Nikolic N, Rudecki AP, King SM, Bowie DJ, Gray SL. PACAP is essential for the adaptive thermogenic response of brown adipose tissue to cold exposure. Journal of Endocrinology 2014;222:327-339.
73. Gray SL, Cummings KJ, Jirik FR, Sherwood NM. Targeted Disruption of the Pituitary Adenylate Cyclase-Activating Polypeptide Gene Results in Early Postnatal Death Associated with Dysfunction of Lipid and Carbohydrate Metabolism. Molecular Endocrinology 2001;15:1739-1747.
74. Hamelink C, Tjurmina O, Damadzic R, et al. Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. Proceedings of the National Academy of Sciences 2002;99:461-466.
75. Jamen F, Persson K, Bertrand G, et al. PAC_1 receptor–deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. Journal of Clinical Investigation 2000;105:1307-1315.
76. Lutz-Bucher B, Monnier D, Koch B. Evidence for the Presence of Receptors for Pituitary Adenylate Cyclase-Activating Polypeptide in the Neurohypophysis that are Positively Coupled to Cyclic AMP Formation and Neurohypophyseal Hormone Secretion. Neuroendocrinology 1996;64:153-161.
77. Hautmann M, Friis UG, Desch M, et al. Pituitary Adenylate Cyclase–Activating Polypeptide Stimulates Renin Secretion via Activation of PAC1 Receptors. Journal of the American Society of Nephrology 2007;18:1150-1156.
78. Nemetz N, Abad C, Lawson G, et al. Induction of colitis and rapid development of colorectal tumors in mice deficient in the neuropeptide PACAP. International Journal of Cancer 2008;122:1803-1809.
79. Babai N, Atlasz T, Tamás A, et al. Degree of damage compensation by various pacap treatments in monosodium glutamate-induced retinal degeneration. Neurotoxicity Research 2005;8:227-233.
80. Chen Y, Samal B, Hamelink CR, et al. Neuroprotection by endogenous and exogenous PACAP following stroke. Regulatory Peptides 2006;137:4-19.
81. Dejda A, Seaborn T, Bourgault S, et al. PACAP and a novel stable analog protect rat brain from ischemia: Insight into the mechanisms of action. Peptides 2011;32:1207-1216.
82. László E, Kiss P, Horváth G, Szakály P, Tamás A, Reglödi D. The effects of pituitary adenylate cyclase activating polypeptide in renal ischemia/reperfusion. Acta Biologica Hungarica 2014;65:369-378.
83. Lazarovici P, Cohen G, Arien-Zakay H, et al. Multimodal Neuroprotection Induced by PACAP38 in Oxygen–Glucose Deprivation and Middle Cerebral Artery Occlusion Stroke Models. Journal of Molecular Neuroscience 2012;48:526-540.
84. Muzzi M, Buonvicino D, De Cesaris F, Chiarugi A. Acute and chronic triptan exposure neither alters rodent cerebral blood flow nor worsens ischemic brain injury. Neuroscience 2017;340:1-7.
85. Ohtaki H, Nakamachi T, Dohi K, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. Proceedings of the National Academy of Sciences 2006;103:7488-7493.
86. Reglodi D, Somogyvari-Vigh A, Vigh S, Kozicz T, Arimura A. Delayed Systemic Administration of PACAP38 Is Neuroprotective in Transient Middle Cerebral Artery Occlusion in the Rat. Stroke 2000;31:1411-1417.
87. Reglodi D, Tamás A, Somogyvári-Vigh A, et al. Effects of pretreatment with PACAP on the infarct size and functional outcome in rat permanent focal cerebral ischemia. Peptides 2002;23:2227-2234.
88. Vaczy A, Reglodi D, Somoskeoy T, et al. The Protective Role of PAC1-Receptor Agonist Maxadilan in BCCAO-Induced Retinal Degeneration. Journal of Molecular Neuroscience 2016;60:186-194.

89. Reglodi D, Vaczy A, Rubio-Beltran E, MaassenVanDenBrink A. Protective effects of PACAP in ischemia. The Journal of Headache and Pain 2018;19:19.
90. Botz B, Bölcskei K, Kereskai L, et al. Differential Regulatory Role of Pituitary Adenylate Cyclase–Activating Polypeptide in the Serum-Transfer Arthritis Model. Arthritis & Rheumatology 2014;66:2739-2750.
91. Warren JB, Larkin SW, Coughlan M, Kajekar R, Williams TJ. Pituitary adenylate cyclase activating polypeptide is a potent vasodilator and oedema potentiator in rabbit skin in vivo. British Journal of Pharmacology 1992;106:331-334.
92. Svensjö E, Saraiva EM, Amendola RS, et al. Maxadilan, the Lutzomyia longipalpis vasodilator, drives plasma leakage via PAC1–CXCR1/2-pathway. Microvascular Research 2012;83:185-193.
93. Watanabe J, Nakamachi T, Matsuno R, et al. Localization, characterization and function of pituitary adenylate cyclase-activating polypeptide during brain development. Peptides 2007;28:1713-1719.
94. Ghzili H, Grumolato L, Thouënnon E, et al. Role of PACAP in the physiology and pathology of the sympathoadrenal system. Frontiers in Neuroendocrinology 2008;29:128-141.
95. Lezak KR, Roelke E, Harris OM, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) in the bed nucleus of the stria terminalis (BNST) increases corticosterone in male and female rats. Psychoneuroendocrinology 2014;45:11-20.
96. King SB, Toufexis DJ, Hammack SE. Pituitary adenylate cyclase activating polypeptide (PACAP), stress, and sex hormones. Stress 2017;20:465-475.
97. Ressler KJ, Mercer KB, Bradley B, et al. Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 2011;470:492.
98. Tajti J, Tuka B, Botz B, Helyes Z, Vecsei L. Role of Pituitary Adenylate Cyclase-Activating Polypeptide in Nociception and Migraine. CNS & Neurological Disorders - Drug Targets 2015;14:540-553.
99. Sándor K, Bölcskei K, McDougall JJ, et al. Divergent peripheral effects of pituitary adenylate cyclase-activating polypeptide-38 on nociception in rats and mice. Pain 2009;141:143-150.
100. Amin FM, Schytz HW. Transport of the pituitary adenylate cyclase-activating polypeptide across the blood-brain barrier: implications for migraine. The Journal of Headache and Pain 2018;19:35.
101. Edvinsson L, Tajti J, Szalárdy L, Vécsei L. PACAP and its role in primary headaches. The Journal of Headache and Pain 2018;19:21.
102. Vollesen ALH, Ashina M. PACAP38: Emerging Drug Target in Migraine and Cluster Headache. Headache: The Journal of Head and Face Pain 2017;57:56-63.
103. Bacchelli E, Cainazzo MM, Cameli C, et al. A genome-wide analysis in cluster headache points to neprilysin and PACAP receptor gene variants. The Journal of Headache and Pain 2016;17:114.
104. Guo S, Petersen AS, Schytz HW, et al. Cranial parasympathetic activation induces autonomic symptoms but no cluster headache attacks. Cephalalgia 2017:0333102417738250.
105. Tuka B, Helyes Z, Markovics A, et al. Alterations in PACAP-38-like immunoreactivity in the plasma during ictal and interictal periods of migraine patients. Cephalalgia 2013;33:1085-1095.
106. Tuka B, Szabó N, Tóth E, et al. Release of PACAP-38 in episodic cluster headache patients – an exploratory study. The Journal of Headache and Pain 2016;17:69.
107. Hou L, Wan D, Dong Z, et al. Pituitary adenylate cyclase-activating polypeptide expression in peripheral blood mononuclear cells of migraineurs. Cell & Bioscience 2016;6:40.
108. Zagami AS, Edvinsson L, Goadsby PJ. Pituitary adenylate cyclase activating polypeptide and migraine. Annals of Clinical and Translational Neurology 2014;1:1036-1040.
109. Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain 2009;132:16-25.
110. Ashina M, Hansen JM, á Dunga BO, Olesen J. Human models of migraine — short-term pain for long-term gain. Nature Reviews Neurology 2017;13:713.
111. Bergerot A, Holland PR, Akerman S, et al. Animal models of migraine: looking at the component parts of a complex disorder. European Journal of Neuroscience 2006;24:1517-1534.
112. Birk S, Sitarz JT, Petersen KA, et al. The effect of intravenous PACAP38 on cerebral hemodynamics in healthy volunteers. Regulatory Peptides 2007;140:185-191.
113. Amin FM, Asghar MS, Guo S, et al. Headache and prolonged dilatation of the middle meningeal artery by PACAP38 in healthy volunteers. Cephalalgia 2012;32:140-149.
114. Amin FM, Hougaard A, Schytz HW, et al. Investigation of the pathophysiological mechanisms of migraine attacks induced by pituitary adenylate cyclase-activating polypeptide-38. Brain 2014;137:779-794.
115. Chan KY, Baun M, de Vries R, et al. Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery. Cephalalgia 2011;31:181-189.

116. Amin FM, Hougaard A, Magon S, et al. Change in brain network connectivity during PACAP38-induced migraine attacks. A resting-state functional MRI study. Neurology 2016;86:180-187.
117. Guo S, Vollesen ALH, Olesen J, Ashina M. Premonitory and nonheadache symptoms induced by CGRP and PACAP38 in patients with migraine. Pain 2016;157:2773-2781.
118. Guo S, Vollesen ALH, Hansen YBL, et al. Part II: Biochemical changes after pituitary adenylate cyclase-activating polypeptide-38 infusion in migraine patients. Cephalalgia 2017;37:136-147.
119. Hassan M, Refai E, Andersson M, Schnell P-O, Jacobsson H. In vivo dynamical distribution of ^{131}I-VIP in the rat studied by gamma-camera. Nuclear Medicine and Biology 1994;21:865-872.
120. Boni LJ, Ploug KB, Olesen I, Jansen-Olesen I, Gupta S. The in vivo Effect of VIP, PACAP-38 and PACAP-27 and mRNA Expression of Their Receptors in Rat Middle Meningeal Artery. Cephalalgia 2009;29:837-847.
121. Akerman S, Goadsby PJ. Neuronal PAC_1 receptors mediate delayed activation and sensitization of trigeminocervical neurons: Relevance to migraine. Science Translational Medicine 2015;7:308ra157-308ra157.
122. Han X, Ran Y, Su M, et al. Chronic changes in pituitary adenylate cyclase-activating polypeptide and related receptors in response to repeated chemical dural stimulation in rats. Molecular Pain 2017;13:1744806917720361.
123. Hoffmann J, Martins-Oliveira M, Akerman S, Supronsinchai W, Xu C, Goadsby PJ. PAC-1 receptor antibody modulates nociceptive trigeminal activity in rat. Cephalalgia 2016;36:141-141.
124. Baun M, Pedersen MHF, Olesen J, Jansen-Olesen I. Dural mast cell degranulation is a putative mechanism for headache induced by PACAP-38. Cephalalgia 2012;32:337-345.
125. Bhatt DK, Gupta S, Olesen J, Jansen-Olesen I. PACAP-38 infusion causes sustained vasodilation of the middle meningeal artery in the rat: Possible involvement of mast cells. Cephalalgia 2014;34:877-886.
126. Ottosson A, Edvinsson L. Release of Histamine from Dural Mast Cells by Substance P and Calcitonin Gene-Related Peptide. Cephalalgia 1997;17:166-174.
127. Study to Evaluate the Efficacy and Safety of AMG 301 in Migraine Prevention [online]. Available at: https://clinicaltrials.gov/ct2/show/NCT03238781. Accessed 02-05-2018.
128. ALD1910 – migraine prevention [online]. Available at: http://www.alderbio.com/ald1910-migraine-prevention/. Accessed 19-15-2018.
129. Martínez C, Juarranz Y, Abad C, et al. Analysis of the role of the PAC_1 receptor in neutrophil recruitment, acute-phase response, and nitric oxide production in septic shock. Journal of Leukocyte Biology 2005;77:729-738.
130. Chang CL, Donaghy M, Poulter N. Migraine and stroke in young women: case-control study. BMJ : British Medical Journal 1999;318:13-18.
131. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.
132. Schurks M, Rist PM, Bigal ME, Buring JE, Lipton RB, Kurth T. Migraine and cardiovascular disease: systematic review and meta-analysis. BMJ: British Medical Journal 2009;339:b3914.
133. Spector JT, Kahn SR, Jones MR, Jayakumar M, Dalal D, Nazarian S. Migraine headache and ischemic stroke risk: an updated meta-analysis. The American journal of medicine 2010;123:612-624.
134. Deen M, Correnti E, Kamm K, et al. Blocking CGRP in migraine patients - a review of pros and cons. J Headache Pain 2017;18:96.
135. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
136. Mitsikostas DD, Reuter U. Calcitonin gene-related peptide monoclonal antibodies for migraine prevention: comparisons across randomized controlled studies. Current Opinion in Neurology 2017;30:272-280.

Chapter XI.

Pharmacological analysis of the inhibition produced by moxonidine and agmatine on the vasodepressor sensory CGRPergic outflow in pithed rats

Based on: ***E Rubio-Beltrán****, A Labastida-Ramírez, O Hernández-Abreu, A MaassenVanDenBrink, CM Villalón (2017) European Journal of Pharmacology; 812:97-103.*

Abstract

Calcitonin gene-related peptide (CGRP) plays a role in several (patho)physiological functions, and modulation of its release is considered a therapeutic target. In this respect, electrical spinal (T_9-T_{12}) stimulation of the perivascular sensory outflow in pithed rats produces vasodepressor responses mediated by CGRP release. This study investigated the role of imidazoline I_1 and I_2 receptors in the inhibition by moxonidine and agmatine of these vasodepressor responses. Male Wistar pithed rats (pretreated i.v. with 25 mg/kg gallamine and 2 mg/kg·min hexamethonium) received i.v. continuous infusions of methoxamine (20 µg/kg·min) followed by physiological saline (0.02 ml/min), moxonidine (1, 3, 10 or 30 µg/kg·min) or agmatine (1000 or 3000 µg/kg·min). Under these conditions, electrical stimulation (0.56-5.6 Hz; 50 V; 2 ms) of the spinal cord (T_9-T_{12}) produced frequency-dependent vasodepressor responses which were: (i) unchanged during saline infusion; and (ii) inhibited during the above infusions of moxonidine or agmatine. Moreover, using i.v. administrations, the inhibition by 3 µg/kg·min moxonidine or 3000 µg/kg·min agmatine (which failed to inhibit the vasodepressor responses by αCGRP; 0.1-1 µg/kg) was: (i) unaltered after saline (1 ml/kg), rauwolscine (300 µg/kg; α_2-adrenoceptor antagonist) or BU224 (300 µg/kg; imidazoline I_2 receptor antagonist); and (ii) reversed after AGN 192403 (3000 µg/kg; imidazoline I_1 receptor antagonist). This reversion was relatively more pronounced after AGN 192403 plus rauwolscine. These blocking doses of antagonists lacked any effects on the electrically-induced vasodepressor responses. Therefore, the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine is mainly mediated by prejunctional imidazoline I_1 receptors on perivascular sensory nerves.

Introduction

Blood pressure is determined by the interaction of neural, humoral and local mechanisms. The neural modulation of the vascular tone is mainly mediated by activation of sympathetic (noradrenergic) and sensory (non-adrenergic non-cholinergic) fibres[1]. In turn, perivascular sensory fibres may release (depending on the vascular bed) different neuromediators, including adenosine triphosphate, vasoactive intestinal peptide, neuropeptide Y, substance P, nitric oxide and calcitonin gene-related peptide (CGRP)[2-5]. Of these neuromediators, CGRP has been shown to play an important role in the modulation of vascular tone, as well as in the pathogenesis of several diseases/disorders, such as migraine, diabetes, arthritis and obesity[6].

Due to the (patho)physiological importance of CGRP, it is crucial to elucidate the mechanisms associated with its release. For example, in the pithed rat model, electrical spinal (T_9-T_{12}) stimulation of the perivascular sensory outflow results in vasodepressor responses mainly mediated by CGRP release, as these responses are blocked by the CGRP receptor antagonists $CGRP_{8-37}$[7] or olcegepant[8]. This vasodepressor sensory CGRPergic outflow is modulated by activation of prejunctional $\alpha_{2A/2C}$-adrenoceptors[9], serotonin $5\text{-}HT_{1B/1D/1F/7}$[10,11], dopamine D_2-like receptors[12] and histamine H_3 receptors[13]. Additional evidence suggests that imidazoline receptors can also modulate neurotransmitter release[14,15]. In the pithed rat model, Cobos-Puc *et al.*[16] have demonstrated that activation of prejunctional imidazoline I_1 receptors inhibits the sympathetic vasopressor outflow. Furthermore, Villalón *et al.*[9] have shown that clonidine, an α_2-adrenoceptor and imidazoline I_1 receptor agonist, inhibits the vasodepressor sensory CGRPergic outflow by activation of $\alpha_{2A/2C}$-adrenoceptors; however, this study could not exclude the possible role of imidazoline receptors.

On this basis, the present study was designed to investigate if moxonidine (imidazoline I_1/α_2-adrenoceptor agonist) and agmatine (endogenous ligand of imidazoline receptors) inhibit the vasodepressor sensory CGRPergic outflow, and the receptors involved in this inhibition by using selective antagonists (see Table 1).

Table 1. Affinity constants (pK_i) of the different drugs considered in this study. Receptor binding affinities (pK_i) of the drugs considered in the present study at α_2 adrenoceptors, imidazoline I_1 and I_2 receptors. Data taken from the following references: [1][Ernsberger and Haxhiu, 1997]; [2][Atlas, 1994] ; [3][Li et al., 1994] ; [4][Hieble and Ruffolo] ; [5][Munk et al., 1996]; [6] [Flamez et al., 1997] . N.D. stands for "not determined".

Agonists	α_2	I_1	I_2
Moxonidine	7.1[1]	8.6[1]	5.0[1]
Agmatine	5.4[2]	6.15[3]	6.0[3]
Antagonists			
Rauwolscine	8.0[4]	4.0[1]	N.D.
AGN 192403	4.6[5]	7.4[5]	N.D.
BU224	4.8[6]	5.7[6]	8.6[6]

Materials and methods

Ethical approval of the study protocol

The experimental protocol of this study was approved by our Institutional Ethics Committee (CICUAL Cinvestav; permission protocol number 507-12) and followed the regulations established by the Mexican Official Norm (NOM-062-ZOO-1999) in accordance with the guide for the Care and Use of Laboratory Animals in the U.S.A.[23], the ARRIVE guidelines for reporting experiments in animals[24] and the Legislation for the protection of animals used for scientific purposes Directive 2010/63/EU[25].

General methods

Experiments were carried out in 135 male Wistar normotensive rats (300-350 g). The animals were maintained at a 12/12h light-dark cycle in a special room at a constant temperature (22±2°C) and humidity (50%), with food and water freely available in their home cages. After anaesthesia with sodium pentobarbital (60 mg/kg, i.p.) and cannulation of the trachea, the rats were pithed as reported earlier [9]. This procedure consists in inserting a stainless steel rod through the orbit and *foramen magnum* and into the vertebral *foramen* to avoid the influence of the central nervous system. Immediately afterwards, the animals were artificially ventilated with room air using an Ugo Basile pump model 7025 (56 strokes/min; stroke volume: 20 ml/kg). After bilateral cervical vagotomy, catheters were placed in: (i) the left and right femoral veins for the continuous infusions of methoxamine and the agonists (moxonidine, agmatine or vehicle), respectively; (ii) the left jugular vein for the continuous infusion of hexamethonium; and (iii) the right jugular vein, for the bolus injections of gallamine, αCGRP or the antagonists. Then, the left carotid artery was connected to a Grass pressure transducer (P23 XL), for the recording of blood pressure. Both heart rate (measured with a 7P4F tachograph) and blood pressure were recorded simultaneously by a model 7D Grass polygraph (Grass Instrument Co., Quincy, MA, U.S.A.). At this point, the 135 rats were divided into two main sets to study the effects of i.v. infusions of either the vehicle (physiological saline), moxonidine or agmatine, on the vasodepressor responses induced by either: (i) electrical stimulation of the vasodepressor sensory CGRPergic outflow (set 1, n=115); or (ii) i.v. bolus of αCGRP (set 2, n=20). The dose-response curves (D–R curves) and vasodepressor stimulus-response curves (S–R curves) elicited by α-CGRP and electrical stimulation, respectively, were completed in about 50 min, with no changes in resting blood pressure. The electrical stimuli (0.56, 1, 1.8, 3.1 and 5.6 Hz), as well as the i.v. doses of αCGRP (0.1, 0.18, 0.31, 0.56 and 1 µg/kg), were given using a sequential schedule at 5–10 min intervals as previously reported [9,16]. The body temperature of each pithed rat was maintained at 37 °C by a lamp and monitored with a rectal thermometer.

Experimental protocols

After the animals had been in a stable hemodynamic condition for at least 20 min, baseline values of diastolic blood pressure (a more accurate indicator of peripheral vascular resistance) and heart rate were determined. Subsequently, the following experimental protocols were applied.

Electrical stimulation of the vasodepressor sensory CGRPergic outflow

In the first set or rats (n=115), the pithing rod was replaced by an electrode enamelled except for 1.5 cm length 9 cm from the tip, placing the uncovered segment at the thoracic segments T_9–T_{12} of the spinal cord to allow selective stimulation of the vasodepressor sensory CGRPergic outflow[7,9,16]. Before electrical stimulation, the animals received (i.v.): (i) gallamine (25 mg/kg) to avoid the electrically-induced muscular twitching; (ii) 10 min later, a continuous infusion of hexamethonium (2 mg/kg·min) to block the vasopressor responses resulting from stimulation of the preganglionic sympathetic outflow; and (iii) 10 min later, a continuous infusion of methoxamine (20 μg/kg·min) to increase diastolic blood pressure at a value of, at least, 135 mm Hg[9]. Then, this set of rats was divided into 4 groups (n=40, 25, 25 and 25).

The first group (n=40) was subdivided into four subgroups that received an i.v. continuous infusion of: (i) nothing (control; n=5); (ii) 0.02 ml/min physiological saline (n=5); (iii) moxonidine (1, 3, 10 or 30 μg/kg·min; n=5 each); or (iv) agmatine (1000 or 3000 μg/kg·min; n=5 each). Twenty min later, the perivascular sensory CGRPergic outflow was electrically stimulated during the above treatments to elicit vasodepressor responses by applying 10-s trains of monophasic rectangular pulses (2 ms, 50 V) at 0.56, 1, 1.8, 3.1 and 5.6 Hz. When diastolic blood pressure returned to baseline levels, the next frequency was applied. This procedure was performed until the S-R curve had been completed.

The second group (n=25) received an i.v. continuous infusion of 0.02 ml/min saline and, 10 min later, it was subdivided into 5 subgroups (n=5 each) that were treated with i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine (α_2-adrenoceptor antagonist); (iii) 3000 μg/kg AGN 192403 (imidazoline I_1 receptor antagonist); (iv) 300 μg/kg BU224 (imidazoline I_2 receptor antagonist); and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S-R curve was elicited as described above, to analyze the effect *per se* of each of these antagonists.

The third group (n=25) received an i.v. continuous infusion of 3 μg/kg·min moxonidine; 10 min later, it was subdivided into 5 subgroups (n=5 each) that received i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine; (iii) 3000 μg/kg AGN 192403; (iv) 300 μg/kg BU224; and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S–R curve was elicited.

The fourth group (n=25) received an i.v. continuous infusion of 3000 μg/kg·min agmatine. As previously, 10 min later, was subdivided into 5 subgroups (n=5 each) that were administered i.v. bolus injections of: (i) 1 ml/kg saline; (ii) 300 μg/kg rauwolscine; (iii) 3000 μg/kg AGN 192403; (iv) 300 μg/kg BU224; and (v) 300 μg/kg rauwolscine+3000 μg/kg AGN 192403. Ten min later, a S–R curve was elicited.

The interval between the different frequencies of stimulation depended on the duration of the resulting vasodepressor responses (5–10 min), as we waited until diastolic blood pressure had returned to baseline values. It is important to highlight that tachyphylaxis of the vasodepressor responses was observed when eliciting a second S–R curve; as a result, only one S–R curve was performed per animal, as previously reported[9,12].

Administration of exogenous αCGRP

The second set of rats (n=20) was pithed as previously described, but the pithing rod was not replaced with an electrode since electrical stimuli were not applied. Consequently, the pithing rod was left throughout the experiment and the administration of both gallamine and hexamethonium was omitted[9,12]. Subsequently, an i.v. continuous infusion of 20 μg/kg·min methoxamine was administered. Ten min later (with diastolic blood pressure maintained at values of, at least, 135 mmHg), this set was divided into four groups (n=5 each) that received i.v. continuous infusions of: (i) nothing (control group); (ii) 0.02 ml/min physiological saline; (iii) 3 μg/kg·min moxonidine; and (iv) 3000 μg/kg·min agmatine. Twenty min later, the vasodepressor responses by i.v. bolus injections of αCGRP (0.1, 0.18, 0.31, 0.56 and 1 μg/kg) were determined during the continuous infusion of the agonists.

Other procedures applied to the experimental protocols

Hexamethonium, methoxamine, moxonidine, agmatine and saline were infused at a rate of 0.02 ml/min by a WPI model sp100i pump (World Precision Instruments Inc., Sarasota, FL, U.S.A.). The continuous infusion of methoxamine (20 μg/kg·min) increased diastolic blood pressure in all cases. The interval between the different frequencies of stimulation or doses of α-CGRP depended on the duration of the resulting vasodepressor responses (5-10 min), as we waited until diastolic blood pressure had returned to baseline values.

Compounds

The compounds used in this investigation were: gallamine triethiodide, hexamethonium chloride, methoxamine hydrochloride, rat α-CGRP, moxonidine hydrochloride, agmatine sulphate, rauwolscine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); (+/-)-2-endo-Amino-3-exo-isopropylbicyclo[2.2.1]heptane hydrochloride (AGN 192403), 2-(4,5-Dihydroimidazol-2-yl) quinoline hydrochloride (BU224 hydrochloride; purchased from Tocris Bioscience, Ellisville, MO, U.S.A.). All compounds were dissolved in physiological saline. The doses of the above compounds refer to the free base of substances, except in the case of gallamine and hexamethonium, where they refer to the corresponding salts.

Data presentation and statistical evaluation

All data in the text and Fig.s are presented as mean±S.E.M. The changes in baseline values of diastolic blood pressure before and during the continuous i.v. infusion of saline, moxonidine or agmatine were compared with a paired Student's *t*-test. Furthermore, the changes in diastolic blood pressure produced by i.v. bolus of αCGRP or electrical stimulation during saline, moxonidine and agmatine-infused animals were determined and expressed as percentage of change from baseline at the steady state effect of methoxamine. The difference between the changes in diastolic blood pressure induced by electrical stimulation or exogenous α-CGRP within the different subgroups of animals was compared with a two-way analysis of variance. Such analysis was followed, if applicable, by the Student-Newman-Keul's *post hoc* test. Statistical significance was accepted at $P<0.05$.

Results

Systemic haemodynamic variables

The baseline values of diastolic blood pressure and heart rate in the 135 rats were 55±4 mm Hg and 237±8 beats/min, respectively. These values did not change ($P>0.05$) after gallamine or

hexamethonium in the rats receiving electrical stimulation (n=115; data not shown). Twenty min after starting the infusion of methoxamine, diastolic blood pressure was significantly increased in all cases (179±8 mm Hg; n=115). The values of diastolic blood pressure and heart rate in the different subgroups before, immediately after and 10 min after i.v. saline or antagonists during methoxamine infusion were not significantly different (data not shown).

Vasodepressor responses elicited by electrical stimulation of the sensory CGRPergic outflow or exogenous α-CGRP

During the continuous infusion of methoxamine, the responses to electrical stimulation or to i.v. bolus of α-CGRP were immediate and resulted in frequency- or dose- dependent decreases in diastolic blood pressure as previously reported [9,10]. In all cases, the vasodepressor responses were considered to be due to selective vasodepressor stimulation (Fig. 1), as only minor and inconsistent effects in heart rate were observed, as previously shown by Villalón *et al*.[9].

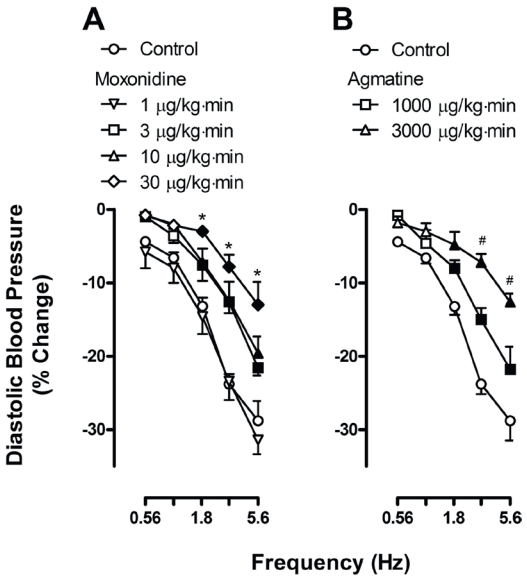

Fig. 1. Effect of continuous i.v. infusions of moxonidine (1, 3, 10 or 30 μg/kg·min) or agmatine (1000 or 3000 μg/kg·min) on the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow. Full symbols represent significant differences *vs* control (P<0.05); *P<0.05 comparing 30 μg/kg·min moxonidine *vs* 3 μg/kg·min moxonidine and *vs* 10μg/kg·min moxonidine; #P<0.05 comparing 3000 μg/kg·min agmatine *vs* 1000 μg/kg·min agmatine. Note that there was no significant difference between the inhibition produced by 3 and 10 μg/kg·min moxonidine.

Effect of i.v. continuous infusions of moxonidine or agmatine on the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow

Fig. 1A shows the inhibition of the vasodepressor responses induced by electrical stimulation of the sensory CGRPergic outflow during i.v. continuous infusions of moxonidine (1, 3, 10 and 30 μg/kg·min). This inhibition, significant as from 3 μg/kg·min moxonidine, was dose-dependent at 10 and 30μg/kg·min moxonidine. On the basis of these results, and considering its selectivity (see Table 1), the dose of 3 μg/kg·min moxonidine was chosen for further pharmacological analysis.

Moreover, the i.v. continuous infusions of agmatine (1000 and 3000 µg/kg·min) dose-dependently inhibited the electrically-induced vasodepressor responses (Fig. 1B). This inhibition was significant at 1.8, 3.1 and 5.6 Hz in all cases (P<0.05).

Effects of compounds per se on the electrically-induced vasodepressor responses

Fig. 2 illustrates that i.v. bolus injections of 1 ml/kg saline (vehicle; Fig. 2A), 300 µg/kg rauwolscine (Fig. 2B), 3000 µg/kg AGN 192403 (Fig. 2C), the combination of 300 µg/kg rauwolscine+3000 µg/kg AGN 192403 (Fig. 2D) or 300 µg/kg BU224 (Fig. 2E) produced no significant effects (P>0.05) on the electrically-induced vasodepressor responses.

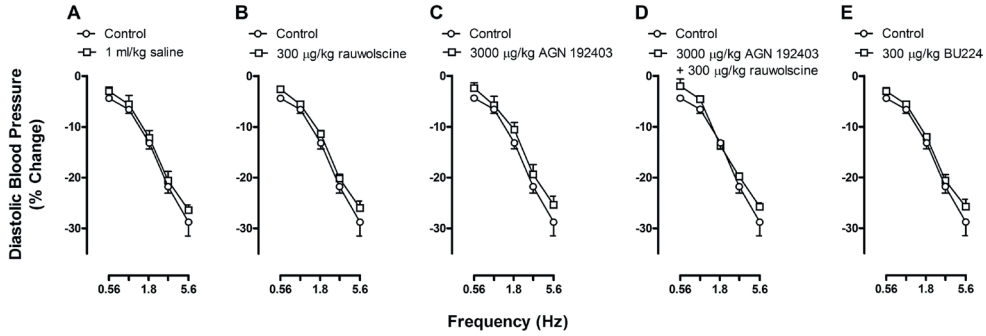

Fig. 2. Effect of i.v. bolus injections of 1 ml/kg saline, 300 µg/kg rauwolscine, 3000 µg/kg AGN192403, 3000 g/kg AGN 192403+300 µg/kg rauwolscine or 300 µg/kg BU224 on the electrically-induced vasodepressor responses. Empty symbols represent no significant difference *vs* control (P>0.05).

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Effect of vehicle or antagonists on the electrically-induced vasodepressor responses during the i.v. continuous infusions of moxonidine

The inhibition by the continuous infusion of 3 µg/kg·min moxonidine on the vasodepressor sensory CGRPergic outflow: (i) was not significantly modified (P>0.05) by the i.v. administration of 1 ml/kg saline (Fig. 3A), 300 µg/kg rauwolscine (Fig. 3B) or 300 µg/kg BU224 (Fig. 3E); and (ii) was significantly (P<0.05) attenuated by the i.v. administration of 3000 µg/kg AGN 192403 (Fig. 3C) or 3000 µg/kg AGN 192403+300 µg/kg rauwolscine (Fig. 3D).

Effect of vehicle or antagonists on the electrically-induced vasodepressor responses during the i.v. continuous infusions of agmatine

The inhibition produced by 3000 µg/kg·min agmatine: (i) was not significantly modified (P>0.05) after the i.v. bolus administration of 1 ml/kg saline (Fig. 3F), 300 µg/kg rauwolscine (Fig. 3G) or 300 µg/kg BU224 (Fig. 3J); and (ii) was reversed by the i.v. administration of 3000 µg/kg AGN 192403 or the combination of 3000 µg/kg AGN 192403+300 µg/kg rauwolscine (Fig. 3H and 3I; P<0.05).

Effect of i.v. continuous infusions of moxonidine or agmatine on the vasodepressor responses induced by i.v. bolus injections of αCGRP

The continuous infusion of: (i) 0.02 ml/min saline (Fig. 4A); (ii) 3 µg/kg·min moxonidine (Fig. 4B); or (iii) 3000 µg/kg·min agmatine (Fig. 4C) did not modify the vasodepressor responses induced by i.v. bolus of αCGRP (P>0.05).

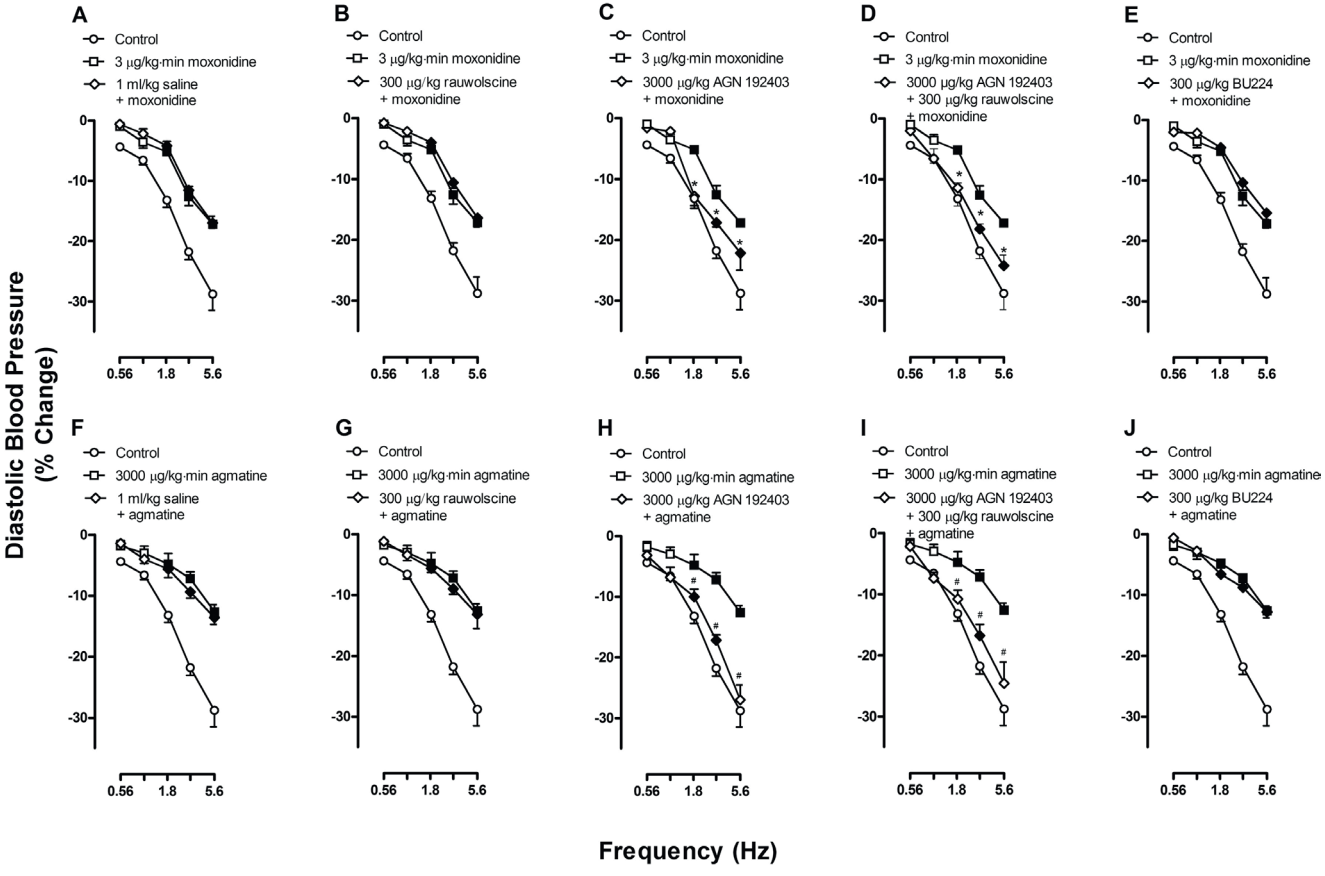


Fig. 3. Effect of i.v. injections of 1 ml/kg saline, 300 μg/kg rauwolscine, 3000 μg/kg AGN 192403, 3000 μg/kg AGN 192403+300 μg/kg rauwolscine or 300 μg/kg BU224 on the inhibition by 3 μg/kg·min moxonidine (upper panel) or 3000 μg/kg·min agmatine (lower panel). Full symbols represent significant differences *vs* control ($P<0.05$); *$P<0.05$ comparing 3 μg/kg·min moxonidine *vs* 3000 μg/kg AGN 192403+moxonidine and *vs* 3000 μg/kg AGN 192403+300 μg/kg rauwolscine+moxonidine; #$P<0.05$ comparing 3000 μg/kg·min agmatine *vs* 3000 μg/kg AGN 192403+agmatine and *vs* 3000 μg/kg AGN 192403+300 μg/kg rauwolscine+agmatine.

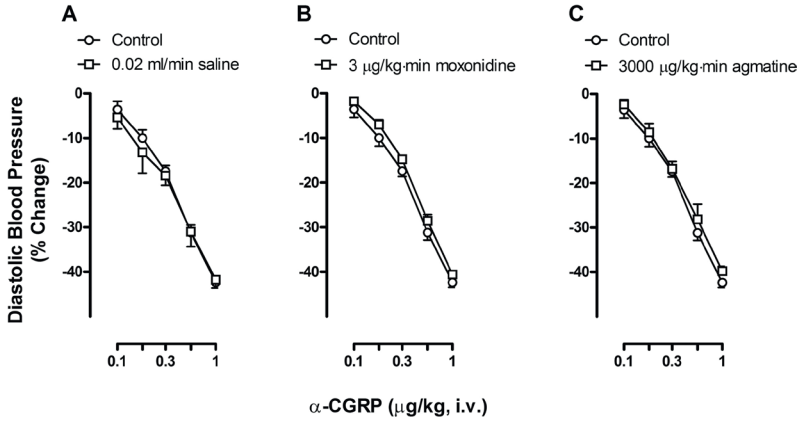


Fig. 4. Effect of i.v. continuous infusions of 0.02 ml/min saline (vehicle), 3 μg/kg·min moxonidine or 3000 μg/kg·min agmatine on the vasodepressor responses induced by i.v. bolus injections of αCGRP. Empty symbols represent no significant differences *vs* control (P>0.05).

Discussion

General

The pithed rat is an experimental model useful for determining the cardiovascular effects of different drugs at the peripheral nervous system (sympathetic and sensory). The nature of this model allows us to discard the influence of the central nervous system, as previously established[9,16]. It is worth mentioning that, although we did not measure sensory nerve activity directly, the electrically-induced neurotransmitter release (i.e. CGRP) was estimated indirectly by the measurement of the evoked vasodepressor responses[9]. Our study shows that the vasodepressor sensory CGRPergic outflow was inhibited by the continuous infusion of moxonidine and agmatine (Fig. 1). These inhibitory responses are mainly mediated by imidazoline I_1 receptors in view that they were: (i) significantly blocked by the imidazoline I_1 receptor antagonist AGN 192403 (Fig. 3); and (ii) resistant to blockade by the α_2-adrenoceptor antagonist rauwolscine or the imidazoline I_2 receptor antagonist BU224, which display very low affinity for imidazoline I_1 receptors (see Table 1). Moreover, the responses to moxonidine and agmatine are considered sensory-inhibitory, since they inhibited the vasodepressor responses to electrical sensory stimulation without affecting those to i.v. bolus injections of exogenous α-CGRP (Fig. 4).

Haemodynamic changes

Our group has previously shown that, in order to observe vasodepressor responses in pithed rats, diastolic blood pressure has to be initially raised and maintained above 135 mm Hg by a continuous i.v. infusion of the α_1-adrenoceptor agonist, methoxamine[9]. Moreover, α_2-adrenoceptors, as well as the imidazoline I_1 and I_2 receptors, do not seem to play a physiological role in the modulation of the vasodepressor sensory CGRPergic outflow as the electrically-induced vasodepressor responses were not significantly affected by i.v. administration of rauwolscine, AGN 192403 or BU224 at doses previously shown to block their respective receptors in the pithed rat model[9,16].

Role of imidazoline I_1 receptors in the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine

Imidazoline I_1 receptors have been shown to inhibit the cardioaccelerator sympathetic outflow[16], but no studies have thus far analyzed whether these receptors also modulate the vasodepressor

sensory CGRPergic outflow. Our experimental approach in pithed rats shows that moxonidine and agmatine inhibited the vasodepressor responses induced by electrical stimulation (Fig. 1) without affecting those by exogenous CGRP (Fig. 4). This finding implies a prejunctional inhibition of the vasodepressor sensory CGRPergic outflow, particularly at the higher stimulation frequencies (1.8, 3.1 and 5.6 Hz). Moreover, considering the binding selectivity of the compounds used (see Table 1), our results suggest a predominant role of the imidazoline I_1 receptors since the inhibitory responses to moxonidine and agmatine were markedly (but not completely) reversed by blocking doses of AGN 192403 (Fig. 3C and 3H).

Pharmacological evidence for the exclusion of the role of α_2-adrenoceptors and imidazoline I_2 receptors

Our findings additionally allow us to exclude the role of α_2-adrenoceptors and imidazoline I_2 receptors. In this respect, since the inhibitory responses to moxonidine and agmatine were resistant to blockade by rauwolscine (Figs. 3B and 3G), an α_2-adrenoceptor antagonist with very low affinity for imidazoline I_1 receptors (see Table 1), the role of α_2-adrenoceptors can be excluded. Nevertheless, in view that moxonidine displays affinity for α_2-adrenoceptors and imidazoline I_1 receptors (Table 1), it was reasonable to assume that the small (though significant) inhibition remaining after AGN 192403 could involve α_2-adrenoceptors. If this were the case, then the combination AGN 192403 plus rauwolscine should have abolished this remaining inhibition. However, the blockade of this response by the above combination (Figs. 3D and 3I) was practically identical to that produced by AGN 192403 alone (Fig. 3C and 3H). Accordingly, the inhibition remaining after AGN 192403 is most likely mediated by novel receptors (probably coupled to $G_{i/o}$) that warrant further experiments falling beyond the scope of the present study.

Finally, the fact that the inhibitory responses to moxonidine and agmatine were resistant to blockade by BU224 (Figs. 3E and 3J), an imidazoline I_2 receptor antagonist with very low affinity for α_2-adrenoceptors and imidazoline I_1 receptors (Table 1), rules out imidazoline I_2 receptors, as previously shown in other experimental models[26].

Potential clinical relevance

As a final point of reflexion, it is important to highlight the potential clinical relevance of the present findings. Indeed, the pharmacological modulation of perivascular CGRP release is of most interest due to the established antimigraine efficacy of the novel CGRP receptor antagonists (i.e. the gepants), with the downside of hepatotoxic side effects and pharmacokinetic limitations[6,27], as well as the potential for cardiovascular side effects[28]. For example, the antimigraine drug olcegepant (a potent CGRP receptor antagonist[29]), has recently been shown to block the vasodepressor sensory CGRPergic outflow and to potentiate the vasopressor sympathetic outflow[8], an effect that might result in an increased vascular resistance and, consequently, in a prohypertensive action. Moreover, the triptans (acute antimigraine 5-$HT_{1B/1D}$ receptor agonists) are capable of inhibiting the trigeminal release of CGRP[30] and also the vasodepressor sensory CGRPergic outflow[10]. On this basis, it is not unreasonable to postulate imidazoline I_1 receptors as a therapeutic target for the development of novel antimigraine drugs with no prohypertensive action. For this purpose, moxonidine (an established central antihypertensive agents[31]), could be explored in other models predictive of antimigraine action, such as the inhibition of neurogenic dural vasodilatation induced by periarterial electrical stimulation in rats, using intravital microscopy[32]. Furthermore, there are other pathologies such as arthritis, obesity and diabetes where the role of CGRP is less clear[6], but its modulation by imidazoline receptors could also be a possible therapeutic target.

Conclusion

Our results suggest that the inhibition of the vasodepressor sensory CGRPergic outflow by 3 µg/kg·min moxonidine and 3000 µg/kg·min agmatine in pithed rats is mainly mediated by a prejunctional activation of imidazoline I_1 receptors on perivascular sensory nerves.

References

1. Westfall TC, Westfall DP. Neurotransmission: The Autonomic and Somatic Motor Nervous Systems. In: Brunton LL, Chabner BA, Knollmann BC, eds. Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 12e. New York, NY: McGraw-Hill Education, 2011.
2. Cowley AW, Jr. Long-term control of arterial blood pressure. Physiol Rev 1992;72:231-300.
3. Rubino A, Burnstock G. Capsaicin-sensitive sensory-motor neurotransmission in the peripheral control of cardiovascular function. Cardiovasc Res 1996;31:467-479.
4. Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 1996;48:113-178.
5. Loscalzo J, Libby P, Epstein JA. Basic Biology of the Cardiovascular System. In: Kasper D, Fauci A, Hauser S, Longo D, Jameson JL, Loscalzo J, eds. Harrison's Principles of Internal Medicine, 19e. New York, NY: McGraw-Hill Education, 2015.
6. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev 2014;94:1099-1142.
7. Taguchi T, Kawasaki H, Imamura T, Takasaki K. Endogenous calcitonin gene-related peptide mediates nonadrenergic noncholinergic depressor response to spinal cord stimulation in the pithed rat. Circ Res 1992;71:357-364.
8. Avilés-Rosas VH, Rivera-Mancilla E, Marichal-Cancino BA, et al. Olcegepant blocks neurogenic and non-neurogenic CGRPergic vasodepressor responses and facilitates noradrenergic vasopressor responses in pithed rats. Br J Pharmacol 2017.
9. Villalón CM, Albarran-Juarez JA, Lozano-Cuenca J, Pertz HH, Gornemann T, Centurión D. Pharmacological profile of the clonidine-induced inhibition of vasodepressor sensory outflow in pithed rats: correlation with alpha($_{2A/2C}$)-adrenoceptors. Br J Pharmacol 2008;154:51-59.
10. González-Hernández A, Manrique-Maldonado G, Lozano-Cuenca J, et al. The 5-HT($_1$) receptors inhibiting the rat vasodepressor sensory CGRPergic outflow: further involvement of 5-HT($_{1F}$), but not 5-HT($_{1A}$) or 5-HT($_{1D}$), subtypes. Eur J Pharmacol 2011;659:233-243.
11. Cuesta C, Garcia-Pedraza JA, Garcia M, Villalon CM, Moran A. Role of 5-HT$_7$ receptors in the inhibition of the vasodepressor sensory CGRPergic outflow in pithed rats. Vascul Pharmacol 2014;63:4-12.
12. Manrique-Maldonado G, González-Hernández A, Altamirano-Espinoza AH, Marichal-Cancino BA, Ruiz-Salinas I, Villalón CM. The role of pre-junctional D_2 -like receptors mediating quinpirole-induced inhibition of the vasodepressor sensory CGRPergic out-flow in pithed rats. Basic Clin Pharmacol Toxicol 2014;114:174-180.
13. Manrique-Maldonado G, Altamirano-Espinoza AH, Marichal-Cancino BA, Rivera-Mancilla E, Avilés-Rosas V, Villalón CM. Pharmacological evidence that histamine H_3 receptors inhibit the vasodepressor responses by selective stimulation of the rat perivascular sensory CGRPergic outflow. Eur J Pharmacol 2015;754:25-31.
14. Ernsberger P. The I1-imidazoline receptor and its cellular signaling pathways. Ann N Y Acad Sci 1999;881:35-53.
15. Gentili F, Bousquet P, Brasili L, et al. Imidazoline binding sites (IBS) profile modulation: key role of the bridge in determining I_1-IBS or I_2-IBS selectivity within a series of 2-phenoxymethylimidazoline analogues. J Med Chem 2003;46:2169-2176.
16. Lozano-Cuenca J, Gonzalez-Hernandez A, Munoz-Islas E, et al. Effect of some acute and prophylactic antimigraine drugs on the vasodepressor sensory CGRPergic outflow in pithed rats. Life Sci 2009;84:125-131.
17. Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous clonidine-displacing substance in the brain. Science 1994;263:966-969.
18. Ernsberger P, Haxhiu MA. The I1-imidazoline-binding site is a functional receptor mediating vasodepression via the ventral medulla. Am J Physiol 1997;273:R1572-1579.
19. Atlas D. Identifying clonidine-displacing substance. Science 1994;266:462-464.
20. Hieble JP, Ruffolo RR, Jr. Subclassification and nomenclature of alpha 1- and alpha 2-adrenoceptors. Prog Drug Res 1996;47:81-130.
21. Munk SA, Lai RK, Burke JE, et al. Synthesis and Pharmacologic Evaluation of 2-endo-Amino-3-exo-isopropylbicyclo[2.2.1]heptane: A Potent Imidazoline$_1$ Receptor Specific Agent. Journal of Medicinal Chemistry 1996;39:1193-1195.

22. Flamez A, De Backer J-P, Czerwiec E, Ladure P, Vauquelin G. Pharmacological characterization of I1 and I2 imidazoline receptors in human striatum. Neurochemistry International 1997;30:25-29.
23. Bayne K. Revised Guide for the Care and Use of Laboratory Animals available. American Physiological Society. Physiologist 1996;39:199, 208-111.
24. McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 2010;160:1573-1576.
25. Legislation for the protection of animals used for scientific purposes Directive 2010/63/EU [online]. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.
26. Cobos-Puc LE, Villalón CM, Ramírez-Rosas MB, et al. Pharmacological characterization of the inhibition by moxonidine and agmatine on the cardioaccelerator sympathetic outflow in pithed rats. Eur J Pharmacol 2009;616:175-182.
27. Edvinsson L, Chan KY, Eftekhari S, et al. Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries. Cephalalgia 2010;30:1233-1240.
28. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
29. Doods H, Hallermayer G, Wu D, et al. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. British Journal of Pharmacology 2000;129:420-423.
30. Goadsby PJ, Lipton RB, Ferrari MD. Migraine — Current Understanding and Treatment. New England Journal of Medicine 2002;346:257-270.
31. van Zwieten PA. Centrally acting antihypertensives: a renaissance of interest˙ Mechanisms and haemodynamics. Journal of hypertension 1997;15:S3-S8.
32. Chan KY, Gupta S, De Vries R, et al. Effects of ionotropic glutamate receptor antagonists on rat dural artery diameter in an intravital microscopy model. British journal of pharmacology 2010;160:1316-1325.

Chapter XII.

Increased mortality and vascular phenotype in a knock-in mouse model of RVCL-S

Based on: IA Mulder, ***E Rubio-Beltrán,*** *K Ibrahimi, M Hoehn, GM Terwindt, MJH Wermer*, A MaassenVanDenBrink* and AMJM van den Maagdenberg*. Submitted. In revision.*

**Authors contributed equally*

Abstract

Background and Purpose: Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by C-terminal frameshift mutations in the *TREX1* gene that encodes the major mammalian 3'to 5'DNA exonuclease. RVCL-S is characterized by vasculopathy, especially in densely vascularized organs, progressive retinopathy, cerebral microvascular disease, white matter lesions and migraine, but the underlying mechanisms are unknown.

Methods: Homozygous transgenic RVCL-S KI mice expressing a truncated *Trex1* protein (similar to what is seen in patients) and wild-type littermates, of various age groups, were subjected to (I) a survival analysis, (II) in vivo post-occlusive reactive hyperemia (PORH) and *ex vivo* Mulvany myograph studies to characterize the micro- and macrovascular reactivity, and (III) experimental stroke after transient middle cerebral artery occlusion (*t*MCAO) with neurological deficit assessment.

Results: The mutant mice show increased mortality starting at midlife (p=0.03 with HR=3.14, 95% CI: 1.05-9.39). The mutants also show a vascular phenotype as evidenced by attenuated PORH responses (across all age groups) (F(1,65)=5.7, p=0.02) and lower acetylcholine-induced relaxations in aortae (in 20- to 24-month-old mice) (RVCL-S KI: E_{max}: 37±8% vs. WT: E_{max}: 65±6%, p=0.01). A vascular phenotype is also suggested by the increased infarct volume seen in 12- to 14-month-old mutant mice at 24 hours after infarct onset (RVCL-S KI: 75.4±2.7 mm^3 vs. WT: 52.9±5.6 mm^3, p=0.01).

Conclusions: Homozygous RVCL-S KI mice show increased mortality, signs of abnormal vascular function, and increased sensitivity to experimental stroke and can be instrumental to investigate the pathology seen in RVCL-S patients.

Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCLS) is an autosomal dominant disorder caused by C-terminal frameshift mutations in the *TREX1* gene,1 which encodes the ubiquitously expressed major three prime repair exonuclease 1 (*Trex1*) protein[1,2]. The mechanisms underlying the etiology of RVCL-S, however, are unknown. RVCL-S is a systemic progressive small vessel disease with middle-age severe symptomatology and decreased life-expectancy, characterized by vasculopathy in various organs, most pronounced in retina, kidney and brain, with white matter lesions and intracerebral mass lesions[3-6]. RVCL-S patients report more often migraine (generally without aura) and have increased risk for ischemic stroke, both suggesting vascular involvement in RVCL-S. Ultrastructural pathological studies revealed thicker endothelial cells with increased vesicles and coarse cytoplasm, and thicker, multi-laminated basement membranes of endothelial cells[5]. Hence it has been hypothesized that the endothelium of small vessels, particularly in highly vascularized organs, is affected in RVCL-S. Endothelial dysfunction (impaired endothelium-dependent vasodilation and endothelial cell damage)[7], which has been proposed as disease mechanism for the vascular phenotype seen in stroke and migraine, was also shown to underlie RVCL-S in patients[4].

While RVCL-S patients are heterozygous for the *TREX1* mutation, here we investigated transgenic homozygous RVCL-S KI mutant mice that express a truncated Trex1 protein (similar to what is seen in RVCL-S patients)[1,5]. The mutant mice express a truncated *mouse* protein (with the endogenous regulation of gene expression still intact), which is slightly different from the existing humanized RVCL-S mouse model in which the human cDNA bearing the same V235fs mutation was introduced in exon 2 of *Trex1*[8]. Here we assessed whether our homozygous RVCL-S KI mice have pathological features in line with the pathology seen in RVCL-S patients, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and induction of experimental stroke).

Methods

Experimental animals

Homozygous transgenic RVCL-S KI and wild-type (WT) littermate mice of different age groups (3-6, 12-14 and 20-24 months) were used. As the heterozygous mutants show a normal survival (Fig. 1) we focused on a comparison between the homozygous mutant and WT mice. For the generation of the mutant mice, a targeting construct was used by which a frameshift mutation was introduced at amino acid position 235 of the Trex1 protein, mimicking the truncation of the most prevalent RVCL-S mutation (for the generation of the mice see Suppl. Methods and Suppl. Fig. I)[1,5]. Homozygous mutant and WT mice were generated by interbreeding heterozygous mice. For the stroke experiments, only male mice were used as this sex is mostly used for such experiments. For the in *vivo* and *in vitro* vascular characterization, results of both sexes were pooled since no sex difference was detected. Animals were randomized for surgical procedure and researchers were blinded for genotype during experimental and analysis procedures. Animals were housed in a temperature-controlled environment with food and water ad libitum. All animal experiments were approved by the local committee for animal health, ethics and research of the Leiden University Medical Center.

Survival analysis

Survival in naive WT and RVCL-S KI mice was assessed in animals who, at 2 months, were randomly assigned to one of the age groups (3-6, 12-14, and 20-24months) to be included in the experimental stroke studies (n=97). Mice were censored for the survival analysis when they reached the age of inclusion for the stroke experiment. In addition, data of 51 heterozygous animals, which were not used in experiments, were added to the survival analysis.

In vivo microvascular characterization

Microvascular characteristics were assessed in vivo using post-occlusive reactive hyperaemia (PORH) measurements of the hind leg with laser Doppler flowmetry (PeriFlux System 5000; Perimed, Järfälla-Stockholm, Sweden). Twenty-four hours before dermal blood flow measurements, the hair of the left hind leg was removed with hair removal cream (Veet®, Reckitt Benckiser Inc., Berkshire, UK). On the day of measurement, mice were anesthetized using 4% isoflurane/O_2 ventilation, and kept on a heating pad regulated by a rectal thermometer to maintain body temperature at 36.7±0.3°C (FHC Inc., Bowdoin, ME, USA). After a 5-min equilibration time period, 10 min of baseline perfusion was recorded. Subsequently, the hind leg circulation was occluded for 2 min with a tourniquet. After release of the tourniquet, blood flow was monitored until return to baseline blood flow (maximally 10 min). The area under the curve and PORH peak were used as readout measures. After the experimental procedure, mice were sacrificed using decapitation where the aorta was collected for *in vitro* measurements.

In vitro macrovascular characterization

Macrovascular characterization was performed *in vitro* by mounting segments of the mouse aorta (inner diameter 0.5-1 mm), in Mulvany myographs with separated 6-mL organ baths[9] containing carbogenated (5% CO_2 and 95% O_2) Krebs-Henseleit buffer solution (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, H_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) at 37°C. Tension was normalized to 90% of the estimated diameter at 100 mmHg of transmural pressure. A reference contractile response of vessel segments was determined with 100 mM KCl. For the acetylcholine (ACh) and sodium nitroprusside (SNP) concentration response curves, aorta segments were pre-contracted with the

thromboxane A2 analogue U46619 (10-100 nM). Relaxant effects of ACh (endothelium-dependent) and SNP (endothelium-independent) and contractile effects of 5-hydroxytryptamine (5-HT) were examined by cumulative application of increasing concentrations of drug.

Transient Middle Cerebral Artery occlusion model

Mice were anesthetized using isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O_2. Carprofen 5 mg/kg, s.c. (Carporal, 50 mg/mL, AST farma B.V., Oudewater, the Netherlands) was administered before surgery for pain relief. During surgery the mouse body temperature was maintained at 36.7±0.3°C as described above. Experimental stroke was induced using the transient middle cerebral artery occlusion (*t*MCAO) model[10]. A silicone-coated nylon monofilament (7017PK5Re, Doccol cooperation, Sharon, MA, USA) was introduced into the internal carotid artery, via an incision in the common carotid artery, to block the middle cerebral artery (MCA) at its origin for 30 min. Cerebral blood flow (CBF) in the MCA territory was measured using laser Doppler flowmetry (PeriFlux System 5000; Perimed) and calculated as%-measure of baseline. In a subset of animals, $tcCO_2$ was measured (TCM4 CombiM monitor with accompanying software version 3.0, Radiometer Medical ApS, Brønshøj, Denmark) to confirm physiological stability during surgery. After surgery, the animal was placed in a temperature-controlled recovery incubator (V1200, Peco Services Ltd, Brough, UK) maintained at 33°C for 2 hr, with easy access to food and water. Postprocedural observation was performed twice daily.

Magnetic resonance imaging

Animals were scanned using a small animal 7T MRI system (Pharmascan, Bruker BioSpin, Ettlingen, Germany) under isoflurane anesthesia. A Multi Slice Multi Echo (MSME) sequence protocol was run with TR/TE of 4.000 ms/9 ms, 20 echoes, 2 averages, matrix 128x128, FOV of 2.50 cm, bandwidth 59523.8 Hz, slice thickness of 0.5 mm and 16 slices (no gap). Quantitative T2 maps were calculated using Paravision 5.1 software (Bruker BioSpin). Scans were performed at 4, 24 and 48 hours after *t*MCAO surgery. An in-house developed automated software pipeline[11] was used to calculate infarct sizes. If automated segmentation showed possible incorrect results, for example due to wrapping artifacts in the MRI data, small manual corrections were made or scans were excluded due to technical failure.

Behavior analysis

Behavior analyses were performed directly before MRI measurements were executed, *i.e.* before and after (at 4, 24, 48 hours, and 5 days) *t*MCAO surgery. During behavioral tests mice were videotaped, and blinded videos were analyzed after completion of the experiments. Neurological deficit scores (NDS) were measured on a 56-point scale[12,13]. The score involved general and focal deficits, where 0 reflected no deficits and 56 reflected the poorest performance. Categories concerning general appearance and performance included fur (0 to 2), ears (0 to 2), eyes (0 to 4), posture (0 to 4), spontaneous activity (0 to 4) and epileptic behavior (0 to 12). Concerning focal deficits, a score was stated for body asymmetry (0 to 4), gait (0 to 4), climbing on a surface inclined at 45° (0 to 4), circling behavior (0 to 4), front-limb symmetry (0 to 4), compulsory circling (0 to 4) and whisker response to light touch (0 to 4).

Vessel anatomy

A subset of mice was used for vessel anatomy analysis[14,15]. In brief, mice were sacrificed using CO_2 and transcardially perfused using 2.5 mL PBS with 50 µL heparin (Heparine natrium 5000 I.U./mL, LEO Pharma B.V., Amsterdam, the Netherlands) and 2.5 mL ink mixture ('Herlitz stempelfarbe ink

and Pelikan Scribtol ink'; Pelikan Vertriebsgesellschaft mbH & Co. KG, Hannover, Germany; ratio 1:9) at a rate of 3 mL/min, pre-heated to 37°C. Brains were removed, photographed and post-fixated in fresh 4% paraformaldehyde. Vessel anatomy was scored as the number of anastomoses between MCA and anterior cerebral artery (ACA), distance between midline and the anastomotic line at 2, 4 and 6 mm from anterior and diameter of the main vessels of the circle of Willis (MCA, ACA, internal carotid artery (ICA), posterior cerebral artery (PCA), basilar artery (BA) and posterior communicating artery (PcomA)). Pial anastomoses were defined as the narrowest part of the vessel or half way between the nearest branch points of the anterior and the middle cerebral artery, localized by tracing the peripheral branches of these major vessels.

Statistical analyses

All statistical analyses were performed in SPSS software (SPSS statistics 23, IBM Corporation, Armonk, NY, USA). Survival analyses were performed using the Log-rank (Mantel-Cox) test and Cox univariable regression analyses was done to calculate the hazard ratio (HR). Animals who reached the inclusion age of the experiment were censored in the analysis. The PORH results were expressed as relative values compared to baseline and included the AUC and the maximal hyperaemia response (E_{max}), expressed as mean±SEM. Vascular relaxant responses to ACh and SNP were expressed as the percentage of contraction induced by 10-100 nM U46619. Data for the vascular responses to 5-HT were expressed as percentage of the contractile response to 100 mM KCl. Both *in vivo* and *in vitro* data were analyzed using univariate ANOVA followed by Bonferroni post-hoc tests. Outcome values were expressed as mean±SEM. Infarct volume and neurologic deficit scores were compared between genotypes using marginal mixed models analyses (data is shown as estimated marginal means±SD). Survival analyses post-surgery were performed using the Log-rank (Mantel-Cox) test. Group comparison concerning vessel diameter and anastomotic distance were analyzed using MANOVA with discriminant function analysis post-hoc test. Absence of PcomA was analyzed using Pearson's Chi Square (2-sided Fisher's exact) test and mean pial number of anastomoses were analyzed with a *t*-test. P-values<0.05 indicate statistical significance.

Results

Increased mortality in RVCL-S KI mice

Homozygous RVCL-S KI mutant mice showed an increased mortality compared to WT controls: 16 (31%) mutant *vs.* 4 (9%) WT mice died during the observation period pre-surgery (p=0.03 with HR=3.14 (95% CI: 1.05-9.39)). The increased mortality in the mutant mice compared to WT mice was apparent from 9 months of age. Heterozygous mutant mice did not show a reduced mortality (p=0.55 with HR=1.25 (95% CI: 0.60-2.59) (Fig. 1). Because of the normal life-span in heterozygous mutants, only homozygous mutants were investigated in subsequent functional experiments. Post-mortem examination, although suboptimal because considerable organ deterioration had occurred at the time of tissue examination, did not reveal a macroscopic cause of death (data not shown). Post-mortem examination also did not show signs of brain atrophy, neither in the heterozygous nor the homozygous mutants (data not shown). Finally, basic histological analyses of various organs (e.g. eye, kidney, skin, liver, brain) of the homozygous and heterozygous mutant mice did not reveal any obvious abnormalities (data not shown). In contrast with RVCL-S patients[5], we observed no structural abnormalities in endothelial cells or the basement membrane of the mutant mice. It remains unclear whether this is because these disease features are not captured in the mouse model or whether these features need longer time to develop than the 2-year maximal age of a mouse.

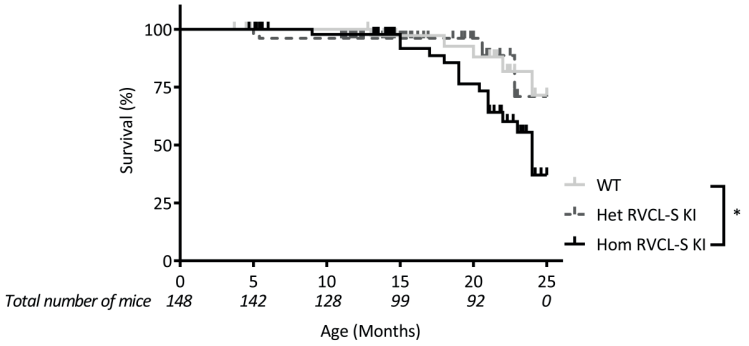


Fig. 1. Survival curves from WT, heterozygous (Het) and homozygous (Hom) RVCL-S KI male mice. At 2 months, homozygous mutant and WT mice were randomly assigned to one of the three age groups for experimental stroke surgery. Mice reaching the age of surgical inclusion were censored from the analysis. WT (n=45, grey line), heterozygous RVCL-S KI (n=51, dotted line), and homozygous RVCL-S KI mice (n=52, black line). Vertical bars along curves represent censored animals. *P<0.05 indicates statistical significance WT *vs.* homozygous RVCL-S KI mice.

Reduced in vivo microvascular reactivity in RVCL-S KI mice

Compared to WT, the RVCL-S KI mice showed a significantly lower area under the reactive hyperemia curve across all age groups (F(1, 60)=5.1, p=0.03) (Fig. 2A). The maximal reactive hyperemia responses, were comparable between genotypes (Fig. 2B). Baseline dermal blood flow and time to maximum value were also similar between genotypes (data not shown).

Increased in vitro macrovascular reactivity in RVCL-S KI mice

No significantly different contraction to 100 mM KCl was seen between genotypes. The concentration response curve to acetylcholine showed statistically significantly attenuated relaxant responses in 20- to 24-month-old RVCL-S KI mice compared to WT mice (E_{max}: 65±6% *vs.* 37±8%, respectively, p=0.01), but not in mice of other age groups (Fig. 2C). No genotypic difference was observed in the response to SNP or 5-HT (Fig. 2C).

Increased susceptibility to experimental stroke with worse outcome in RVCL-S KI mice

Overall infarct volume was higher in RVCL-S KI compared to WT mice (p=0.02), with a significant overall effect of age (p=0.02) and time after surgery (p<0.001). As shown in Fig. 3, the effects were mainly driven by the increased infarct volume in RVCL-S KI mice at the age of 12-14 months (*at 4 hours*: RVCL-S KI: 60.4±5.4 *vs.* WT: 39.8±4.8 mm^3, p=0.007; *24 hours:* RVCL-S KI: 84.3±9.5 *vs.* WT: 52.9±7.8 mm^3, p=0.01; *48 hours:* RVCL-S KI: 114.0±13.3 *vs.* WT: 58.1±10.7 mm^3, p=0.03). There was no significant genotypic effect on any cerebral vessel anatomy parameter (Suppl. Fig. II and Suppl. Table I). The overall NDS was worse in RVCL-S KI mice compared to WT mice for all age groups (p=0.002) (Fig. 4A). This effect was mainly driven by the 12- to 14-month-old group. In that age group NDS was worse in RVCL-S KI than WT after surgery for an extended period of time (*at 4 hours:* RVCL-S KI: 29.3±3.4 *vs.* WT: 11.1±2.9, p<0.001; *24 hours:* RVCL-S KI: 24.9±3.4 *vs.* WT: 9.9±2.7, p=0.001; *48 hours:* RVCL-S KI: 28.5±3.8 *vs*. WT: 10.0±2.6, p<0.001; *5 days:* 24.2±3.7 *vs.* 7.5±2.4, p=0.001), whereas there was no difference before surgery (*pre-surgery:* RVCL-S KI: 0.6±0.4 *vs.* WT: 0.5±0.2, p=0.20). Mortality within 5 days after stroke onset was also increased in the 12- to 14-month-old group (RVCL-S KI: 7 (58%) *vs*. WT: 4 (25%), p=0.04), an effect that was also observed for the 20- to 24-month-old group (RVCL-S KI: 8 (57%) *vs.* WT: 2 (13%), p=0.02); the young age group only showed a non-significant trend (RVCL-S KI: 4 (57%) *vs.* WT: 2 (33%)). There were no differences in basic parameters such as body weight (Suppl. Table II) or activity levels (Fig. 4) (determined from pre-surgery analyses) between mutant and WT mice that could explain the difference in age-related survival.

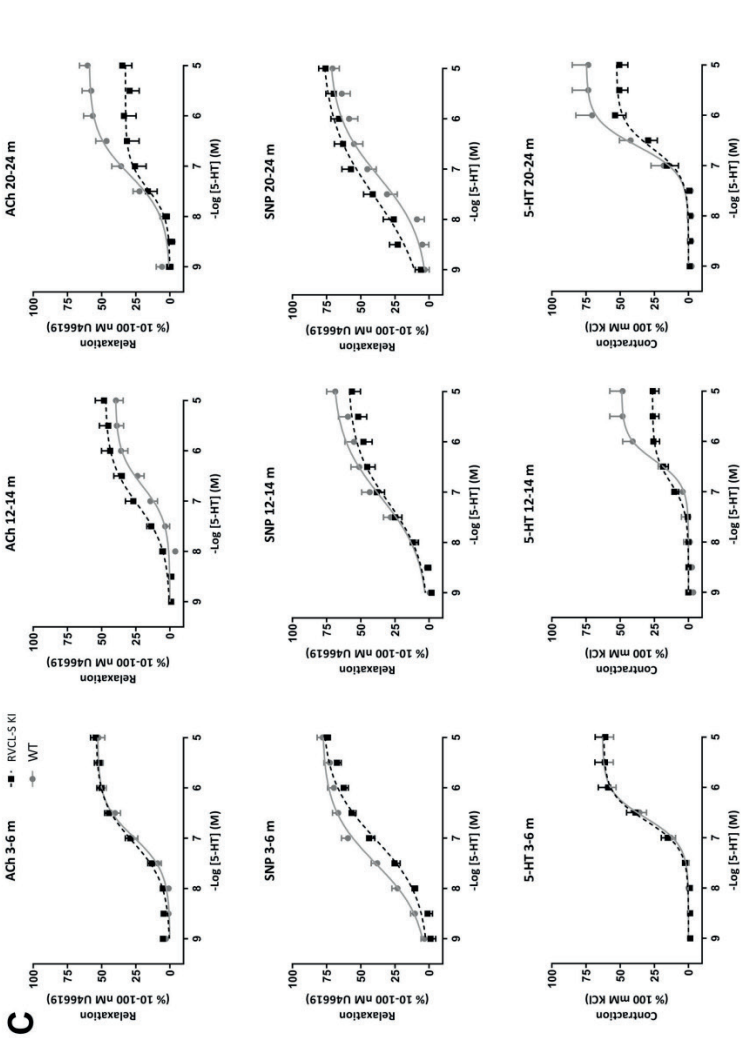


Fig. 2. Micro- and macrovascular characterization of WT and homozygous RVCL-S KI mice. *In vivo* microvascular characterization using post-occlusive reactive hyperemia (PORH) measurements of the hind leg with the area under the reactive hyperemia curve (A) and maximal reactive hyperemia (B) responses for WT and RVCL-S KI mice of the three age groups (3-6, 12-14, and 20-24 months). *P<0.05 indicates a statistical significant difference between RVCL-S KI and WT mice across all age groups. Number of animals are indicated within each bar. (C) *In vitro* macrovascular characterization of isolated aortae obtained from WT (grey circles) and homozygous RVCL-S KI (black squares) mice. Panels depict concentration response curves to acetylcholine (ACh, n=7-16), sodium nitroprusside (SNP, n=7-17) and serotonin (5-HT, n=5-15). *P<0.05 indicates statistical significance for attenuated relaxant responses to ACh in 20- to 24-month-old RVCL-S KI mice compared to WT mice. m=months, M=molar.

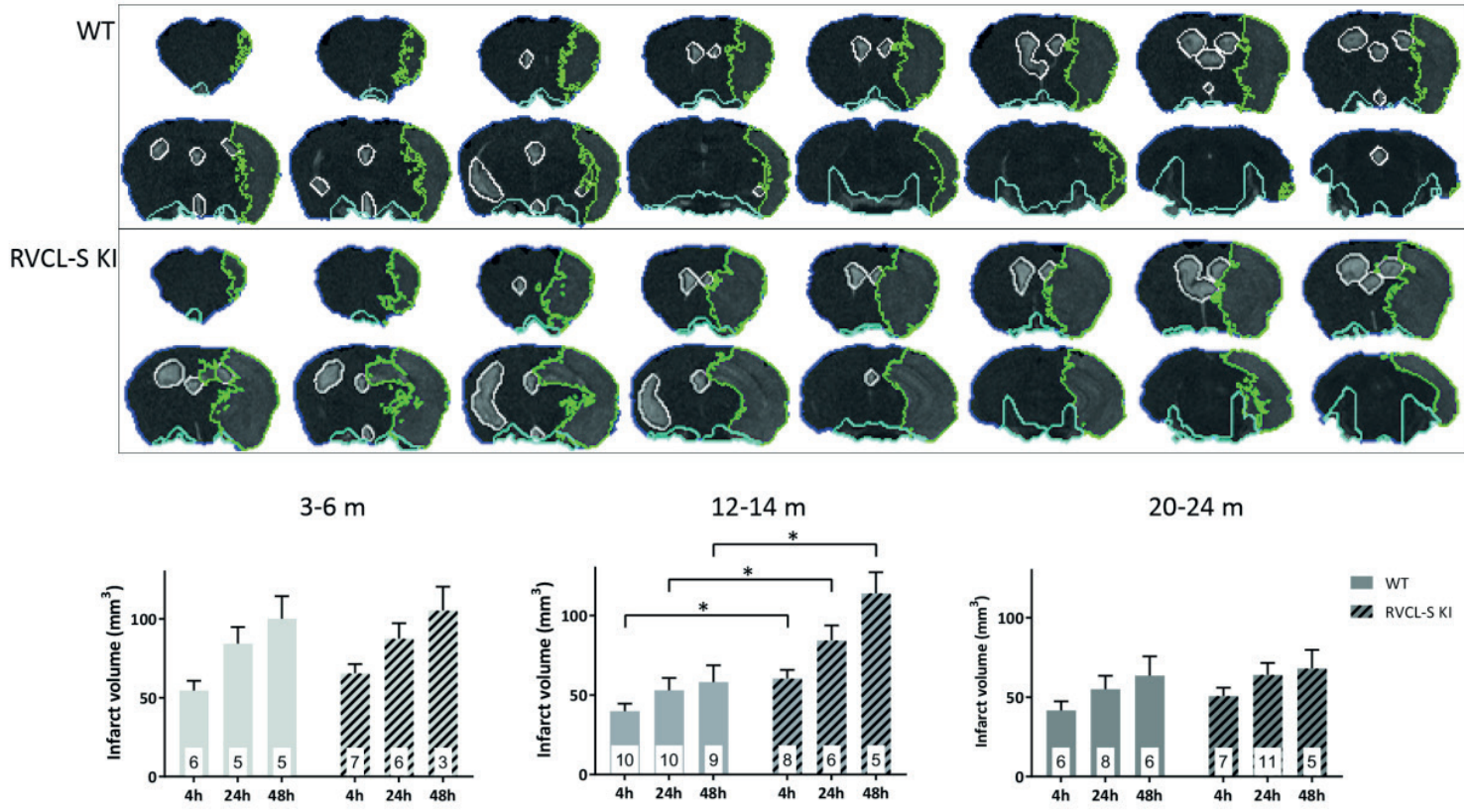


Fig. 3. Infarct volume in WT and homozygous RVCL-S KI male mice. (A) Example traces of automated stroke segmentation in T_2-weighted MRI images at 24 hours after infarct induction in 12- to 14-month-old WT and RVCL-S KI mice. (B) Stroke volumes at 4, 24 and 48 hours after infarct induction in WT and RVCL-S mice of the three age groups (3-6, 12-14, and 20-24 months) (estimated marginal mean±SD, *P<0.05 indicates a statistical significant difference between 12- to 14-month-old RVCL-S KI and WT mice for each individual time point. Number of animals are indicated within each bar. Numbers can vary due to death of the animal or technical failure of the MRI scan.

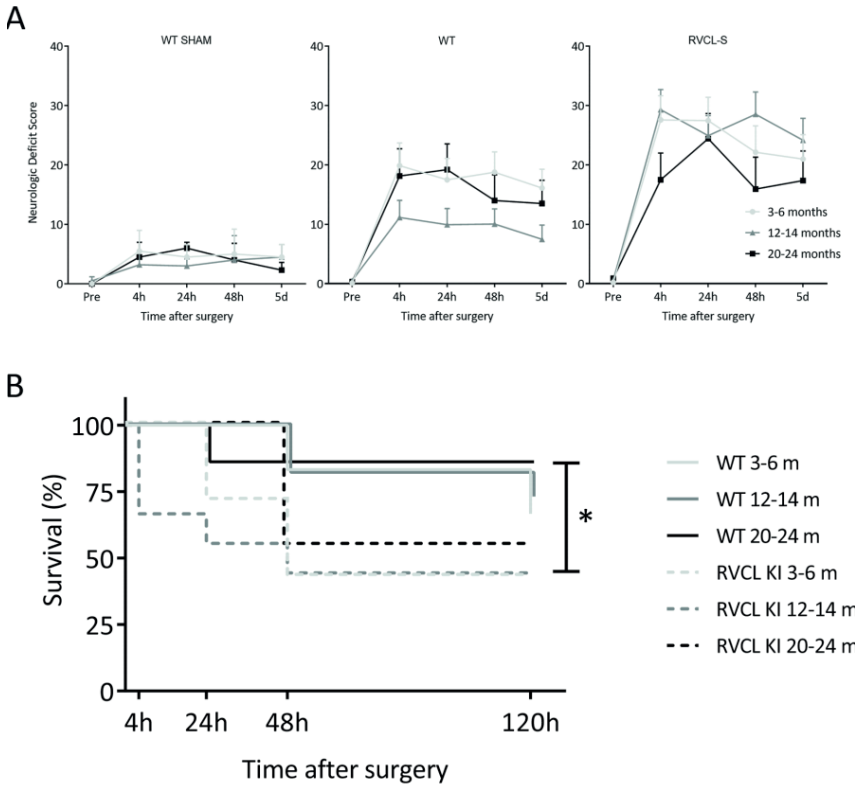


Fig. 4. Behavior analysis after infarct induction in WT and homozygous RVCL-S KI male mice. (A) Neurologic deficit score (NDS) (estimated marginal mean±SD) for WT SHAM surgery and WT and RVCL-S post-*t*MCAO and (B) survival curves at 4, 24, 48 hours and 5 days after *t*MCAO or SHAM surgery in WT (continuous line) and RVCL-S KI mice (discontinuous line) of the three age groups (3-6, 12-14, and 20-24 months). *P<0.05 indicates a statistical significant difference (A) between 12- to 14-month-old RVCL-S KI and WT mice for NDS and (B) between RVCL-S KI and WT mice concerning survival post-surgery.

Discussion

Here we investigated whether RVCL-S KI mice, which express a truncated mouse Trex1 protein that mimics the truncated protein seen in patients with the RVCL-S V235fs mutation[1,5], exhibit features in line with the phenotype of patients with RVCL-S. We demonstrated that homozygous RVCL-S KI mice have (I) a shorter life-expectancy, (II) a vascular phenotype, as evidenced by abnormal post-occlusive reactive hyperemia (PORH) (microvascular characterization; *in vivo* in hind leg) and acetylcholine-induced relaxant (macrovascular characterization; *in vitro* in aortic segments) responses, and (III) an increased susceptibility to experimental stroke with a worsened outcome. Except for outcome measures of experimental stroke, the other features can be considered spontaneous phenotypes. Except for signs of abnormal microvascular responses that were already observed from a young age (3-6 months), all other abnormalities were only observed in old mice, *i.e.* in mice of 12-14 months and/or 20-24 months. Our study in mice is relevant because it is not understood how vasculopathy, at middle-age, is brought about in patients with RVCL-S and how it is related to the lower life-expectancy and increased risk for ischemic stroke[3-6]. Similar to earlier observations in RVCL-S patients, our data point to decreased functional endothelial responses in

mice. In patients this is exemplified by reduced flow-mediated dilatation responses[4], and in mice by reduced blood flow after PORH across all investigated ages and by reduced macrovascular relaxant responses to the endothelium-dependent vasodilator acetylcholine.

RVCL-S KI mice have a vascular phenotype

This study is the first to show a vascular phenotype in RVCL-S KI mice. The results of the *in vivo* PORH experiments point towards a microvascular dysfunction in RVCL-S KI mice, with a decreased AUC of the PORH but a normal maximal PORH peak. As the effect was seen in all age groups it shows that a vascular phenotype is developing already before the mortality and increased stroke susceptibility become apparent. The PORH response[16] represents an interplay between multiple vasodilation pathways such as the endothelium-derived hyperpolarizing factor (EDHF) pathway[17] (which is characterized by the AUC) and the release of neuropeptides from perivascular nerves during the hyperemia peak (which is characterized by the maximal hyperaemia response (E_{max}))[18]. Notably, the *in vitro* examinations of macrovasculature showed abnormal endothelial function in response to ACh, but only in very old RVCL-S KI mice. The decreased relaxant response to ACh seems due to diminished endothelial function, as responses to the endothelium-independent NO donor SNP were normal in RVCL-S KI mice of the same age category. As mentioned above, the *in vivo* PORH AUC data suggest the involvement of an affected EDHF pathway response. EDHF acts as vasorelaxant and is known to act in microvessels rather than in larger vessels[19,20]. Thus, the results of our *in vitro* macrovascular studies are not in contrast with the microvascular PORH results. Testing 5-HT[21] showed no abnormal vasoconstrictive response in RVCL-S KI mice, although concentration response curves tended to be lower in the 12-14 and 20-24 months age groups compared to those in WT mice.

Increased ischemic vulnerability in RVCL-S KI mice

The vascular dysfunction seen in RVCL-KI mice may be the underlying cause of the increased stroke risk in RVCL-S patients. Therefore, we investigated whether RVCL-S KI mice had a worse stroke outcome. Although the infarct area is still evolving days to weeks after *t*MCAO, we used 48 hours as final MRI read out point since infarct size on T2-weighted MRI is maximized at 48 hours post *t*MCAO[22,23]. An overall increased infarct volume was observed in RVCL-S KI mice, compared to WT mice; albeit driven by the 12- to 14-month-old group. Given the decreased survival in the very old mutant mice, one can envisage that no genotypic difference in infarct volume was seen in the 20- to 24-month-old group because mutant mice that are most affected had already died before reaching the age of inclusion. Endothelial dysfunction was shown to lead to a procoagulatory, pro-inflammatory and proliferative state, which predisposes to atherosclerosis and increases stroke risk[7], but it remains to be seen whether this mechanism explains the increased infarct size in RVCL-S KI mice. There remains the, in our view unlikely, possibility that thrombus formation in the vessel after *t*MCAO may have influenced infarct outcome but we have no *a priori* reason to assume that thrombus formation would be different between mutant and WT mice; although this, admittedly, has not been demonstrated, which would require the use of different methodology than used in the present study.

There are other striking differences for the various age groups, when considering infarct size and outcome. Not finding a genotypic effect in young mice may be a reflection that the pathology has not advanced enough, although *in vivo* microvascular reactivity was already abnormal at that age. As an alternative explanation, a genotypic effect may be masked by the seemingly unusually large stroke volume in the respective WT mice; as volumes in WT mice from that age analyzed in our lab usually are 5-20% smaller (previously published data[11]).

It is even more surprising that mutant mice of 20-24 months have a worse outcome after *t*MCAO but no increased infarct size. One plausible explanation may have been an inclusion bias. Since animals with large infarcts have a higher *a priori* chance of dying within 48 hours from infarct induction, mice that do survive, tend to have smaller infarcts. Apparently, the mutant mice that could be included still exhibit mutation-associated pathology, as reflected by the increased mortality. Such underlying pathology may also explain why a total of 50% of the 20- to 24-month-old RVCL-S KI mice (compared to 15% of the WT group; Fig. 1) had already died before scheduled surgery. Of relevance, WT aged male mice (and rats) have smaller infarct volumes[24-28], but worse functional outcome[29,30] when compared to young animals. It remains enigmatic why aged male mice have a decreased infarct size[28,31-34]. Aged vessels may have undergone morphological and pathological changes[35], such as structural alterations in the vessel wall, atherosclerosis and vessel wall calcification[36,37], small vessel disease[38], and a reduced impairment of post-stroke synaptic plasticity[34,39].

RVCL-S phenotype in mice versus *humans*

One always needs to be cautious when extrapolating data from transgenic mouse models to human disease. Although RVCL-S in humans is an autosomal dominant disorder, so the presence of one mutated allele causes disease, in our experiments we focused on homozygotes mice instead to increase chances of capturing disease aspects by doubling the genetic load. It is not uncommon to focus investigations on homozygous mutant mice first as the lifespan in mice (max 2 years) is much shorter than in humans, so a mutation has less time to exert its effect.

In humans, RCVL-S is characterized by various structural abnormalities, foremost the vasculopathy of the retina and other organs, micro-infarcts, and white matter lesions[5,6]. A basic histological analysis of the eye and other organs in our mutant mice did not reveal such abnormalities, even not in mice that were far older than 12 months. This can be regarded a shortcoming of our model but this disease feature may not be captured because of the shorter lifespan of a mouse. Not finding histological abnormalities in our mutant mice is in accordance with findings in a previously described RVCL-S mouse model[8,40], that also expresses V235fs-truncated, albeit human, Trex1 protein. In that model, DNase activity, a hallmark of Trex1, was maintained in truncated protein[40]. Both heterozygous and homozygous mutants were reported to be grossly normal with no signs of tissue inflammation; the mutant mice did exhibit striking elevations of autoantibodies against non-nuclear antigens in the serum[40]. Of note, the authors did not report the decreased survival phenotype, likely because they did not investigate very old mice. We would consider both RVCL-S mouse models equally relevant and having multiple transgenic models will allow replication in separate models.

RVCL-S is considered a small vessel disease[5]. Abnormal functionality of the microvasculature, exemplified by the abnormal response to *in vivo* PORH, was observed in mutant mice already of young age. Moreover, in the experimental ischemic stroke experiments, outcome (as in human ischemic stroke) is influenced by the status of the microvasculature bed. Both types of experiments show that abnormal functionality of the microvasculature is captured in our mouse model. We recently found that functional endothelial responses were decreased in RVCL-S patients, while microvascular responses to capsaicin were normal (analyzed using flow-mediated vasodilatation (FMD))[4]. This points to an endothelial dysfunction in RVCL-S, which is in line with our micro- and macrovascular observations in the homozygous mutant mice. While FMD responses are generally considered as a measure for macrovascular function, it should be kept in mind that FMD is a physiologic response to the increased shear stress during reactive hyperemia and is thus critically dependent on the reactivity of the downstream microvasculature to transient ischemia. Therefore, we consider our findings in mice in line with the findings in RVCL-S patients.

In conclusion, we found that the homozygous RVCL-S KI mice exhibit early mortality in line with the reduced life expectancy seen in (heterozygous) patients with RVCL-S. Moreover, we observed a blunted (cerebral) microvascular response suggestive of endothelial involvement, which seems to occur already in young mutant mice, as well as an abnormal macrovascular response in old mutants. Finally, we observed an increased infarct size and worse outcome in homozygous RVCL-S KI mice in response to experimentally induced stoke, but it is at present unclear how this relates to the phenotype in RVCL-S patients. Regardless, our RVCL-S KI mouse model seems very promising to help unravel the pathophysiology of RVCL-S.

References

1. Richards A, van den Maagdenberg AMJM, Jen JC, Kavanagh D, Bertram P, Spitzer D, et al. C-terminal truncations in human 3'-5' DNA exonuclease *TREX1* cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. Nat Genet. 2007;39:1068-1070.
2. Mazur DJ, Perrino FW. Structure and expression of the Trex1 and Trex2 3'-5' exonuclease genes. J Biol Chem. 2001;276:14718-14727.
3. Pelzer, Bijkerk R, Reinders MEJ, van Zonneveld AJ, Ferrari MD, et al. Circulating endothelial markers in retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations. Stroke. 2017;48:3301-3307.
4. Boer I, Stam AH, Buntinx L, Zielman R, van der Steen I, van den Maagdenberg AMJM, et al. RVCL-S and CADASIL display distinct impaired vascular function. Neurology. 2018;91:e956-e963.
5. Stam AH, Kothari PH, Shaikh A, Gschwendter A, Jen JC, Hodgkinson S, et al. Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations. Brain. 2016;139:2909-2922.
6. Terwindt GM, Haan J, Ophoff RA, Groenen SM, Storimans CW, Lanser JB, et al. Clinical and genetic analysis of a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon. Brain. 1998;121 (Pt 2):303-316.
7. Lee S, Kim W, Park J, Jang HH, Lee SM, Woo JS, et al. Effects of electroacupuncture on endothelial function and circulating endothelial progenitor cells in patients with cerebral infarction. Clin Exp Pharmacol Physiol. 2015;42:822-827.
8. Hasan M, Fermaintt CS, Gao N, Sakai T, Miyazaki T, Jiang S, et al. Cytosolic nuclease Trex1 regulates oligosaccharyltransferase activity independent of nuclease activity to suppress immune activation. Immunity. 2015;43:463-474.
9. Chan KY, Edvinsson L, Eftekhari S, Kimblad PO, Kane SA, Lynch J, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (mk-0974) in human isolated coronary arteries. J Pharmacol Exp Ther. 2010;334:746-752.
10. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral-artery occlusion without craniectomy in rats. Stroke. 1989;20:84-91.
11. Mulder IA, Khmelinskii A, Dzyubachyk O, de Jong S, Rieff N, Wermer MJ, et al. Automated ischemic lesion segmentation in mri mouse brain data after transient middle cerebral artery occlusion. Front Neuroinform. 2017;11:3.
12. De Simoni MG, Storini C, Barba M, Catapano L, Arabia AM, Rossi E, et al. Neuroprotection by complement (c1) inhibitor in mouse transient brain ischemia. J Cereb Blood Flow Metab. 2003;23:232-239.
13. Storini C, Rossi E, Marrella V, Distaso M, Veerhuis R, Vergani C, et al. C1-inhibitor protects against brain ischemia-reperfusion injury via inhibition of cell recruitment and inflammation. Neurobiol Dis. 2005;19:10-17.
14. Eikermann-Haerter K, Lee JH, Yuzawa I, Liu CH, Zhou Z, Shin HK, et al. Migraine mutations increase stroke vulnerability by facilitating ischemic depolarizations. Circulation. 2012;125:335-345.
15. Hasan MR, Herz J, Hermann DM, Doeppner TR. Intravascular perfusion of carbon black ink allows reliable visualization of cerebral vessels. J Vis Exp. 2013 doi: 10.3791/4374.
16. Ibrahimi K, De Graaf Y, Draijer R, Jan Danser AH, Maassen VanDenBrink A, van den Meiracker AH. Reproducibility and agreement of different non-invasive methods of endothelial function assessment. Microvasc Res. 2018;117:50-56.
17. Cracowski JL, Gaillard-Bigot F, Cracowski C, Sors C, Roustit M, Millet C. Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans. J Appl Physiol (1985). 2013;114:245-251.
18. Larkin SW, Williams TJ. Evidence for sensory nerve involvement in cutaneous reactive hyperemia in humans. Circ Res. 1993;73:147-154.
19. Danser AH, Tom B, de Vries R, Saxena PR. L-name-resistant bradykinin-induced relaxation in porcine coronary arteries is no-dependent: Effect of ace inhibition. Br J Pharmacol. 2000;131:195-202.

20. Hwa JJ, Ghibaudi L, Williams P, Chatterjee M. Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. Am J Physiol. 1994;266:H952-958.
21. Gupta S, Mehrotra S, Villalon C, De Vries R, Garrelds I, Saxena P, et al. Effects of female sex hormones on responses to CGRP, acetylcholine, and 5-HT in rat isolated arteries. Headache. 2007;47:564-575.
22. Neumann-Haefelin T, Kastrup A, de Crespigny A, Yenari MA, Ringer T, Sun GH, et al. Serial MRI after transient focal cerebral ischemia in rats: dynamics of tissue injury, blood-brain barrier damage, and edema formation. Stroke. 2000;31:1965-1973.
23. Li F, Silva MD, Sotak CH, Fisher M. Temporal evolution of ischemic injury evaluated with diffusion-, perfusion-, and T2-weighted MRI. Neurology. 2000;54:689-696.
24. Chen Y, Sun FY. Age-related decrease of striatal neurogenesis is associated with apoptosis ofneural precursors and newborn neurons in rat brain after ischemia. Brain Res. 2007;1166:9-19.
25. DiNapoli VA, Huber JD, Houser K, Li X, Rosen CL. Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats. Neurobiol Aging. 2008;29:753-764.
26. Liu F, McCullough LD. Interactions between age, sex, and hormones in experimental ischemic stroke. Neurochem Int. 2012;61:1255-1265.
27. Manwani B, Liu F, Xu Y, Persky R, Li J, McCullough LD. Functional recovery in aging mice after experimental stroke. Brain Behav Immun. 2011;25:1689-1700.
28. Liu F, Yuan R, Benashski SE, McCullough LD. Changes in experimental stroke outcome across the life span. J Cereb Blood Flow Metab. 2009;29:792-802.
29. Badan I, Buchhold B, Hamm A, Gratz M, Walker LC, Platt D, et al. Accelerated glial reactivity to stroke in aged rats correlates with reduced functional recovery. J Cereb Blood Flow Metab. 2003;23:845-854.
30. Popa-Wagner A, Badan I, Walker L, Groppa S, Patrana N, Kessler C. Accelerated infarct development, cytogenesis and apoptosis following transient cerebral ischemia in aged rats. Acta Neuropathol. 2007;113:277-293.
31. Wang RY, Wang PS, Yang YR. Effect of age in rats following middle cerebral artery occlusion. Gerontology. 2003;49:27-32.
32. Shapira S, Sapir M, Wengier A, Grauer E, Kadar T. Aging has a complex effect on a rat model of ischemic stroke. Brain Res. 2002;925:148-158.
33. Correa F, Gauberti M, Parcq J, Macrez R, Hommet Y, Obiang P, et al. Tissue plasminogen activator prevents white matter damage following stroke. J Exp Med. 2011;208:1229-1242.
34. Sieber MW, Claus RA, Witte OW, Frahm C. Attenuated inflammatory response in aged mice brains following stroke. PLoS One. 2011;6:e26288.
35. Manwani B, Friedler B, Verma R, Venna VR, McCullough LD, Liu F. Perfusion of ischemic brain in young and aged animals: A laser speckle flowmetry study. Stroke. 2014;45:571-578.
36. Lakatta EG. Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises: Part iii: Cellular and molecular clues to heart and arterial aging. Circulation. 2003;107:490-497.
37. Herrera MD, Mingorance C, Rodriguez-Rodriguez R, Alvarez de Sotomayor M. Endothelial dysfunction and aging: An update. Ageing Res Rev. 2010;9:142-152.
38. Popa-Wagner A, Pirici D, Petcu EB, Mogoanta L, Buga AM, Rosen CL, et al. Pathophysiology ofthe vascular wall and its relevance for cerebrovascular disorders in aged rodents. Curr Neurovasc Res. 2010;7:251-267.
39. Sieber MW, Guenther M, Jaenisch N, Albrecht-Eckardt D, Kohl M, Witte OW, et al. Agespecific transcriptional response to stroke. Neurobiol Aging. 2014;35:1744-1754.
40. Sakai T, Miyazaki T, Shin DM, Kim YS, Qi CF, Fariss R, et al. DNase-active TREX1 frame-shift mutants induce serologic autoimmunity in mice. J Autoimmun. 2017;81:13-23.

SUPPLEMENTAL METHODS

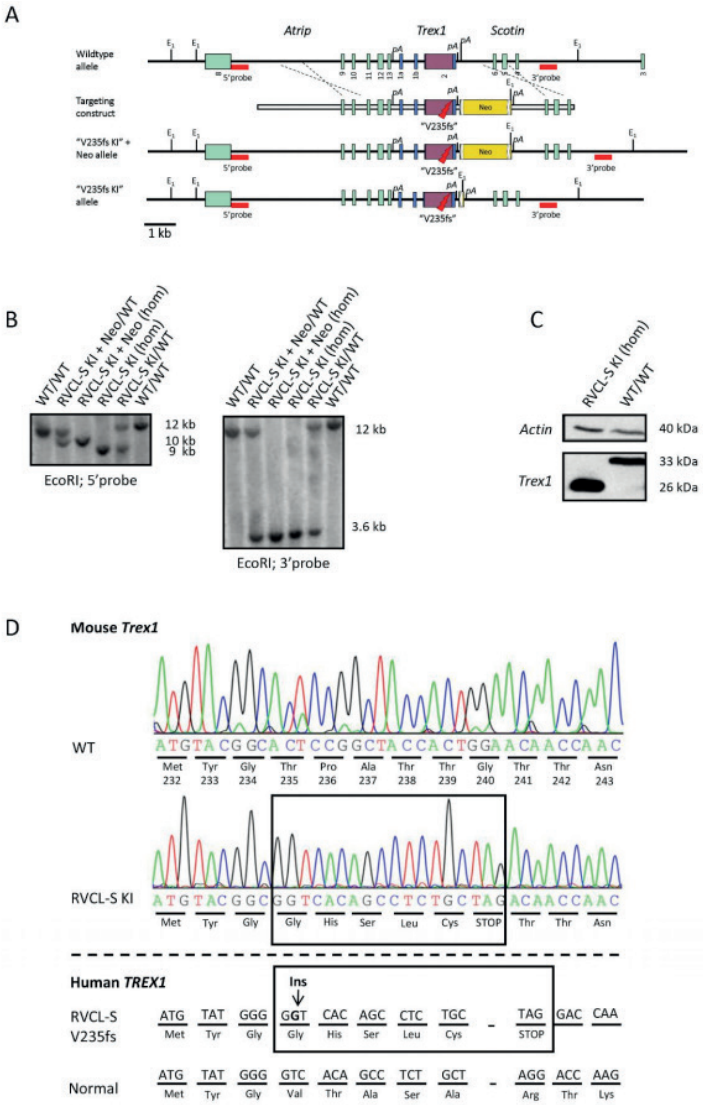
Generation and characterization of transgenic Trex1 V235fs knock-in mice The mouse Trex1 gene was modified using a gene targeting approach, in such a manner that the gene product was truncated at amino acid position 235 (which is a Threonine codon in the mouse gene and a Valine codon in the human gene) and contained the same abnormal amino acid sequence until the new stop codon as seen in RVCL-S patients with the V235fs mutation (for details on the sequences, see Supplemental Fig. ID).

-Targeting construct for the generation of RVCL-S KI mice (Supplemental Fig. IA). The targeting construct contained the RVCL-S mutation and a PGK-driven neomycin selection cassette (Neo), flanked by LoxP sites, which had been introduced downstream of Trex1 and upstream of neighbouring Scotin, between the polyA (pA) sequences of both genes.

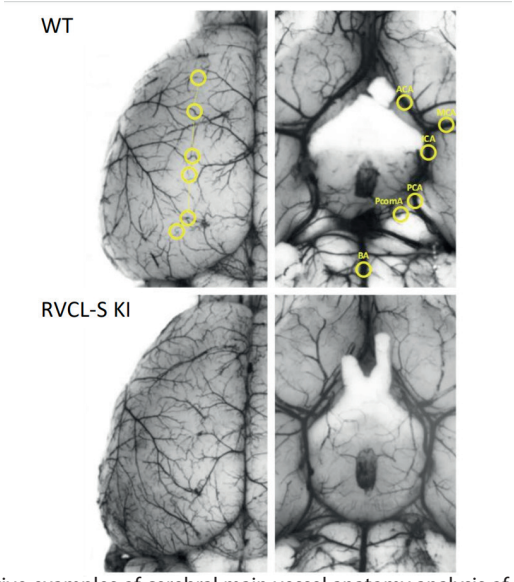
-Targeting ES cells and confirmation of correctly targeted alleles in mice by Southern blot analysis (Supplemental Fig. IB). E14 ES cells were targeted according to standard procedures and selected, correctly targeted, ES cells were injected into C57BL/6J blastocysts. Obtained chimeras were bred with C57BL/6J mice and germline transmission of the mutant allele was obtained, thus creating mice with the "RVCL-S + Neo" allele. The selection cassette was then removed by crossing the mutant mice with mice of the EIIA-Cre deleter strain1 (in which Cre recombinase expression is driven by the EIIA early promoter), which resulted in mice with the RVCL-S KI allele. Heterozygous RVCL-S KI mice were subsequently interbred to provide mice with homozygous mutant and wildtype genotypes. Confirmation of correct targeting was obtained by Southern blotting of EcoRI-digested genomic DNA (from cortex) of mice of the various genotypes (i.e. before and after Cre-mediated recombination). Upstream (5') and downstream (3') probes (as depicted in Supplemental Fig. IA) were used for Southern blotting.

-Verification of truncated Trex1 protein by Western blot analysis (Supplemental Fig. IC). Expression of truncated Trex1 protein was confirmed by semi-quantitative Western blotting. To this end, cortex tissue was used of wildtype and homozygous RVCL-S KI mice that had been killed by cervical dislocation. Whole cell lysates were homogenized with a potter (icecooled), followed by short sonication, using RIPA-buffer in the presence of protease inhibitor cocktail (Cat. No. 1836 170, Roche, Mannheim, Germany). Concentrations were measured (Pierce BCA Protein Assay Kit (Thermo, Cat#23225)) and 2 µg was loaded on a 15% PAA gel. After separation, proteins were transferred onto a nitrocellulose membrane. Blots were blocked with PBS/5% low fat milk/0.05% Tween-20 and subsequently incubated for 2 hours at room temperature with primary anti-mouse Trex1 antibody (BD transduction material number 611986). (1:2,500 diluted in incubation buffer (PBS/0.05% Tween-20). Secondary peroxidase-labelled Rabbit anti-mouse antibody (DAKO, P0260) (1:2,000 diluted in incubation buffer) incubation was performed for 1 hour at room temperature. Primary actin antibody incubation was performed for 2 hours at room temperature (A2066, Sigma, St Louis, MO) (1:2,000 diluted in incubation buffer). Secondary peroxidase-labelled Swine anti-rabbit antibody (DAKO, P0217) (1:2,000 diluted in incubation buffer) incubation was performed for 1 hour at room temperature. Western blotting was done according to the enhanced chemiluminescence ECL protocols (Amersham). Semi-quantification was based on equal β-actin signal intensity (loading control) and revealed Trex1 protein bands of 26 KDa for the mutant allele (as predicted from the truncation) and 33 KDa for the wildtype gene product.

-Direct Sanger sequencing of RT-PCR products verified expression of V235fs mutation containing RNA (Supplemental Fig. ID). The presence of the truncation mutation at the RNA level was confirmed by direct sequence analysis of RT-PCR product. To this end, RNA was extracted from cortex of wildtype and homozygous RVCL-S KI mice that had been killed by cervical dislocation. Total RNA was isolated using RNA Instapure (Eurogentec, Seraing, Belgium). For RT-PCR, first-strand cDNA was synthesized using random primers, and subsequent PCR was performed using Trex1-specific primers (primer sequences are available from the authors upon request). PCR products were sequenced using standard conditions. Details on the nature of human and mouse wildtype and mutant TREX1/Trex1 sequences are provided for comparison. In brief, the relevant part of wildtype mouse Trex1 sequence starting with the Valine codon at position 235, followed by a sequence of Thr-Ala-Ser-Ala, was changed to a sequence of His-Ser-Leu-Cys, followed by the premature stop codon, in the mutant sequence.



Suppl. Fig. I. Generation and molecular characterization of RVCL-S KI mice. A) The relevant part of the genomic structure of the wildtype *Trex1* allele, the targeting vector, and the predicted gene structure after homologous recombination ("V235fs KI + Neo" allele), and after Cre-mediated deletion of the Neo-cassette (Neo) (depicted as a yellow box) ("V235fs KI" allele) are shown. Numbered boxes indicate exons of *Atrip, Scotin* and *Trex1* genes. The position of the "V235fs" mutation in *Trex1's* coding exon 2 (purple box) is indicated. Red horizontal lines indicate probes for Southern blot analysis. EcoRI restriction sites are marked as E1, polyadenylation signal sites are marked as pA. B) Southern blots of EcoRI-digested genomic DNA obtained from mice with the indicated combinations of the RVCL-S KI + Neo, RVCL-S KI, and wildtype alleles. Respective bands obtained with either the 5'- or the 3'-probe are indicated. WT: wildtype; Hom: homozygote. C) Western blot of cortex protein lysate isolated from wildtype (WT/WT) and homozygous (hom) RVCL-S KI mice probed with Trex1 or actin antibody reveals expression of truncated (26 KDa) or normalsized (33 KDa) Trex1 protein; note that higher levels of Trex1 protein are present in the mutant compared to wildtype mice. D) Electropherograms of relevant parts (black boxes) of *Trex1* obtained from direct sequencing of RT-PCR products of cortex. Total RNA isolated from wildtype (WT) and homozygous RVCL-S KI mice are shown. For comparison, the respective sequences of mutant (RVCL-S) and normal human *TREX1* are shown below the dotted horizontal line.



Suppl. Fig. II. Representative examples of cerebral main vessel anatomy analysis of the Circle of Willis and pial collaterals in wildtype (WT) and homozygous RVCL-S KI mice. Representative ventral and dorsal views of WT and RVCL-S KI brains show the circle of Willis anatomy and pial arterial anastomoses between middle and anterior cerebral arteries. Pictures were taken after transcardiac ink perfusion under deep isoflurane anesthesia. Circles on the dorsal surface indicate examples of analyzed pial anastomoses (number and distance to midline were analyzed), whereas circles on the ventral surface indicate measurement areas for arterial diameters. None of these endpoints significantly differed between WT and RVCL-S KI mice (Suppl. Table I).

PART V:

Summary and general conclusions

Chapter XIII.

Summarizing discussion and future perspectives

...and *ends* with reason.

There is nothing higher than reason."

Migraine is a highly prevalent and complex neurovascular disorder[1]. It has been shown that during migraine, (dysfunctional) activation of the trigeminovascular system leads to cranial vasodilation mediated by the release of calcitonin gene-related peptide (CGRP)[2]. Despite the high prevalence of migraine, current treatment options are not effective for all patients. Therefore, a need for novel acute and prophylactic antimigraine drugs exists.

In **Chapter III** we reviewed a new class of acutely acting antimigraine drugs, "ditans", and compared them to the current gold standard for the acute treatment, the triptans. Triptans are 5-$HT_{1B/1D/(1F)}$ receptor agonists that in the past already have been shown to inhibit the release of CGRP from trigeminal fibers via 5-HT_{1D} receptor activation and to constrict cranial and coronary arteries via 5-HT_{1B} receptor activation[3-6]. Due to the coronary vasoconstriction, they are contraindicated in patients with cardiovascular disease[7]; however, it is important to also consider that migraineurs have an increased cardiovascular risk[8,9], therefore it is important to develop antimigraine drugs devoid of vascular effects. On the other hand, lasmiditan was developed as a selective 5-HT_{1F} receptor agonist, which has been shown to be effective for the acute treatment of migraine and with no reported vasoconstrictive properties, suggesting no vascular (side) effects. To corroborate the specificity and vascular profile of lasmiditan, in **Chapter IV**, we set out to investigate its pharmacological properties, in particular, to assess the selectivity, second messenger activity and its potential to induce vasoconstriction *in vitro* (in human isolated coronary, internal mammary and middle meningeal arteries) and *in vivo* (carotid and coronary artery diameters in anesthetized dogs). We compared our results to the responses obtained with sumatriptan, the most commonly prescribed triptan. Our results showed that lasmiditan is a selective agonist of the *human* 5-HT_{1F} receptor that is devoid of contractile properties *in vitro* and *in vivo*. The latter may represent a cardiovascular safety advantage when compared to the triptans.

Due to the important role of CGRP in migraine pathophysiology small-molecule antagonists against the CGRP receptor (gepants) were developed. Unfortunately, due to pharmacokinetic issues and hepatotoxicity reports[10], their development was temporarily halted. In the recent years, novel gepants have been developed and have been shown to be effective for the acute (ubrogepant) and prophylactic (atogepant) treatment of migraine[11]. In **Chapter V**, we analyzed the effects of ubrogepant and atogepant on the relaxations induced by CGRP in human isolated middle meningeal, cerebral and coronary arteries, as well as their antagonistic profile. As triptans are contraindicated in patients with cardiovascular disease due to their contractile properties, we also studied the effects *per se* in proximal and distal coronary arteries and compared them to the responses elicited by zolmitriptan. Our results showed that both gepants antagonized the CGRP-induced relaxations in all vessels studied, being more potent in the cranial arteries when compared to the inhibition observed in human coronary arteries. This property may be of clinical relevance and an advantage for these antagonists, since they seem to be less potent where the antagonism of CGRP-induced vasodilation is not desired. Moreover, atogepant was shown to be more potent in inhibiting the CGRP-mediated vasodilatory responses in all vessels tested. The analysis of the antagonistic profile in human coronary arteries showed that ubrogepant presents a competitive profile and, interestingly, atogepant a non-competitive one. Furthermore, while zolmitriptan elicited concentration-dependent contractions, neither of the gepants had significant vasoconstrictor responses in human coronary arteries.

As the initial trials with gepants (namely, telcagepant)[10] for the acute and prophylactic treatment of migraine were halted due to hepatotoxicity reports, a new class of prophylactic antimigraine drugs was developed: the antibodies against CGRP (eptinezumab, fremanezumab, galcanezumab) or its receptor (erenumab). This represents a milestone in prophylactic treatment, as previous

preventive treatments were not originally developed for migraine but were only later shown to be effective in this disorder. Nevertheless, the antibodies against CGRP (receptor) raise a concern, as CGRP is an ubiquitous peptide, not only involved in migraine pathophysiology, but also in physiological/homeostatic processes[12]. In light of this, in **Chapter VI**, we discussed the pros and cons of long-term CGRP blockade in migraine patients. In support of this therapeutic approach, clinical trials have shown that this treatment is efficacious, with results that seem comparable to the currently used prophylactic drugs. Furthermore, no major adverse effects related to CGRP (receptor) blockade have been reported. Additionally, the liver toxicity induced by some of the gepants is not present with the antibodies, which are well tolerated. On the other hand, as previously mentioned, there are concerns about the long-term effects, as CGRP and its receptor are abundantly present in both the vasculature, and in the peripheral and central nervous system, and are involved in several physiological processes and in the homeostatic response to ischemic events. Therefore, blocking CGRP may pose a risk in subjects with comorbidities such as cardiovascular diseases. In addition, evidence from animal studies suggests that CGRP blockade may induce constipation, affect the homeostatic functions of the pituitary hormones and/or attenuate wound healing, though, none of these effects have so far been reported in clinical trials. Taking into consideration the abovementioned aspects, the pros of blocking CGRP in migraine patients seem to exceed the cons, but adequate (cardiovascular) safety studies are required.

In line with the cardiovascular concerns, Depre *et al.*[13], recently addressed this matter by evaluating the effect of erenumab (CGRP receptor antibody) on exercise time during a treadmill test in patients with stable angina, concluding that the inhibition of the canonical CGRP receptor does not seem to worsen myocardial ischemia, contrary to theoretical concerns. Nevertheless, in **Chapter VII**, we commented on this study, as we considered that the design, the chosen patient population and the interpretation of the results do not entirely support such conclusions. To begin with, the authors of the study stated that the concentrations of exogenous CGRP required to increase total exercise time or protect against myocardial ischemia far exceed the endogenous physiological levels of CGRP that are released during a response to ischemia. This is not completely correct, as it is not the *systemic* plasma concentration that is relevant, but the concentration of CGRP at the *neuro-vascular junction*, where CGRP is released. Furthermore, the authors quoted the lack of vasoconstrictive properties of erenumab[14] as supporting argument of their conclusion, when the main concern is whether inhibition of the (vasodilatory) actions of CGRP is detrimental during an ischemic event. Regarding the study population, the patients included (78% male) suffered from stable angina pectoris, a typically considered "male" form of cardiac pathology[15], often caused by stenosis of the epicardial conducting portions of the coronary artery, where the importance of CGRP is limited. In contrast, in females, coronary artery disease often presents without an angiographically detectable stenosis but as diffuse atherosclerosis in the intramyocardial, smaller (distal) sections of the coronary artery bed[15-18] where the role of CGRP vasodilation seems more pronounced. As the majority of migraine sufferers are females, and therefore represent the main patient population under treatment with the CGRP (receptor) antibodies, the study failed to represent a relevant population. Moreover, the pharmacokinetic and pharmacodynamic considerations of this study were not adequate, since the plasma concentrations obtained 30 minutes after intravenous infusion could not ensure blockade of the CGRP receptor because it may well have taken several hours before the drug reached sufficiently high levels at the level of the receptor (located in the smooth muscle wall[16]), able to induce an effective blockade. Unfortunately, the authors did not confirm the blockade of the CGRP receptor and in previous studies the earliest time point shown for such blockade is 2 days after intravenous administration[19]. Overall, the study lacked to provide

evidence of effective CGRP receptor blockade and did not represent the patients that will benefit the most from the use of erenumab (*i.e.* females with microvascular disease) and might potentially be at the highest risk. As migraine patients have an increased cardiovascular risk, there will be cases of patients with ischemic complaints, even without a causal relationship. In fact, in the most recent interim analysis of the open-label extension study of erenumab, a patient died of what researchers called an "arteriosclerosis event"[20]. Therefore, appropriate studies in relevant study populations are needed to avoid sudden distress, such as happened with the triptans in the past.

Considering the abovementioned concerns, in **Chapter VIII** we characterized the relaxant responses to CGRP *in vitro,* in the absence and presence of erenumab, the human CGRP-receptor antibody approved for the prophylactic treatment of migraine, in isolated human middle meningeal, internal mammary of cardiovascularly compromised patients undergoing bypass surgery and (proximal and distal) coronary arteries. Furthermore, in mammary arteries, we also assessed whether the vasodilatory responses to acetylcholine, sodium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) and nicardipine, as well as the vasoconstrictor responses to dihydroergotamine (DHE), were modified by erenumab. Our results showed that the CGRP-mediated vasodilatory responses were significantly antagonized in the presence of erenumab with no significant difference in potency among tissues, contrary to what we observed with the gepants in **Chapter V**. Moreover, in mammary arteries, erenumab did not affect the responses to the other vasoactive compounds, suggesting functional specificity.

Even though the development of the antibodies against CGRP or its receptor for the prophylactic treatment of migraine are a major breakthrough, for the past decades the preventive treatment has consisted of drugs initially developed for other diseases, such as hypertension, epilepsy and depression. Although these drugs have been proven to be effective[21], their exact mechanism of action has not been described. As the trigeminovascular system is currently the main target for migraine treatment, in **Chapter IX**, we set out to investigate whether propranolol, one of the most widely prescribed drugs for the prophylactic treatment of migraine, modulates the activation of the trigeminovascular system. For this purpose, we investigated the effect of propranolol on the rise of dermal blood flow (DBF) of the forehead skin (innervated by the trigeminal nerve) by capsaicin application and electrical stimulation before and after placebo and propranolol in a randomized, double-blind, cross-over study, including healthy females on contraceptives and males. Additionally, we correlated our results with data from a Dutch prescription database by analyzing the change in triptan use after propranolol prescription in a population similar to our DBF study subjects. Our results showed that the DBF responses to capsaicin were attenuated after propranolol, but not after placebo. Interestingly, when we stratified by sex, no changes in the DBF responses to capsaicin were observed in females after propranolol, whereas a significant decrease remained present in males. DBF responses to electrical stimulation were not modified in any of the cases. Furthermore, when comparing the change in triptan use after propranolol, a more pronounced decrease was observed in male patients than in female patients on contraceptives. Our results suggest that propranolol modulates the trigeminovascular system activation in a sex-dependent manner, as in female subjects, that unfortunately represent the great majority of migraine patients, no significant DBF inhibition was observed after propranolol (80 mg) and in our retrospective study, a seemingly lower decrease in triptan use was observed in female patients.

As the understanding of migraine pathophysiology increases, novel therapeutic targets are proposed. In **Chapter X**, we reviewed one of the most recently proposed targets: the pituitary adenylate cyclase activating polypeptide-38 (PACAP) and one of its receptors, the PAC_1 receptor. PACAP is a neuropeptide described to be involved in neuroprotection, neurodevelopment,

nociception and inflammation. Interestingly, PACAP and its receptors are present in the different components of the trigeminovascular system, and intravenous infusion of PACAP induces migraine-like attacks. This led to the development of antibodies against PACAP (ALD 1910) and also against the receptor considered to be the most likely involved in the pathophysiology of migraine, the PAC_1 receptor (AMG 301), with the latter drug already being in Phase II clinical trials. No results have been published so far, but preclinical studies with AMG 301 have shown positive results comparable to those observed with triptans. However, as previously discussed with the CGRP (receptor)-antibodies, if these antibodies prove to be effective for the treatment of migraine, several considerations must be addressed, such as the potential side effects of long-term blocking of the PACAP (receptor) pathway and whether these antibodies will really represent a therapeutic advantage for the patients that do not respond to the CGRP (receptor)-antibodies.

Since inhibition of CGRP release is one of the proposed mechanisms of action of triptans[22] and imidazoline receptors have been described to inhibit neurotransmitter release[23,24], in **Chapter XI** we investigated whether moxonidine, an imidazoline I_1/α_2-adrenoceptor agonist, and agmatine, the endogenous ligand of the imidazoline receptors, inhibit the vasodepressor sensory CGRPergic outflow in pithed rats, and we further characterized the receptors involved. Our results showed that the infusion of moxonidine or agmatine inhibited the vasodepressor responses induced by stimulation of the sensory CGRPergic outflow, but not the responses to i.v. bolus of CGRP. Moreover, the inhibition of the vasodepressor responses was reversed after administration of the imidazoline I_1 receptor antagonist, and relatively more pronounced after administration of the combination of the imidazoline I_1 receptor antagonist plus the $\alpha_2$adrenoceptor antagonist. Therefore, the inhibition of the vasodepressor sensory CGRPergic outflow by moxonidine and agmatine is mainly mediated by prejunctional imidazoline I_1 receptors on perivascular sensory nerves and could represent a therapeutic target for migraine treatment.

Despite the major therapeutic advances in the last decades with the triptans and, more recently, with the novel ditans, gepants and the antibodies against CGRP or its receptor, not all migraine patients respond to treatment, and thus, new therapeutic targets are needed. For this, we need to understand migraine pathophysiology, as it still remains largely unknown. Animal models have contributed greatly to our current knowledge, but only represent certain features of this rather complex disorder. There are, however, monogenic diseases such as Autosomal dominant Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL, a vasculopathy caused by a mutation in the *TREX1* gene that presents migraine as the earliest manifestation), that provide an opportunity to study the genetic and vascular mechanisms involved in migraine pathophysiology. In **Chapter XII**, we assessed whether a new mouse model of RVCL (RVCL-KI), has features in line with the pathology seen in patients, such as a reduced life expectancy and a vascular phenotype (as assessed by functional vascular measurements and induction of experimental stroke). Our results showed that, in line with the phenotype in patients, mutant mice showed increased mortality, signs of abnormal vascular function and increased sensitivity to experimental stroke, suggesting that this transgenic mouse model can be instrumental to study the mechanisms underlying RVCL, as well as to further understand the pathophysiology of migraine.

Future perspectives

In *Chapter IV*, we demonstrated that lasmiditan is a selective agonist of the *human* 5-HT_{1F} receptor, devoid of contractile properties *in vitro* and *in vivo*. Currently, the exact mechanism(s) of action of lasmiditan remains elusive. As the therapeutic efficacy of triptans for the acute treatment of migraine is believed to be mediated via vasoconstriction of the trigeminal-innervated vasculature

and inhibition of CGRP release from the trigeminal fibers[22], future studies should now assess whether lasmiditan modulates the activation of the trigeminovascular system. For this, our human model of trigeminal nerve-mediated vasodilation is an excellent option, as the results could be compared to the results obtained previously by our group in healthy volunteers with sumatriptan[25]. Furthermore, the response to lasmiditan could also be studied in migraine patients, to analyze whether in their case, modulation of the trigeminovascular system is altered.

As mentioned above, our *human* model of trigeminal nerve-mediated vasodilation is an excellent option to study modulation of the trigeminovascular system *in vivo*. In *Chapter XI* we showed that moxonidine inhibits the CGRPergic outflow in pithed rats by activation of prejunctional (imidazoline I_1) receptors; therefore, it would be of great interest to further confirm our results by analyzing the effect of moxonidine in our human model of trigeminovascular activation. This could reinforce moxonidine (and the I_1 imidazoline receptors), as a therapeutic option for migraine treatment. Moreover, in this thesis, we characterized the inhibition of the CGRP-induced relaxations in human cranial and coronary arteries by ubrogepant, atogepant (*Chapter V*) and erenumab (*Chapter VIII*). Further studies could evaluate in our human model the modulation of the trigeminovascular system by both gepants and erenumab and assess whether the responses differ depending on the type of drug (gepants *vs.* antibodies), the intended type of treatment (acute *vs.* prophylactic) or whether there are no differences at all. Especially as at the moment, the only rationale behind choosing atogepant and erenumab for the prophylactic and ubrogepant for the acute treatment of migraine seems to be their half-life.

Besides investigating the mechanisms underlying the therapeutic efficacy of ubrogepant, atogepant and erenumab, future well-designed studies should evaluate the cardiovascular safety of these drugs. In the case of the gepants, experiments can be performed in a porcine *in vivo* model of ischemia-reperfusion, where one might assess cardiac function and infarct size in the absence and presence of cumulative doses of ubrogepant and, more importantly, atogepant (developed for the prophylactic treatment). In the case of erenumab, studies cannot be performed in animal models as it is a selective antibody against the *human* CGRP receptor, unless an antibody with affinity for the porcine (or rat/mice) receptor is developed. Alternatively, we could evaluate the effect of erenumab in a human model of microvascular function. Our group has previously assessed microvascular function with local thermal hyperemia (LTH) of the skin, measured with a laser Doppler flow imager[26] in healthy volunteers. Prospective studies should assess microvascular function before and after erenumab in a population representative of migraine patients, taking into consideration the pharmacokinetics of erenumab.

Our group has previously evaluated the vasodilatory responses to PACAP in human meningeal and coronary arteries[27], however, due to the complex pharmacology of the $VPAC_{1/2}$ and PAC_1 receptors[28], the exact receptor mediating the vasodilatory responses remained unclear. As discussed in *Chapter X*, if clinical trials show that the novel antibody against the PAC_1 receptor (AMG 301) is effective for the prophylactic treatment of migraine, this would suggest a peripheral site of action. Therefore, studies should evaluate whether the observed vasodilatory responses to PACAP in human meningeal and coronary arteries are inhibited in the presence of AMG 301. Otherwise, it would mean that the most likely site of action of the antibodies against the PAC_1 receptor is the trigeminal ganglion rather than the dural vasculature.

Finally, CGRP does not only bind to the canonical CGRP receptor, but also to the adrenomedullin (AM_1, AM_2) and amylin (AMY_1, AMY_3) receptors. Similarly, adrenomedullin and amylin can bind to the canonical CGRP receptor[29]. In migraine patients under erenumab treatment, CGRP may stimulate the amylin receptors described in the trigeminovascular system[30], reducing its efficacy. Conversely,

the amylin receptor has been proposed as the 'second' CGRP receptor previously reported by our group in the human coronary artery[31,32], which may represent a cardiovascular safety benefit for erenumab. Future studies should evaluate the vasodilatory responses to adrenomedullin and amylin in human isolated meningeal and coronary arteries in the presence of erenumab and whether these responses differ between meningeal and coronary arteries.

References

1. Stovner LJ, Nichols E, Steiner TJ, et al. Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurology 2018;17:954-976.
2. Edvinsson L. The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine. Headache 2017;57 Suppl 2:47-55.
3. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.
4. MaassenVanDenBrink A, Reekers M, Bax WA, Saxena PR. Human Isolated Coronary Artery Contraction to Sumatriptan Characterised by the Selective 5-$HT_{1B/1D}$ Receptor Antagonist GR55562. Pharmacology & Toxicology 2000;86:287-290.
5. MaassenVanDenBrink A, Saxena PR. Coronary Vasoconstrictor Potential of Triptans: A Review of In Vitro Pharmacologic Data. Headache: The Journal of Head and Face Pain 2004;44:S13-S19.
6. MaassenVanDenBrink A, van den Broek RW, de Vries R, Bogers AJ, Avezaat CJ, Saxena PR. Craniovascular selectivity of eletriptan and sumatriptan in human isolated blood vessels. Neurology 2000;55:1524-1530.
7. Dodick D, Lipton RB, Martin V, et al. Consensus statement: cardiovascular safety profile of triptans (5-HT agonists) in the acute treatment of migraine. Headache 2004;44:414-425.
8. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ : British Medical Journal 2005;330:63-63.
9. Sacco S, Kurth T. Migraine and the risk for stroke and cardiovascular disease. Curr Cardiol Rep 2014;16:524.
10. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert Opinion on Investigational Drugs 2012;21:807-818.
11. Tepper SJ. Anti-Calcitonin Gene-Related Peptide (CGRP) Therapies: Update on a Previous Review After the American Headache Society 60th Scientific Meeting, San Francisco, June 2018. Headache: The Journal of Head and Face Pain 2018;58:276-290.
12. MaassenVanDenBrink A, Meijer J, Villalón CM, Ferrari MD. Wiping Out CGRP: Potential Cardiovascular Risks. Trends Pharmacol Sci 2016;37:779-788.
13. Depre C, Antalik L, Starling A, et al. 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: The Journal of Head and Face Pain 2018;58:715-723.
14. Rubio-Beltran E, Labastida A, de Vries R, et al. Effects of AMG 334 on human isolated coronary artery. Cephalalgia; 2016: 41-41.
15. Humphries KH, Pu A, Gao M, Carere RG, Pilote L. Angina with "normal" coronary arteries: Sex differences in outcomes. American Heart Journal 2008;155:375-381.
16. Maas AHEM, Stam HCG, Eizema K, et al. Red alert for women's heart: the urgent need for more research and knowledge on cardiovascular disease in women: Proceedings of the Workshop held in Brussels on Gender Differences in Cardiovascular disease, 29 September 2010. European Heart Journal 2011;32:1362-1368.
17. Duncker DJ, Koller A, Merkus D, Canty JM. Regulation of Coronary Blood Flow in Health and Ischemic Heart Disease. Progress in Cardiovascular Diseases 2015;57:409-422.
18. Chan KY, Edvinsson L, Eftekhari S, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. J Pharmacol Exp Ther 2010;334:746-752.
19. Vu T, Ma P, Chen JS, et al. Pharmacokinetic-Pharmacodynamic Relationship of Erenumab (AMG 334) and Capsaicin-Induced Dermal Blood Flow in Healthy and Migraine Subjects. Pharmaceutical Research 2017;34:1784-1795.
20. Ashina M, Dodick D, Goadsby PJ, et al. Erenumab (AMG 334) in episodic migraine. Interim analysis of an ongoing open-label study 2017;89:1237-1243.
21. Jackson JL, Cogbill E, Santana-Davila R, et al. A Comparative Effectiveness Meta-Analysis of Drugs for the Prophylaxis of Migraine Headache. PloS one 2015;10:e0130733-e0130733.

22. Rubio-Beltran E, Labastida-Ramirez A, Villalon CM, MaassenVanDenBrink A. Is selective 5-HT_{1F} receptor agonism an entity apart from that of the triptans in antimigraine therapy? Pharmacol Ther 2018.
23. Cobos-Puc LE, Villalón CM, Ramírez-Rosas MB, et al. Pharmacological characterization of the inhibition by moxonidine and agmatine on the cardioaccelerator sympathetic outflow in pithed rats. Eur J Pharmacol 2009;616:175-182.
24. Villalón CM, Albarran-Juarez JA, Lozano-Cuenca J, Pertz HH, Gornemann T, Centurión D. Pharmacological profile of the clonidine-induced inhibition of vasodepressor sensory outflow in pithed rats: correlation with alpha(2A/2C)-adrenoceptors. Br J Pharmacol 2008;154:51-59.
25. Ibrahimi K, Danser AHJ, Terwindt GM, van den Meiracker AH, MaassenVanDenBrink A. A human trigeminovascular biomarker for antimigraine drugs: A randomised, double-blind, placebo-controlled, crossover trial with sumatriptan. Cephalalgia 2016;37:94-98.
26. Ibrahimi K, De Graaf Y, Draijer R, Jan Danser AH, Maassen VanDenBrink A, van den Meiracker AH. Reproducibility and agreement of different non-invasive methods of endothelial function assessment. Microvascular Research 2018;117:50-56.
27. Chan KY, Baun M, de Vries R, et al. Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery. Cephalalgia 2011;31:181-189.
28. Rubio-Beltrán E, Correnti E, Deen M, et al. PACAP38 and PAC1 receptor blockade: a new target for headache? The Journal of Headache and Pain 2018;19:64.
29. Hay Debbie L, Walker Christopher S, Gingell Joseph J, Ladds G, Reynolds Christopher A, Poyner David R. Receptor activity-modifying proteins; multifunctional G protein-coupled receptor accessory proteins. Biochemical Society Transactions 2016;44:568-573.
30. Walker CS, Eftekhari S, Bower RL, et al. A second trigeminal CGRP receptor: function and expression of the AMY1 receptor. Annals of Clinical and Translational Neurology 2015;2:595-608.
31. Gupta S, Mehrotra S, Villalon CM, et al. Characterisation of CGRP receptors in human and porcine isolated coronary arteries: evidence for CGRP receptor heterogeneity. Eur J Pharmacol 2006;530:107-116.
32. Haanes KA, Chan KY, MaassenVanDenBrink A. Comment on "A second trigeminal CGRP receptor: function and expression of the AMY1 receptor". Annals of clinical and translational neurology 2016;3:307-308.

Nederlandse samenvatting

Migraine is een complexe neurovasculaire aandoening met een hoge prevalentie[1]. Het is aangetoond dat, tijdens een migraine aanval, (dysfunctionele) activatie van het trigeminovasculaire systeem tot craniale vasodilatatie leidt, opgewekt door de afgifte van calcitonin gene-related peptide (CGRP)[2]. Ondanks de hoge prevalentie van migraine zijn de huidige behandelopties niet bij alle patiënten effectief. Daarom bestaat er een behoefte aan de ontwikkeling van nieuwe acute en profylactische antimigraine middelen.

In **hoofdstuk III** geven we een overzicht van een nieuwe klasse acuut-werkende antimigraine middelen, "ditanen", en vergeleken deze met de huidige gouden standaard voor de acute behandeling, de triptanen. Triptanen zijn 5-$HT_{1B/1D/(1F)}$ receptor agonisten waarvan in het verleden al aangetoond is dat ze de afgifte van CGRP in trigeminale vezels remmen door middel van 5-HT_{1D} receptor activatie en craniële en coronaire arteriënvernauwen door middel van 5-HT_{1B} receptor activatie[3-6]. Vanwege de coronaire vasoconstrictie worden deze middelen gecontra-indiceerd bij patiënten met cardiovasculaire aandoeningen[7]. Het is belangrijk om ook rekening te houden met het feit dat migraineurs een verhoogd risico op cardiovasculaire aandoeningen hebben[8, 9]. Om deze reden is het belangrijk dat antimigraine middelen zonder vasculaire effecten ontwikkeld worden. Lasmiditan is ontwikkeld als een selectieve 5-HT_{1F} receptor agonist, hetgeen aangetoond effectief was bij de acute behandeling van migraine en zonder vermelde vasoconstrictieve eigenschappen, wat erop wijst dat lasmiditan geen vasculaire (bij)werkingen heeft. Om de specificiteit en het vasculair profiel van lasmiditan te onderzoeken, hebben we in **hoofdstuk IV** de farmacologische eigenschappen van dit middel onderzocht. We hebben de selectiviteit en second messenger activiteit bestudeerd, net als de potentie om *in vitro* vasoconstrictie te induceren (in de humane geïsoleerde coronair arterie, de a. mammaria interna en de a. meningea media), net als *in vivo* (diameters van de a. carotis en de coronair arterie in honden onder anesthesie). We vergeleken onze resultaten met die verkregen met sumatriptan, de triptaan die het vaakst voorgeschreven wordt. Onze resultaten toonden aan dat lasmiditan een selectieve agonist van de *humane* 5-HT_{1F} receptor is, die zowel *in vitro* als *in vivo* geen vaatvernauwende eigenschappen heeft. Dit laatste vormt mogelijk een cardiovasculair veiligheidsvoordeel ten opzichte van de triptanen.

Gezien de belangrijke rol van CGRP in de pathofysiologie van migraine zijn kleine molecuul antagonisten tegen de CGRP receptor (gepanten) ontwikkeld. Helaas is hun ontwikkeling tijdelijk stopgezet wegens farmacokinetische en hepatotoxische tekortkomingen[10]. Recent zijn nieuwe gepanten ontwikkeld en deze zijn aangetoond effectief voor de acute (ubrogepant) en profylactische (atogepant) behandeling van migraine[11]. In **hoofdstuk V** analyseerden we de effecten van ubrogepant en atogepant op de door CGRP veroorzaakte relaxaties in de humane geïsoleerde a. meningea media, cerebrale en coronaire arteriën, evenals hun antagonistische profiel. Aangezien triptanen gecontraïndiceerd zijn bij patiënten met hart- en vaatziekten vanwege hun contractiele eigenschappen hebben we ook de effecten *per se* bestudeerd in proximale en distale coronair arteriën en deze vergeleken met de contractiele effecten van zolmitriptan. Onze resultaten lieten zien dat beide gepanten de CGRP-geïnduceerde relaxaties in alle bestudeerde bloedvaten remden, waarbij de remming potenter was in de craniële arteriën in vergelijking met die in humane kransslagaders. Deze eigenschap is mogelijk klinisch relevant , aangezien een voordeel van deze antagonisten is dat deze minder sterk werkzaam lijken te zijn op plaatsen waar de remming van CGRP-geïnduceerde vasodilatatie niet wenselijk is. Bovendien toonde atogepant zich meer potent in het remmen van de CGRP-gemedieerde vasodilatoire response in alle geteste bloedvaten. De analyse van het antagonistische profiel in humane kransslagaders laat zien dat ubrogepant zich profileert als een competitieve antagonist, terwijl atogepant opmerkelijk genoeg een non-competitieve antagonist

lijkt. Terwijl zolmitriptan dosis-afhankelijke contracties teweeg bracht, gaf geen van de gepanten na toediening een significant vaatvernauwende respons in humane kransslagaders.

Aangezien de eerste studies met gepanten (namelijk telcagepant)[10] voor de acute en profylactische behandeling van migraine stopgezet waren wegens gerapporteerde hepatotoxicitet, werd een nieuwe klasse profylactische antimigrainemiddelen ontwikkeld: de antlichamen tegen CGRP (eptinezumab, fremanezumab, galcanezumab) of de CGRP receptor (erenumab). Dit is een mijlpaal in de profylactische behandeling van migraine, aangezien eerdere preventieve behandelingen niet specifiek voor migraine ontwikkeld waren, maar later pas effectief bleken te zijn voor de behandeling van deze aandoening. Desondanks zijn er zorgen over de antilichamen tegen de CGRP (receptor), omdat CGRP een alom aanwezig peptide is dat niet alleen betrokken is bij de pathofysiologie migraine, maar ook bij fysiologische/homeostatische processen[12]. In het licht hiervan bespreken we in **hoofdstuk VI** de voor- en nadelen van lange-termijn CGRP remming bij migraine patiënten. In het voordeel van deze therapeutische aanpak spreken klinische studies waarin werd aangetoond dat deze behandeling effectief is, met resultaten die vergelijkbaar lijken te zijn met de profylactische middelen die op dit moment gebruikt worden. Bovendien zijn er geen grote schadelijk effecten rondom CGRP (receptor) remming gevonden. Daarnaast induceren de antilichamen niet de levertoxiciteit die bij sommige gepanten optreedt. Aan de andere kant zijn er, zoals eerder genoemd, zorgen over de lange-termijn effecten, gezien het feit dat CGRP en de CGRP receptor in overvloed aanwezig zijn in zowel de vasculatuur als in het perifere en centrale zenuwstelsel, en dit peptide betrokken is bij meerdere fysiologische processen en in de homeostatische respons op ischemische gebeurtenissen. Om deze reden kan de remming van CGRP een risico vormen bij patiënten met comorbiditeiten zoals cardiovasculaire ziekten. Bovendien wijzen dierstudies uit dat CGRP remming kan leiden tot constipatie, een negatieve invloed heeft op de homeostatische functies van de hypofyse hormonen en/of wondheling afzwakt, alhoewel geen van deze effecten tot dusver gevonden worden bij klinische studies. De voorgenoemde zaken in ogenschouw nemend, lijken er meer voordelen aan aan CGRP remming bij migraine patiënten verbonden te zijn dan nadelen, maar adequate (cardiovasculaire) veiligheidsstudies zijn noodzakelijk.

In lijn met de cardiovasculaire bezwaren, zijn Depre *et al.*[13] recentelijk op deze kwestie ingegaan met een evaluatie van het effect van erenumab (CGRP receptor antilichaam) op de trainingstijd tijdens een inspanningstest bij patiënten met stabiele angina pectoris. Hieruit kwam naar voren dat de remming van de canonieke CGRP receptor de myocard ischemie niet lijkt te verergeren, in tegenstelling tot de theoretische bezwaren. Desalniettemin geven wij in **hoofdstuk VII** commentaar op deze studie, aangezien wij vinden dat de opzet, de gekozen patiëntenpopulatie en de interpretatie van de resultaten dit soort conclusies niet geheel ondersteunen. Om te beginnen beweren de auteurs van de studie dat de concentraties van exogeen CGRP die nodig zijn om de totale trainingstijd te verhogen of om te beschermen tegen myocard ischeme veel hoger zijn dan de endogene fysiologische CGRP spiegels die afgegeven worden tijdens een respons op ischemie. Dit is niet geheel juist, aangezien het niet de *systemische* plasma concentratie is die relevant is, maar de concentratie van CGRP bij de *neurovasculaire verbinding*, waarbij CGRP vrijkomt. Voorts halen de auteurs het gebrek aan vasoconstrictieve eigenschappen van erenumab[14] aan als ondersteunend bewijs voor hun conclusie, terwijl het belangrijkste probleem is of remming van de (vasodilatoire) werking van CGRP tijdens een ischemische gebeurtenis nadelig is. Wat de studiepopulatie betreft: er waren patiënten geïncludeerd (78% mannelijk) die leden aan stabiele angina pectoris, wat als een typisch "mannelijke" vorm van cardiale pathologie[15] beschouwd wordt, welke veelal veroorzaakt wordt door stenose in de epicardiale, geleidende delen van de kransslagader, waar het belang van

CGRP beperkt is. In tegenstelling tot mannen openbaren coronaire hartziekten zich bij vrouwen vaak zonder een angiografisch meetbare stenose, maar als diffuse atherosclerose in de intra-myocardiale, kleinere (distale) secties van het coronaire vaatbed[15-18] waar de rol van CGRP vasodilatatie veel groter lijkt te zijn. Gezien het feit dat de meerderheid van de patiënten die aan migraine ljiden vrouw is, en daarom de belangrijkste patiëntenpopulatie is die onder behandeling zal staan van de CGRP (receptor) antilichamen, werd in de bovengenoemde studie niet de relevante populatie onderzocht. Daarnaast waren de farmacokinetische en farmacodynamische redeneringen in deze studie niet adequaat, aangezien de plasma concentraties, die 30 minuten na intraveneuze infusie verkregen zijn, geen remming van de CGRP receptor kunnen garanderen omdat het een paar uur kan duren voordat het middel een voldoende hoge concentratie bereikt heeft op het niveau van de receptor (die zich in de gladde spiercel wand[16] bevindt), om zo een effectieve remming teweeg te brengen. Helaas hebben de auteurs de remming van de CGRP receptor niet bevestigd, en in eerdere studies werd een dergelijke remming op zijn vroegst 2 dagen na intraveneuze toedieningvastgesteld[19]. Samengevatwerd in de studie geen bewijs voor effectieve CGRP receptor remming geleverd en omvatte de studie niet de groep patiënten die het meeste baat zouden hebben bij het gebruik van erenumab (d.w.z. vrouwen, veelal met microvasculaire aandoeningen) en wellicht het grootste risico lopen bij gebruik van het middel. Aangezien migraine patiënten een verhoogd risico op hart- en vaatziekten hebben, zullen er gevallen van patiënten met ischemische klachten zijn, zelfs zonder een causaal verband. Er is zelfs in de meest recente interim-analyse van de open-label extensiestudie van erenumab een patiënt overleden aan wat onderzoekers een "manifestatie van arteriosclerose" noemden[20]. Daarom zijn passende studies in relevante studiepopulaties noodzakelijk om plotselinge problemen, zoals in het verleden met triptanen, te voorkomen.

De voorgenoemde bedenkingen in ogenschouw nemend, typeren we in *hoofdstuk VIII* de relaxerende respons op CGRP *in vitro,* in de aan- en afwezigheid van erenumab, het humane CGRP-receptor antilichaam geregistreerd voor de profylactische behandeling van migraine. We hebben de studie uitgevoerd in de humanegeïsoleerde a. meningea media, a. mammaria interna van cardiovasculair gecompromitteerde patiënten die een bypass operatie ondergingen en (proximale en distale) coronair arteriën. Bovendien hebben we in de a. mammaria interna onderzocht of de vaatverwijdende respons op acetylcholine, natrium nitroprusside, pituitary adenylate cyclase activating polypeptide-38 (PACAP), vasoactive intestinal peptide (VIP) en nicardipine, evenals de vaatvernauwende respons op dihydroergotamine (DHE), beïnvloed werden door erenumab. Onze resultaten lieten zien dat de CGRP-gemedieerde vaatverwijdende responsen significant afnemen in de aanwezigheid van erenumab, waarbij geen significant verschil in werkzaamheid is opgemerkt tussen de verschillende weefsels, in tegenstelling tot wat we bij de gepanten zagen in *hoofdstuk V.* In de a. mammaria interna liet erenumab bovendien geen effect zien op de responsen op de andere vasoactieve stoffen, wat wijst op een functionele specificiteit.

Waar de ontwikkeling van de antilichamen tegen CGRP of de CGRP receptor voor de profylactische behandeling van migraine een enorme doorbraak markeren, bestond de preventieve behandeling in de afgelopen decennia voornamelijk uit middelen die in eerste instantie ontwikkeld waren voor andere ziekten, zoals hypertensie, epilepsie en depressie. Ofschoon deze middelen bewezen effectief zijn[21], is hun exacte werkingswijze nog niet bekend. Aangezien het trigeminovasculaire systeem op dit moment het voornaamste doelwit is bij de behandeling van migraine, onderzoeken we in *hoofdstuk IX* of propranolol, één van de meest voorgeschreven middelen voor de profylactische behandeling van migraine, de activatie van het trigeminovasculaire systeem reguleert. Voor dit doel onderzochten we het effect van propranolol op de verhoging van de doorbloeding van de huid (dermal blood flow, DBF) in het voorhoofd (geprikkeld door de n. trigeminus) door

de toediening van capsaïcine en electrische stimulatie voor en na toediening van placebo en propranolol in een gerandomiseerde dubbelblinde cross-over studie bij gezonde vrouwen die contraceptie gebruiken en bij mannen. Daarnaast correleerden we onze resultaten met data uit een Nederlandse doktersreceptendatabase door de verandering in triptaangebruik na voorschrijven van propranolol te analyseren in een populatie overeenkomstig met onze DBF proefpersonen. Onze resultaten toonden aan dat de DBF respons op capsaïcine na toediening van de propranolol werd afgezwakt, maar niet na toediening van de placebo. Interessant genoeg zagen we dat, wanneer we de proefpersonen op sekse indeelden, er bij vrouwen geen veranderingen in de DBF respons op capsaïcine was na toediening van propranolol, terwijl bij mannen een significante afname aanwezig bleef. In geen van de gevallen trad een verandering in DBF respons op bij electrische stimulatie. Bij een vergelijking van de verandering in triptaangebruik na propranolol zagen we bovendien een sterkere afname bij mannelijke patiënten dan bij vrouwelijke patiënten die contraceptie gebruiken. Onze resultaten wijzen erop dat propranolol de trigeminovasculaire systeem activatie op een sekse-afhankelijke wijze reguleert, aangezien bij vrouwelijke proefpersonen, die het overgrote deel van de migraine patiënten vertegenwoordigen, geen significante DBF remming werd gezien na toediening van propranolol (80 mg). In onze retrospectieve studie werd een ogenschijnlijk lagere afname van triptaangebruik gevonden bij vrouwelijke patiënten.

Met het toenemen van het begrip van de pathofysiologie van migraine worden nieuwe therapeutische aangrijpingspunten gepostuleerd. In *hoofdstuk X* beoordelen we één van de meest recentelijk voorgestelde doelen: pituitary adenylaat cyclase activerend polypeptide-38 (PACAP) en één van de receptoren, de PAC_1 receptor. PACAP is een neuropeptide waarvan de literatuur stelt dat dit betrokken is bij neuroprotectie, neuro-ontwikkeling, nociceptie en ontstekingen. Het is interessant dat PACAP en de bijbehorende receptoren aanwezig zijn in de verschillende componenten van het trigeminovasculaire systeem, en dat intraveneuze infusie van PACAP migraine-achtige aanvallen teweegbrengt. Dit leidde tot de ontwikkeling van antilichamen tegen PACAP (ALD 1910) en ook tegen de receptor die het meest verdacht wordt van betrokkenheid bij de pathofysiologie van migraine, de PAC_1 receptor (AMG 301). AMG 301 bevindt zich reeds in Fase II klinische studies. Er zijn tot dusver nog geen resultaten van de klinische studies gepubliceerd, maar pre-klinische studies met AMG 301 hebben positieve resultaten laten zien die vergelijkbaar zijn met de resultaten met triptanen. Echter, zoals eerder opgemerkt in het kader van de CGRP (receptor)-antilichamen, zijn er ook voor de remming van PACAP verschillende zaken die eerst in overweging genomen moeten worden, zoals de mogelijke bijwerkingen bij langdurige remming van de PACAP (receptor) route en of deze antilichamen daadwerkelijk therapeutische voordelen bieden bij de groep patiënten die niet reageert op de CGRP (receptor)-antilichamen.

Aangezien remming van de CGRP afgifte één van de voorgestelde werkingsmechanismen is van de triptanen[22] en imidazoline receptoren volgens de literatuur afgifte van neurotransmitters remmen[23,24], hebben we in *hoofdstuk XI* onderzocht of moxonidine, een imidazoline I_1/α_2-adrenoceptor agonist, en agmatine, de endogene liganden van de imidazoline receptoren, de vasodepressor sensorische CGRP uitstroom in verdoofde ratten onderdrukken. Voorts hebben we de betrokken receptoren gekarakteriseerd. Uit onze resultaten blijkt dat de infusie van moxonidine of agmatine de vasodepressor responsen, opgewekt door stimulatie van de sensorische CGRP uitstroom, onderdrukten, maar niet de respons op een intraveneus toegediende bolus met CGRP. Daarnaast werd de onderdrukking van de vasodepressor responsen ongedaan gemaakt na toediening van een imidazoline I_1 receptor antagonist, en werd deze relatief verhoogd na toediening van de combinatie van de imidazoline I_1 receptor antagonist plus de α_2-adrenoceptor antagonist. We concluderen dat de onderdrukking van de vasodepressor sensorische CGRP uitstroom door moxonidine en agmatine

hoofdzakelijk gemedieerd wordt door prejunctionele imidazoline I_1 receptoren op perivasculaire sensorische zenuwen, dit zou een therapeutisch doel voor de behandeling van migraine kunnen zijn.

Ondanks de grote therapeutische vooruitgang in de afgelopen decennia met de triptanen en, meer recentelijk, met de nieuwe ditanen, gepanten en de antilichamen tegen CGRP of de CGRP receptor, reageren niet alle migraine patiënten op de behandeling en zijn nieuwe therapeutische doelen nodig. Hiervoor is een beter begrip van de pathofysiologie van migraine noodzakelijk, aangezien deze nog steeds grotendeels onbekend is. Diermodellen hebben veel bijgedragen aan onze huidige kennis, maar vertegenwoordigen slechts bepaalde kenmerken van deze redelijk complexe ziekte. Er bestaan echter monogenische ziekten zoals Autosomaal dominante Retinale Vasculopathie met Cerebrale Leukodystrophie (RVCL, een vasculopathie veroorzaakt door een mutatie in het TREX1 gen, waarbij migraine één van de vroegste manifestaties is), die mogelijkheden bieden om de genetische en vasculaire mechanismen die betrokken zijn bij de pathofysiologie van migraine nader te onderzoeken. In *hoofdstuk XII* bekeken we of een nieuw muismodel van RVCL (RVCL-KI) kenmerken heeft die parallellen vertonen met de pathologie die bij patiënten gezien wordt, zoals een verkorte levensverwachting en een vasculair fenotype (beoordeeld door middel van functionele vasculaire metingen en het induceren van een experimenteel herseninfarct). Onze resultaten toonden aan dat, overeenkomstig het fenotype in patiënten, mutante muizen een verhoogd sterftecijfer hadden, tekenen van abnormale vasculaire functie vertoonden en een verhoogde gevoeligheid hadden voor een experimenteel herseninfarct, wat erop wijst dat dit transgene muismodel kan bijdragen aan de bestudering van de onderliggende mechanismen bij RVCL, alsook voor het beter doorgronden van de pathofysiologie van migraine.

Toekomstperspectief

In *hoofdstuk IV* toonden we aan dat lasmiditan een selectieve agonist van de *humane* 5-HT_{1F} receptor is, zonder vaatvernauwende eigenschappen *in vitro* en *in vivo*. Momenteel is het precieze werkingsmechanisme van lasmiditan nog onbekend. Aangezien de therapeutische effictiviteit van triptanen voor de acute behandeling van migraine lijkt te worden gemedieerd door vasoconstrictie in de trigeminale-geïnnerveerde vasculatuur en remming van de CGRP afgifte vanuit de trigeminale vezels[22], zouden toekomstige studies moeten uitwijzen of lasmiditan de activatie van het trigeminovasculaire systeem reguleert. Hiervoor is ons humane model voor trigeminale zenuw-gemedieerde vasodilatatie een zeer geschikte optie, daar in dat geval de resultaten vergeleken kunnen worden met de eerdere resultaten bij onze groep gezonde vrijwilligers met sumatriptan[25]. Bovendien kan de respons op lasmiditan ook bestudeerd worden bij migraine patiënten, om te analyseren of in hun geval regulatie van het trigeminovasculaire systeem verandert.

Zoals eerder genoemd, is ons *humane* model van trigeminale zenuw-gemedieerde vasodilatatie een zeer geschikte manier om de regulatie van het trigeminovasculaire systeem *in vivo* te onderzoeken. In *hoofdstuk XI* lieten we zien dat moxonidine de CGRP-uitstroom remt in verdoofde ratten door activatie van prejunctionele (imidazoline I_1) receptoren. Om deze reden zou het erg interessant zijn onze resultaten verder te bevestigen door middel van een analyse van het effect van moxonidine in ons humane model voor trigeminovasculaire activatie. Dit versterkt mogelijk de positie van moxonidine (en de I_1 imidazoline receptoren) als een therapeutische optie voor de behandeling van migraine. Bovendien hebben wij in dit proefschrift de remming van de CGRP-geïnduceerde relaxaties in humane craniële arteriën en kransslagaders met ubrogepant, atogepant (*hoofdstuk V*) en erenumab (*hoofdstuk VIII*) getypeerd. Nader onderzoek in ons humane model is nodig om de regulatie van het trigeminovasculaire systeem door zowel gepanten als erenumab te evalueren en om vast te stellen of de responsen anders zijn afhankelijk van het type middel

(gepanten *vs.* antilichamen), het bedoelde behandeltype (acuut *vs.* profylactisch) of dat er wellicht helemaal geen verschillen optreden. Dit is zeker interessent omdat op dit moment de halfwaardetijd de enige gedachte achter de keuze tussen atogepant en erenumab voor de prophylactische en ubrogepant voor de acute behandeling van migraine lijkt te zijn.

Naast het onderzoek naar de onderliggende mechanismen van de therapeutische effectiviteit van ubrogepant, atogepant en erenumab, zouden toekomstige, goed opgezette studies dienen te bepalen wat de cardiovasculaire veiligheid van deze middelen is. In het geval van de gepanten, kunnen experimenten gedaan worden in een varkens *in vivo* model van ischemie en reperfusie, waarin gekeken kan worden naar de hartfunctie en infarctgrootte in de aan- en afwezigheid van cumulatieve doses ubrogepant en, nog belangrijker, atogepant (ontwikkeld voor de profylactische behandeling). In het geval van erenumab, kunnen studies niet in diermodellen worden uitgevoerd omdat het een selectief antilichaam tegen de *humane* CGRP receptor betreft, tenzij een antilichaam met affiniteit voor het varkens- (of rat-/muis-) receptor ontwikkeld wordt. Als alternatief zou gekeken kunnen worden naar het effect van erenumab in een human model voor microvasculaire functie. Onze groep heeft eerder de microvasculaire functie onderzocht met lokale thermale hyperemie (LTH) van de huid, gemeten met een laser Doppler flow imager[26] in gezonde vrijwilligers. Toekomstige studies zouden de microvasculaire functie voor en na toediening van erenumab dienen te onderzoeken in een populatie die representatief is voor migraine patiënten, rekening houdend met de farmacokinetische eigenschappen van erenumab.

Onze groep heeft eerder onderzoek gedaan naar de vaatverwijdende responsen op PACAP in humane meningeale en coronaire arteriën[27], maar door de complexe farmacologie van de $VPAC_{1/2}$ en PAC_1 receptoren[28] blijft het onduidelijk welke receptor precies verantwoordelijk is voor het opwekken van de vaatverwijdende respons. Zoals besproken in *hoofdstuk X* zou, indien kllinische studies aantonen dat het nieuwe antilichaam tegen de PAC_1 receptor (AMG 301) effectief is voor de profylactische behandeling van migraine, dit wijzen op een perifere plaats van werking. Daarom dienen nadere studies na te gaan of de geobserveerde vaatverwijdende responses op PACAP in humane meningeale en coronaire arteriën geremd worden in de aanwezigheid van AMG 301. Anders zou dit betekenen dat de meest waarschijnlijke plaats van werking van de antilichamen tegen de PAC_1 receptor de trigeminale ganglion is in plaats van de durale vasculatuur.

Tot slot bindt CGRP zich niet alleen aan de canonieke CGRP receptor, maar ook aan de adrenomedulline (AM_1, AM_2) en amyline (AMY_1, AMY_3) receptoren. Evenzo kunnen adrenomedulline en amyline zich binden aan de canonieke CGRP receptor[29]. Bij migraine patiënten die behandeld worden met erenumab kan CGRP de amyline receptoren stimuleren, zoals beschreven in het trigeminovasculaire systeem[30], hetgeeen de effectiviteit vermindert. Daartegenover wordt de amyline receptor voorgesteld als de 'tweede' CGRP receptor die eerder werd gerapporteerd door onze groep in de humane coronair arterie[31, 32], hetgeen kan betekenen dat er een cardiovasculair veiligheidsvoordeel bestaat voor erenumab. Toekomstige studies dienen de vaatverwijdende responses op adrenomedulline en amyline in humane geïsolateerde meningeale en coronaire arteriën in de aanwezigheid van erenumab nader te onderzoeken en vast te stellen of er verschillen zijn in de respons tussen meningeale en coronaire arteriën.

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In 2007 she started her studies in Chemical Pharmaceutical Biology at the Autonomous University of Querétaro. In 2011 she initated her Bachelor internship at the Department of Pain and Epilepsy of the National Institute of Neurobiology under the supervision of Prof. dr. Miguel Condés-Lara. After completing her Bachelor, she joined the Master degree program in Neuropharmacology and Experimental Therapeutics in the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV) in Mexico City, under the supervision of Prof. dr. Carlos M. Villalón. In 2015 she started her PhD training at the Department of Internal Medicine, Division of Vascular Medicine and Pharmacology of Erasmus MC under the supervision of dr. Antoinette Maassen van den Brink.

Rubio-Beltrán E, Labastida-Ramírez A, Haanes KA, van den Bogaerdt A, Bogers AJJC, Zanelli E, Meeus L, Danser AHJ, Gralinski MR, Senese PB, Johnson KW, Kovalchin J, Villalón CM, MaassenVanDenBrink A. *Characterization of binding, functional activity and contractile responses of the selective 5-HT$_{1F}$ receptor agonist lasmiditan.* Br J Pharmacol. 2019; In press.

Rubio-Beltrán E, Labastida-Ramírez A, Haanes KA, van den Bogaerdt A, Bogers AJJC, Dirven C, Danser AHJ, Xu C, Snellman J, MaassenVanDenBrink A. *Characterisation of vasodilatory responses in the presence of the CGRP receptor antibody erenumab in human isolated arteries.* Cephalalgia. 2019; In press.

Haanes KA, Labastida-Ramírez A, Blixt FW, **Rubio-Beltrán E,** Dirven CM, Danser AHJ, Edvinsson L, MaassenVanDenBrink A. *Exploration of purinergic receptors as potential anti-migraine targets using established pre-clinical migraine models*. Cephalalgia. 2019; In press.

Bussiere JL, Davies R, Dean C, Xu C, Kim KY, Vargas HM, Chellman GJ, Balasubramanian G, **Rubio-Beltran E,** MaassenVanDenBrink A, Monticello TM. *Nonclinical safety evaluation of erenumab, a CGRP receptor inhibitor for the prevention of migraine.* Regul Toxicol Pharmaco. 2019;106:224-238.

Labastida-Ramírez A, **Rubio-Beltrán E,** Haanes KA, de Vries R, Dammers R, Bogers AJJC, van den Bogaerdt A, Daugherty BL, Danser AHJ, Villalón CM, MaassenVanDenBrink A. *Effects of two isomethеptene enantiomers in isolated human blood vessels and rat middle meningeal artery – potential antimigraine efficacy*. J Headache Pain. 2019; 19:47.

Rubio-Beltrán E, MaassenVanDenBrink A. *Understanding CGRP and Cardiovascular Risk*. In: Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg. 2019.

Rubio-Beltrán E, Labastida-Ramírez A. *Sex Hormones and CGRP*. In: Maassen van den Brink A., MacGregor E. (eds) Gender and Migraine. Headache. Springer, Cham. 2019.

MaassenVanDenBrink A, **Rubio-Beltrán E,** Duncker D, Villalón CM. *Is CGRP Receptor Blockade Cardiovascularly Safe? Appropriate Studies Are Needed.* Headache. 2018; 58:1257-1258.

Rubio-Beltrán E, Correnti E, Deen M, Kamm K, Kelderman T, Papetti L, Vigneri S, MaassenVanDenBrink A, Edvinsson L; European Headache Federation School of Advanced Studies (EHF-SAS). *PACAP38 and PAC$_1$ receptor blockade: a new target for headache?* J Headache Pain. 2018; 19:64.

Reglodi D, Vaczy A, **Rubio-Beltrán E,** MaassenVanDenBrink A. *Protective effects of PACAP in ischemia.* J Headache Pain. 2018; 19:19.

Rubio-Beltrán E*, Labastida-Ramírez A*, Villalón CM, MaassenVanDenBrink A. *Is selective 5-HT$_{1F}$ receptor agonism an entity apart from that of the triptans in antimigraine therapy?* Pharmacol Ther. 2018; 186:88-97.

Condés-Lara M, Martínez-Lorenzana G, Rojas-Piloni G, Tello-García IA, Manzano-García A, **Rubio-Beltran E,** González-Hernández A. A*xons of individual dorsal horn neurons bifurcate to project in both the anterolateral and the postsynaptic dorsal column systems.* Neuroscience. 2018; 371:178-190.

Labastida-Ramírez A*, **Rubio-Beltrán E*,** Villalón CM, MaassenVanDenBrink A. *Gender aspects of CGRP in migraine*. Cephalalgia. 2019; 39:435-444.

Deen M*, Correnti E*, Kamm K*, Kelderman T*, Papetti L*, **Rubio-Beltrán E*,** Vigneri S*, Edvinsson L*, Maassen Van Den Brink A*; European Headache Federation School of Advanced Studies (EHF-SAS). *Blocking CGRP in migraine patients - a review of pros and cons.* J Headache Pain. 2017; 18:96.

Rubio-Beltrán E, Labastida-Ramírez A, Hernández-Abreu O, MaassenVanDenBrink A, Villalón CM. *Pharmacological analysis of the inhibition produced by moxonidine and agmatine on the vasodepressor sensory CGRPergic outflow in pithed rats.* Eur J Pharmacol. 2017; 812:97-103.

Labastida-Ramírez A, **Rubio-Beltrán E,** Hernández-Abreu O, Daugherty BL, MaassenVanDenBrink A, Villalón CM. *Pharmacological analysis of the increases in heart rate and diastolic blood pressure produced by (S)-isometheptene and (R)-isometheptene in pithed rats.* J Headache Pain. 2017; 18:52.

Condés-Lara M, Martínez-Lorenzana G, **Rubio-Beltrán E,** Rodríguez-Jiménez J, Rojas-Piloni G, González-Hernández A. *Hypothalamic paraventricular nucleus stimulation enhances c-Fos expression in spinal and supraspinal structures related to pain modulation.* Neurosci Res. 2015; 98:59-63.

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PhD Portfolio

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PhD period:	2015-2019
Erasmus MC Department:	Department of Internal Medicine, Division of Vascular Medicine and Pharmacology
Promotor:	Prof. dr. A. H. J. Danser
Supervisor:	Dr. A. Maassen van den Brink
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

In-depth Courses (13.7 ECTS)

2018	*Homeostatic regulation and migraine, a view from the bench*, London, England
	iHead Meeting, The International Headache Society, London, England
	Experimental Neurophysiology: Theory and Practice, Amsterdam, The Netherlands
2017	*Headache research for young scientists*, Vancouver, Canada
	1st School of Advanced Studies of the European Headache Federation, Rome, Italy
2016	Laboratory animal science, Erasmus MC, Rotterdam, The Netherlands
2015-2019	*COEUR Courses and Research Seminars*, Erasmus MC, Rotterdam, The Netherlands

Teaching activities (6 ECTS)

2015-2019	Pharmacology practical courses, scientific internship of Junior Med School students, Autonomic Nervous system practicum
2018	Supervising Master's thesis of a Biomedical Science's student
	Supervising Master's thesis of a NIHES student
2016-2017	Supervising Bachelor's thesis of Psychobiology students

Presentations (5.7 ECTS)

2019	19th Congress of the International Headache Society, Dublin, Ireland. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (E-poster).

61th Annual Scientific Meeting of the American Headache Society, Philadelphia, United States. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (Oral presentation).

13th European Headache Federation Congress, Athens, Greece. *Characterization of the effects of the CGRP receptor antagonists, atogepant and ubrogepant, on isolated human coronary, cerebral, and middle meningeal arteries* (Oral presentation).

2018

12th European Headache Federation Congress, Florence, Italy. *Effect of propranolol in a non-invasive human model of trigeminovascular activation* (Poster presentation).

12th European Headache Federation Congress, Florence, Italy. *PACAP38 and PAC_1 receptor blockade: a new target for headache?* (Oral presentation).

17th Biennial Migraine Trust International Symposium, London, England. *Pharmacological selectivity of inhibition of CGRP-induced relaxations by erenumab studied in human isolated internal mammary artery* (E- poster presentation).

4th School of Advanced Studies of the European Headache Federation. *Blocking CGRP in migraine patients, pros and cons* (Oral presentation).

28th ADMA Annual Meeting, Zwolle, The Netherlands. *Effect of propranolol in a non-invasive human model of trigeminovascular activation* (Oral presentation).

NVF Spring Meeting, Utrecht, The Netherlands. *Functional activity of antimigraine drugs at 5-HT receptors. Coronary artery contraction correlation* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Oral presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Disturbed Prelamin-A processing in VSMC causes aortic root dilation and aging-resembling macrovascular motor dysfunction* (Poster presentation).

2017

11th European Headache Federation Congress, Rome, Italy. *Blocking CGRP in migraine patients, pros and cons* (Oral presentation).

18th Congress of the International Headache Society, Vancouver, Canada. *In vitro characterization of agonist binding and functional activity at a panel of serotonin receptor subtypes for lasmiditan, triptans and other 5-HT receptor ligands and activity relationships for contraction of human isolated coronary artery* (Poster presentation).

18th Congress of the International Headache Society, Vancouver, Canada. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

27th ADMA Annual Meeting, Norwich, England. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Oral presentation).

FIGON Dutch Medicine Days 2017, Ede, The Netherlands. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

2016 FIGON Dutch Medicine Days 2016, Ede, The Netherlands. *Effects of AMG 334 on human isolated coronary artery* (Poster presentation).

5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland. *Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries* (Poster presentation).

5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland. *Effects of AMG 334 on human isolated coronary artery* (Poster presentation).

NVF Spring Meeting, Groningen, The Netherlands. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

2015 FIGON Dutch Medicine Days 2016, Ede, The Netherlands. *Pharmacological analysis of the inhibitory effects produced by moxonidine and agmatine on the sensory vasodepressor CGRPergic outflow in pithed rats* (Poster presentation).

International conferences (8.1 ECTS)

2019 19th Congress of the International Headache Society, Dublin, Ireland.

61th Annual Scientific Meeting of the American Headache Society, Philadelphia, United States.

13th European Headache Federation Congress, Athens, Greece.

2018 12th European Headache Federation Congress, Florence, Italy.

17th Biennial Migraine Trust International Symposium, London, England.

28th ADMA Annual Meeting, Zwolle, The Netherlands.

2017 11th European Headache Federation Congress, Rome, Italy.

18th Congress of the International Headache Society, Vancouver, Canada.

27th ADMA Annual Meeting, Norwich, England.

2016 5th European Headache and Migraine Trust International Congress – EHMTIC, Glasgow, Scotland.

26th ADMA Annual Meeting, Dordrecht, The Netherlands.

Grants & awards

Fellowship of the International Headache Society for the International Headache Academy (iHEAD) 2019 (Dublin, 2-4 September).

Fellowship of the International Headache Society for the International Headache Academy (iHEAD) 2018 (London, 3-5 September).

Travel grant for the 28th ADMA Annual Meeting 2017 (Zwolle, 8-10 June).

IHS travel grant to attend the IHC 2017 (Vancouver, 7-10 September).

Travel grant for the 27th ADMA Annual Meeting 2017 (Norwich, 25-27 May).

Fellowship of the European Headache Federation for the 1st School of Advanced Studies 2017 (Rome, 7-9 April).

1st prize Poster competition with the poster "*Lasmiditan and Sumatriptan: comparison of in vivo vascular constriction in the dog and in vitro contraction of human arteries*" Wetenschapsdagen Interne Geneeskunde 2017 (Antwerpen, 12-13 January).

Abbreviations

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
AMY_1	amylin type 1 receptor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BBB	blood brain barrier
CGRP	calcitonin gene-related peptide
CH	cluster headache
CLR	calcitonin-like receptor
CNS	central nervous system
DAP	diastolic arterial pressure
DBF	dermal blood flow
DHSC	dorsal horn of the spinal cord
E_{max}	maximal response
HCA	human coronary artery
HCxA	human cerebral artery
HMMA	human middle meningeal artery
LCX	left circumflex coronary artery
$logD_{pH7.4}$	distribution coefficient at physiological pH
mABs	monoclonal antibodies
MAP	mean arterial pressure
MCA	middle cerebral artery
MMA	middle meningeal artery
MRA	magnetic resonance angiography
ND	not defined
NTS	nucleus tractus solitarius
PACAP27	pituitary adenylate cyclase activating polypeptide-27
PACAP38	pituitary adenylate cyclase activating polypeptide-38
PAG	periaqueductal grey area
pEC_{50}	negative logarithm of the half maximal effective concentration
PLC	phospholipase C
PTSD	post-traumatic stress disorder
PVN	hypothalamic pararaventricular nucleus
RAMP1	receptor activity-modifying protein 1
RANCOVA	repeated measure analysis of covariance
RCP	receptor component protein
SAP	systolic arterial pressure
TG	trigeminal ganglion
TNC	trigeminal nucleus caudalis
VIP	vasoactive intestinal peptide

"All our knowledge *begins* with the senses,
proceeds then to the understanding,
and *ends* with reason.
There is nothing higher than reason."

Immanuel Kant
Critique of Pure Reason